Familial Abdominal Aortic Aneurysm

Clinical Features and Genetics

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Familial Abdominal Aortic Aneurysm

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CONTENTS

General Introduction

GENERAL INTRODUCTION

Cardiovascular disease is the most important cause of death in the world and the second cause of death in the Netherlands after cancer.[1, 2] Prevention and adequate treatment is therefore needed in order to reduce mortality. Cardiovascular disease encompasses ischemic heart disease, cerebrovascular disease, peripheral arterial occlusive disease and aneurysmal disease. The first three subgroups are characterized by narrowing of the artery, generally expressed by the term 'occlusive arterial disease'. On the contrary, aneurysmal disease, originating from the Greek word ἀνευρύνειν meaning "to dilate", is characterized by widening of the artery. The most common aneurysm in humans is the abdominal aortic aneurysm (AAA), for which annually 2000 patients receive operative treatment and 500 die from aneurysm rupture in the Netherlands.[3, 4] Abdominal aortic aneurysms are often asymptomatic until rupture and early detection saves lives. The question is why the aorta dilates in aneurysmal disease and occludes in arterial occlusive disease, despite overlapping atherosclerotic risk profiles. Apparently there is a predisposition leading to dilatation in AAA and genetic factors may play an important role herein. The focus of this thesis is to establish the clinical and genetic risk profiles for AAA, to provide appropriate treatment for AAA patients and to determine the need for screening of families of AAA patients.

Definition

Diagnostic criteria for AAA are based on absolute measurements of the aorta or a ratio to the expected normal diameter of the aorta in order to compensate for individual variation.[5] Today, the most used definition of AAA is an abdominal aortic diameter of 3.0 cm or more measured in either anterior-posterior or transverse planes with ultrasound, computed tomography (CT), or magnetic resonance imaging (MRI).[6] No consensus has been reached whether aneurysm diameters should be established using outer-to-outer or inner-to-inner aortic wall measurements, although conventionally outer-to-outer wall measurements are used.[7, 8]

Epidemiology

The prevalence of AAA is approximately 6% (range 4%-8%) in men and 2% (range 1%-2%) in women over 50 years of age based on several large international population screening studies.[9-14] The prevalence of AAA in recent screening programs seems declining and a Swedish population screening study reported recently a prevalence as low as 1.7%.[15-17] The reason why is not completely understood, but reduced risk factor exposure may be the cause.[17] When aortic aneurysms dilate over-time, the risk for rupture increases and approximately 25% of the patients receiving operative repair are treated for ruptured AAA.[18] Ruptured AAA is frequently fatal with mortality up to 80%.[19] Risk factors for AAA

identified in large-scale cross sectional studies include high age, and AAA under the age of 50 is rarely seen aside from patients with a genetic aortic aneurysm.[20, 21] Another risk factor is gender, as prevalence in men is four times higher than in women.[22] The reason for this remains unknown, but may be related to genetics, lifestyle or risk factor exposure. Smoking, especially the duration of smoking, is one of the modifiable risk factors for AAA and increases the risk by eightfold.[23, 24] Interestingly, to date no causative link has been found between smoking and AAA, although some studies suggest it is due to disruption of collagen synthesis and expression of metalloproteinases.[25] Lipids also play an important role in AAA as most observational studies show elevated lipids in patients with AAA,[12, 26] which may be related to the formation of atherosclerosis.[27] A reduction in aneurysm growth has been reported in AAA patients treated with statins,[28-30] but this result could not be reproduced by another study.[31] The association between high blood pressure and AAA is weak,[32, 33] and accelerated aneurysm growth due to hypertension has only been established in experimental studies.[34] Obesity has been reported to be a risk factor for AAA and increases the risk by one to twofold.[35, 36] Diabetes mellitus reduces the risk of AAA,[37] and is also associated with reduced aneurysm growth rate.[38] A proposed explanation for this phenomenon is that medical therapy and hyperinsulinemia result in stabilization of the thrombus and increased stiffness of the aortic wall.[39, 40] Overall, the risk factors for aneurysmal disease largely overlap the risk factors for occlusive arterial disease except for diabetes mellitus, which have led to the traditional view that both aneurysmal and occlusive arterial disease are both caused by atherosclerosis.

Pathophysiology

To understand the pathophysiology of AAA, knowledge of the components of the aorta is essential. The aortic wall consists of three layers; the tunica intima, tunica media and tunica adventitia. The intima consists of endothelial cells, while the media consists mainly of smooth muscle cells, elastic fibers and collagen. The adventitia consists mainly of collagen and fibroblasts. Traditionally it was believed that AAA was caused by advanced atherosclerosis,[41] but in contrast to occlusive arterial disease where mainly the intima and media are affected, all layers of the aortic wall are affected in aneurysmal disease.[27] In addition, if atherosclerosis would have caused AAA, the severity of atherosclerosis should correlate with AAA formation, which is not the case.[42] Although atherosclerosis remains associated with abdominal aneurysmal disease, current evidence suggests a different pathophysiology between aneurysmal and occlusive arterial disease.

The pathophysiologic mechanism in AAA is complex and characterized by two processes: extracellular matrix (ECM) degeneration,[43] and inflammation.[44] The ECM is essential for the strength of the aortic wall and elastin and collagen are its main components. Elastin is flexible and can normally sustain pressure from pulsation, whereas collagen, present

throughout most aortic layers, provides stability for axial forces. Proteolysis is the main cause for degradation of the aortic wall during aneurysm formation. Responsible proteases include matrix metalloproteinases and cysteine, among others.[45, 46] Aortic wall degradation decreases not only its stability but also triggers an inflammatory response.[47] Inflammation is the second most important mechanism in aneurysm formation, and histologic studies showed large amounts of inflammatory cells in the aortic wall, including macrophages and pro-inflammatory cytokines.[48, 49] The inflammatory cells promote smooth muscle cell apoptosis and elastin and collagen degradation.[44] Whether inflammation is the cause for proteolysis or is a response, remains elusive.

Other findings in patients with AAA include generalized arteriomegaly,[50] and AAA has also been associated with aneurysms at other vascular sites. For example, up to 15% of patients with AAA have a concomitant popliteal aneurysm,[51] and up to 60% of the patients with popliteal aneurysms have concomitant AAA.[6] Associations between AAA with inguinal hernias,[52] incisional hernias,[53] and chronic pulmonary disease (COPD) are also described and support the evidence of a systematic pathologic effect of weakened ECM in aneurysmal disease.[54]

Genetics

A genetic susceptibility for abdominal aneurysms was first suggested by Clifton et al. who described three brothers presenting with ruptured AAA.[55] A large number of studies have investigated the prevalence of familial AAA since then. Familial AAA can be ascertained by information from family history alone or by screening families, but both methods have their shortcomings. Family history alone usually underestimates the true prevalence of familial AAA because AAA is usually asymptomatic, patients may not be aware that relatives are diagnosed with AAA, affected relatives might not have been diagnosed correctly with AAA, or relatives are too young to have developed an aneurysm yet. Screening relatives with aortic imaging is limited as well, since it is cross-sectional and patients may develop aneurysms over time, and it can only be performed in relatives who are alive, willing and capable of undergoing screening. The average familial occurrence of AAA is approximately 13% (range 6%-36%) based on family history studies, and increases to around 20% in more systematic ascertained family histories.[56] Ultrasound screening studies show a similar percentage of familial occurrence in AAA of approximately 20%. Although the genetic predisposition is clear, genetic defects for AAA remain largely elusive. Genome wide association studies (GWAS), comparing single nucleotide polymorphisms in large numbers of unrelated AAA patients with controls, have not been able to identify single nucleotide polymorphisms associated with AAA or causes for AAA.[57] This endorses the heterogenic nature of genetic susceptibility for AAA and indicates the importance of family based genome studies to find genetic causes for AAA.

Abdominal versus thoracic aortic aneurysms

There are several factors that have led to the believe that abdominal and thoracic aortic aneurysms (TAA) are two different disease entities. For instance, the aortic wall originates from various embryologic cells. The thoracic aorta is formed from cells from the neural crest, while the abdominal aorta is formed from cells from the mesoderm, resulting in regional variation of the aortic wall.[58] The elastin/collagen ratio is higher in the thoracic aortic wall as compared to the abdominal wall and the abdominal aorta has less fibromuscular layers.[59] Also, the media of the abdominal aorta is avascular and therefore completely dependent on nutrition from the smooth muscle cells for survival in contrast to the thoracic aorta.[58] On the other hand, there are overlapping clinical characteristics between both diseases. In TAA, men are also more affected than females, although less than in AAA (70% versus 85%, respectively), and both diseases have a similar age distribution with a mean age at diagnosis of approximately 65 years.[60] Conventional risk factors for atherosclerosis are also present in TAA,[61] although these occur less frequently in patients with genetic aortic aneurysm syndromes. Furthermore, the percentage of familial occurrence in TAA is similar as found in patients with AAA, being approximately 20%, and importantly many AAA patients have concomitant TAA.[62] Taken together, for years it was believed that TAA is different from AAA because it originates from different embryologic cells, despite the overlapping clinical characteristics. This hypothesis is supported by the fact that the pathologic mechanisms in TAA are different from AAA, as they are generally associated with aortic dissections and histologic studies show medial necrosis and less inflammation and atherosclerosis.[63]

Regarding genetics, a part of the patients with TAA are caused by inherited connective tissue disorders such as Marfan and Loeys-Dietz syndrome.[20, 64] These connective tissue disorders are mainly caused by alterations in the TGF-beta pathway, caused by defects in several genes including *FBN1*, *TGFBR1*, *TGFBR2*, *TGFB2*, and *SMAD3*.[20] This pathway plays an important role in vascular and ECM remodeling.[65-68] Histologically, TGF-beta mediated aortic aneurysms show loss of elastin and disarrayed elastic fibers in the aortic media.[69] The role of alterations in in the TGF-beta pathway and AAA however, are currently unknown.[70-73] Other genetic defects causing TAA have also been described including genes affecting smooth muscle cells, such as *MYH11*,[74-76] *MYLK,*[77] and *ACTA2,*[78] or affecting the connective tissue, such as *COL3A1*.[79] Table 1 provides an overview of all genes currently associated with aortic aneurysms.

Interestingly, in families with familial syndromic and non-syndromic TAA, isolated AAA has occasionally been observed. Genes associated with TAA may therefore also play a role in the degenerative changes in the abdominal aortic wall causing AAA. It would therefore be very interesting to learn from this and to investigate whether there is overlap between TAA and AAA genetics, or whether they are complete indeed different disease entities.

Table 1 – Aortic aneurysm genes

Aortic aneurysm genes	Phenotype OMIM	OMIM Gene Number
ACTA2	Aortic aneurysm, familial thoracic 6	*102620
COL3A1	Ehlers-Danlos syndrome, type IV	*120180
COL5A1	Ehlers-Danlos syndrome, classic type	*120215
EFEMP2	Cutis laxa, autosomal recessive, type IB	*604633
ELN	Cutis laxa, autosomal recessive; Supravalvar aortic stenosis	*130160
FBN1	Aortic aneurysm, ascending, and dissection	*134797
FBN2	Marfan syndrome	*154700
FLNA	Cardiac valvular dysplasia, X-linked	*300017
MFAP5	Aortic aneurysm, familial thoracic 9	*601103
MTHFR	Vascular disease, susceptibility to	*607093
MYH11	Aortic aneurysm, familial thoracic 4	*160745
MYLK	Aortic aneurysm, familial thoracic 7	*600922
PRKG1	Aortic aneurysm, familial thoracic 8	*176894
SKI	-	*164780
SLC2A10	Arterial tortuosity syndrome	*606145
SMAD3	Loeys-Dietz syndrome, type 3	*603109
TGFB2	Loeys-Dietz syndrome, type 4	*190220
TGFB3	Loeys-Dietz syndrome, type 5	*190230
TGFBR1	Loeys-Dietz syndrome, type 1	*190181
TGFBR2	Loeys-Dietz syndrome, type 2	*190182
PKD1	Adult polycystic kidney disease	*173900

Diagnosis

Diagnosing AAA may be easy with non-invasive abdominal ultrasound.[80] Most AAAs remain undiagnosed however, because they are asymptomatic unless screening is performed, allowing to detect asymptomatic aortic dilatations.[81] Identification of AAA patients may also be made on abdominal imaging performed for other reasons. In case of aneurysm rupture, patients mostly present with abdominal and back pain associated with signs of shock.[6] Genetic risk stratification and targeted family screening of relatives of AAA patients may allow early identification an elective repair. So far no treatment to reduce dilatation is available to prevent rupture.[6]

Treatment

Treatment for AAA encompasses two components; aneurysm-related treatment in order to prevent death from rupture and optimizing cardiovascular status in order to improve perioperative care and for secondary prevention.

Aneurysm-related treatment: Treatment for the aneurysm itself is needed when the diameter reaches 5.5 cm, a moment where the perioperative risk is lower than the chance of rupture, or when there is evidence of rapid aneurysm growth (>1 cm/year).[6] In women, aneurysms might be treated when diameter reache 5.0 cm due to their relative smaller arterial system. Until patients reache treatment size, patients should be included in a AAA surveillance program, which was shown to be safe.[82] No consensus is reached on screening interval but patients should in general receive annual abdominal imaging in case of aneurysm size between 3.0 and 4.0 cm, every 6 months in case of aneurysm size between 4.0 and 5.0 cm and every three months in case of aneurysm size between 5.0 and 5.5 cm.[6] Surveillance can be performed by using duplex ultrasound. Operative treatment is recommended in case aneurysm size reaches 5.5 cm and may be considered from 5.0 cm in women. Endovascular repair has been introduced in 1991,[83] and is now, along with conventional open repair, standard of care.[6] Postoperative surveillance depends on type of treatment. After open repair, it is recommended to perform duplex ultrasound in order to identify possible pseudo-aneurysms, as well as a regular ankle-brachial index to identify arterial or graft occlusion.[6] Follow-up after endovascular repair is under much debate.[84] For now, a 30 days and 1 year CT-angiography and thereafter yearly duplex imaging seems adequate in order to identify aneurysm sac growth and is recommended as standard of care.[6]

Optimizing cardiovascular status: Cardiovascular status should be optimized to improve perioperative care as well as for secondary prevention. Respiratory, cardiac and renal comorbidities are the most important elements for improving cardiovascular status.[85] In order to improve perioperative care, respiratory function should be optimized by smoking cessation, which showed to reduce postoperative cardiac complications,[86] and reduces postoperative wound infections.[6] To improve cardiac and renal status, all patients should receive anti-platelet therapy and blood pressure control which has shown to be effective to reduce perioperative coronary events.[87, 88] Furthermore, all AAA patients should receive statin therapy to improve postoperative cardiac outcome and this may reduce aneurysm growth.[28, 89] Regarding secondary prevention, it is necessary that all atherosclerotic risk factors should be treated adequately.[6] For example, smoking cessation is important to reduce COPD development and blood pressure control should be continued in order to reduce cardiovascular comorbidity. Besides these patient specific therapies, it is important that all patients should continue anti-platelet therapy and statin therapy for an indefinite period of time.[6]

OUTLINE OF THE THESIS

The aim of this thesis is to investigate the differences between aneurysmal and occlusive arterial disease in order to provide evidence whether or not they are separate entities of cardiovascular disease, and to delineate the clinical features of familial abdominal aortic aneurysm and find evidence for a genetic origin of abdominal aortic aneurysm.

In **PART I,** the clinical and genetic features of patients with aneurysmal disease are compared to patients with occlusive arterial disease. In **Chapter 1**, the common carotid artery intima-media thickness (CIMT), a marker for generalized atherosclerosis, is assessed to investigate whether there is a difference in atherosclerotic burden in patients with aneurysmal and occlusive arterial disease. In **Chapter 2**, the vitamin D status is assessed in a large group of patients with aneurysmal and occlusive arterial disease, since recent evidence suggests that vitamin D deficiency might play a role in the pathogenesis of arterial disease. **Chapter 3** explores the molecular processes that differentiate aneurysmal from occlusive arterial disease by comparing RNA expression profiles from aortic wall samples from patients with aneurysmal and occlusive arterial disease. **Chapter 4** investigates the relationship between COPD and aortic aneurysms based on a clinical and experimental study, since both diseases may be caused by an inherited deficiency in connective tissue.

PART II describes the clinical and genetic features of familial abdominal aortic aneurysm. In **Chapter 5**, the CIMT is compared between patients with familial and sporadic AAA to investigate the role of atherosclerosis in familial AAA. And **Chapter 6** presents the first genetic analysis of aneurysm genes associated with TAA in a sample of familial and sporadic AAA patients, since isolated AAA is also observed in families with TAA.

PART III is dedicated to determine the clinical outcome after operative treatment of patients with familial abdominal aortic aneurysm. In **Chapter 7,** the aneurysm-related complications after endovascular aneurysm repair (EVAR) are investigated in patients with familial AAA. **Chapter 8** shows the clinical outcome of patients with a positive family history derived from a large worldwide EVAR registry, using a single late generation stent graft.

PART IV addresses the clinical management of familial abdominal aortic aneurysm. **Chapter 9** summarizes what a vascular surgeon should now about familial AAA. **Chapter 10** presents the risk for relatives of AAA patients, in relation to the gender of the patients and of the relatives. This chapter also presents the prevalence of familial AAA based on a family history study and describes whether patients with familial AAA can be identified on the basis of specific clinical characteristics. Finally, **Chapter 11** demonstrates the importance of genetic testing in patients with complex aortic pathology.

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PART I

Clinical and Genetic Features of Aneurysmal and Occlusive Arterial Disease

1

Aneurysmal Disease is Associated with Lower Carotid Intima-media Thickness than Occlusive Arterial Disease

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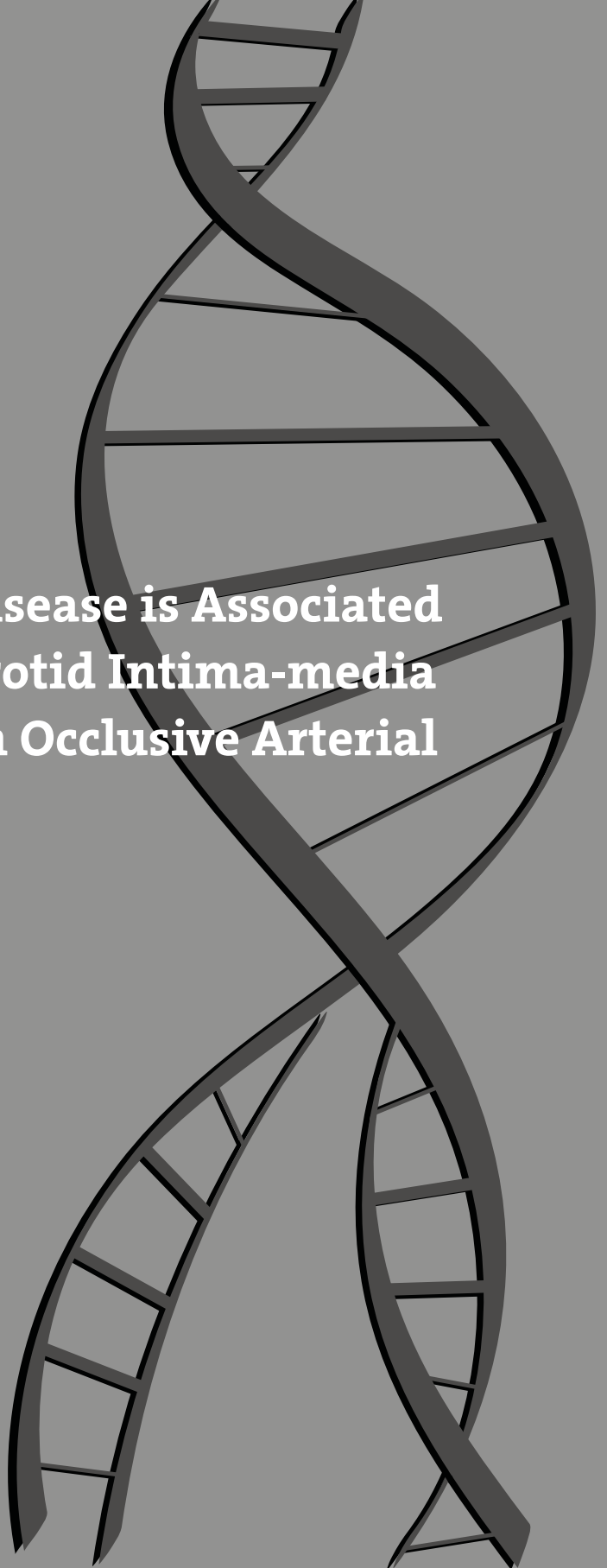

ABSTRACT

Objective: Patients with aneurysmal and occlusive arterial disease have overlapping cardiovascular risk profiles. The question remains how atherosclerosis is related to the formation of aortic aneurysms. Common carotid artery intima-media thickness (CIMT) is an easily accessible and objective marker of early atherosclerosis. The aim of the current study was to investigate whether there is a difference in atherosclerotic burden as measured by CIMT between patients with aneurysmal and those with occlusive arterial disease.

Methods: From 2004 to 2011, the CIMT was measured using B-mode ultrasonography in patients undergoing vascular surgery for aortic aneurysmal or occlusive arterial disease in the Erasmus University Medical Center. Cardiovascular risk factors, comorbidities, and medication were recorded. Patients treated for combined aneurysmal and occlusive arterial disease and patients diagnosed with a genetic aneurysm syndrome were excluded. Univariable and multivariable analyses were used to calculate differences in CIMT between aneurysmal and occlusive arterial disease.

Results: In total 904 patients were included in the study; 502 patients with aneurysmal disease (85% male, mean age 72 years) and 402 patients with occlusive arterial disease (65% male, mean age 64 years). The mean CIMT in patients with aneurysmal disease was 0.97±0.29 mm and 1.07±0.38 mm in patients with occlusive arterial disease (P<.001). Adjustment for cardiovascular risk factors, comorbidities, and medication showed a mean difference in CIMT of 0.15 mm (95%CI: 0.10:0.20, P<.001).

Conclusions: The present study shows a lower CIMT in patients with aneurysmal disease than those with occlusive arterial disease, indicating a lower atherosclerotic burden in patients with aneurysmal disease. These findings endorse the idea that additional pathogenic mechanisms are involved in aortic aneurysm formation. Further studies are needed to clarify the role of atherosclerosis in aortic aneurysm formation.

INTRODUCTION

It is well known that aortic aneurysm disease is correlated with systemic atherosclerotic disease. Patients with aneurysmal and those with occlusive arterial diseases, including ischemic heart disease, carotid artery stenosis, and peripheral arterial disease, have overlapping cardiovascular risk profiles. Furthermore, patients with an abdominal aortic aneurysm (AAA) have a high degree of coronary artery disease as well as atherosclerotic changes in the aortic wall.[1, 2]

However, a causal relation between atherosclerosis and aneurysmal disease has not been established. Most patients with atherosclerotic disease do not develop an aneurysm, and aneurysmal disease is not always associated with peripheral arterial, carotid or coronary disease.[1] Secondly, although conventional cardiovascular risk factors, like age, male gender, diabetes mellitus, hypertension, smoking, and dyslipidemia are often observed in patients with aneurysmal disease, a number of studies report conflicting results on the relationship between these risk factors and AAA.[3-5] Next, atherosclerosis affects predominantly the intimal and medial layers of the vascular wall, whereas in aneurysmal disease pathologic changes also involve the adventitial layer. High proteolytic activity, destruction of collagen and elastin, and massive infiltration of inflammatory cells are characteristics of an aneurysmal arterial wall.[6] Given the epidemiologic, biochemical, and structural differences between aneurysmal and occlusive arterial disease, the conventional view of aneurysm formation as a consequence of atherosclerosis is currently challenged.[7]

The aim of the current study was to investigate whether there is a difference in atherosclerotic burden between patients with aneurysmal and those with occlusive arterial disease. The atherosclerotic burden was determined by measuring the common carotid artery intima-media thickness (CIMT) using B-mode ultrasonography. Thickening of the intimal and medial layers of the common carotid artery is an early expression of generalized atherosclerosis.[8, 9] In addition, CIMT serves as a risk predictor for cardiovascular and cerebrovascular events.[10, 11]

METHODS

Study population

The study population consisted of patients undergoing elective open or endovascular surgery for aortic aneurysmal disease or for occlusive arterial disease, between 2004 and 2011 in the Erasmus University Medical Center in Rotterdam, the Netherlands. The aortic aneurysmal disease population was defined as patients having an aortic and/or thoracic aortic aneurysm with a diameter >55 or >60 mm, respectively. The occlusive arterial disease population was defined as patients with symptomatic peripheral arterial

disease (PAD), which included patients with intermittent claudication or critical limb ischemia in combination with a resting and/or post-exercise ankle-brachial index (ABI) <0.9 and/or imaging findings compatible with the clinical symptoms. Patients treated for combined aneurysmal and occlusive arterial disease and patients diagnosed with a genetic aneurysm (e.g. Marfan, Loeys-Dietz, or vascular Ehlers-Danlos syndrome) were excluded. The study complied with the declaration of Helsinki and was approved by the Institutional Review Board.

Patient characteristics

Patients were prospectively enrolled and the following characteristics were recorded for all patients as part of routine clinical practise prior to surgery, including the cardiovascular risk factors: age, gender, body mass index (BMI), hypertension (blood pressure ≥140/90 mmHg in non-diabetics, ≥130/80 mmHg in diabetics, or use of antihypertensive medication), hypercholesterolemia (low-density lipoprotein [LDL] cholesterol ≥3.5 mmol/L, or use of lipid lowering medication), diabetes mellitus (fasting plasma glucose ≥7.0 mmol/L, non-fasting glucose ≥11.1 mmol/L, or use of anti-diabetic medication), smoking status, and kidney disease (serum creatinine ≥2.0 mg/dL). Furthermore, cardiovascular comorbidities were recorded, including congestive heart failure, ischemic heart disease (a history of angina pectoris, myocardial infarction, coronary revascularisation, or pathologic Q-waves on the electrocardiogram), and cerebrovascular disease (a history of ischemic/hemorrhagic stroke or transient ischemic attack). Furthermore, prescription medications were recorded, including statins, beta-blockers, renin-angiotensin system (RAAS) inhibitors, diuretics, and antiplatelet drugs.

Atherosclerotic marker

The severity of atherosclerotic disease was assessed by measurements of the CIMT prior to surgery using B-mode ultrasonography according to the guidelines from the 'Mannheim Carotid Intima-Media Thickness Consensus'.[12, 13] Patients were examined in the supine position with the head turned 45° away from the side being scanned and the neck extended slightly. A longitudinal view of the right and left common carotid artery was obtained by a portable Sonosite Titan Ultrasound System (Sonosite Inc., Bothell, WA, USA) with a L38-10-5 MHz linear ultrasound transducer or a portable Vivid-I Ultrasound System (Vivid-I, GE Healthcare, Solingen, Germany) with an 8L-RS transducer. Several measurements were made along a minimum of 10 mm at the posterior wall of the right and left common carotid artery. The intima-media thickness was calculated online by built-in software of the ultrasound system from the interface between lumen and intima to the interface between media and adventitia. The maximum CIMT value of both common carotid arteries was used for the analysis. Atherosclerotic plaques, defined as

focal structures of at least 0.5 mm encroaching into the arterial lumen, were excluded from analysis.[13] The sonographers who performed the measurements were blinded for the clinical characteristics of the patients and had an interobserver correlation of 96.2%.[12]

Statistical analysis

Dichotomous data are presented as numbers and percentages. Continuous variables are presented as mean ± standard deviation or median with interquartile range [IQR] when not normally distributed. Categorical data were analysed with chi-square test, and continuous variables with ANOVA or Kruskal-Wallis test, as appropriate. Linear univariable and multivariable regression analyses were performed to evaluate the difference in CIMT between patients with aneurysmal and those with occlusive arterial disease. Multivariable analyses were adjusted for age, gender, BMI, congestive heart failure, ischemic heart disease, cerebrovascular disease, kidney disease, diabetes mellitus, hypercholesterolemia, hypertension, current smoking, and the use of statins, beta-blockers, rennin-angiotensin system (RAAS) inhibitors, diuretics, and antiplatelet drugs. Additionally, we imputed interaction between significant differences in medication use (statins, beta-blockers and antiplatelets) and CIMT was tested and, because of nonsignificance, not included in the final model. Covariates were chosen on the basis of biological plausibility. Multivariable binary logistic regression analysis was used to calculate associations between aneurysmal and occlusive arterial disease. Covariates in the model were the same cardiovascular risk factors, comorbidities, and medication as used in the multivariable linear analyses.

For all tests, a P-value <.05 (two-sided) was considered significant. All analyses were performed using IBM SPSS Statistics version 20.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

A total of 904 patients were included in the study; 502 patients with aneurysmal disease and 402 patients with occlusive arterial disease. The characteristics of the patient populations are presented in Table 1.

Atherosclerotic marker

The mean CIMT in the population was 1.02±0.33 mm (Table 1). Patients with aneurysmal disease had a mean CIMT of 0.97±0.29 mm (median 0.96 mm [0.8-1.2]), whereas patients with occlusive arterial disease had a mean CIMT of 1.07±0.38 mm (median 1.0 mm [0.8-1.3]). Univariate regression analysis showed a mean difference in CIMT of 0.09 mm (95%CI: 0.05:0.14, P<.001, Table 2). After adjustment for potentially confounding factors, this difference increased to 0.15 mm (95%CI: 0.10:0.20, P<.001).

Table 1 – Baseline characteristics of patients with aneurysmal and occlusive arterial disease

	Total population	Aneurysmal disease	Occlusive disease	P-value
	n=904	n=502	n=402	
Baseline characteristics				
Male gender (%)	685 (75.8)	425 (84.7)	260 (64.7)	<.001
Age (years, mean ± SD)	68.4 ± 10.1	71.6 ± 7.8	64.4 ± 11.2	<.001
Body mass index (kg/m^2, mean ± SD)	26.0 ± 4.2	26.0 ± 3.8	26.0 ± 4.5	.920
Cardiovascular comorbidities (%)				
Congestive heart failure	106 (11.7)	51 (10.1)	55 (13.6)	.428
Ischemic heart disease	380 (42.0)	217 (43.2)	163 (40.5)	.417
Cerebrovascular disease	142 (15.7)	70 (13.9)	72 (17.9)	.103
Cardiovascular risk factors (%)				
Kidney disease	141 (15.5)	75 (14.9)	66 (16.4)	.543
Diabetes mellitus	213 (23.5)	77 (15.3)	136 (33.8)	<.001
Hypertension	610 (67.4)	342 (68.1)	268 (66.6)	.655
Hypercholesterolemia	800 (88.4)	436 (86.8)	364 (98.0)	.084
Smoking – current	393 (43.4)	197 (39.2)	196 (48.7)	.004
Smoking – ever	683 (75.5)	392 (78.0)	329 (81.8)	.163
Medication (%)				
Statins	688 (76.1)	363 (72.3)	325 (80.8)	.004
Beta-blockers	748 (82.7)	428 (85.2)	320 (79.6)	.022
RAAS inhibitors	415 (45.9)	226 (45.0)	189 (47.0)	.577
Diuretics	214 (23.6)	108 (21.5)	106 (26.3)	.092
Antiplatelets	591 (65.3)	297 (59.1)	294 (73.1)	<.001
Atherosclerotic marker				
CIMT (mm, mean ± SD)	1.02 ± 0.33	0.97 ± 0.29	1.07 ± 0.38	<.001

Abbreviations: RAAS inhibitors; renin-angiotensin system inhibitors, CIMT; common carotid intima-media thickness

The presence of cerebrovascular disease correlated with the CIMT in multivariable linear regression analysis (beta 0.03, 95%CI: 0.01:0.13, P=.022). In addition, the cardiovascular risk factors age (beta 0.01, 95%CI: 0.00:0.01, P<.001), male gender (beta 0.09, 95%CI: 0.04:0.14, P=.001), and hypercholesterolemia (beta 0.10, 95%CI: 0.01:0.19, P=.025) correlated also with CIMT as did statin use (beta -0.08, 95%CI: -0.14:-0.01, P=.020).

Table 2 – Differences in CIMT between aneurysmal and occlusive arterial disease

Atherosclerotic marker		Beta	95% CI for Beta	P-value
CIMT	Unadjusted	0.09	0.05 : 0.14	<.001
	Adjusted[a]	0.15	0.10 : 0.20	<.001

Abbreviations: CIMT; common carotid intima-media thickness
[a] Adjusted for: age, gender, BMI, congestive heart failure, ischemic heart disease, cerebrovascular disease, kidney disease, diabetes mellitus, hypertension, current smoking, and the use of statins, beta-blockers, renin-angiotensin system (RAAS) inhibitors, diuretics, and antiplatelet drugs.

Table 3 – Studies presenting mean difference in CIMT between patients with aneurysmal disease and peripheral arterial disease

	Year of publication	Number of patients	Mean CIMT difference
	year	n	mm
Simons et al.	1999	172	0.18[b]
Spring et al.	2006	67	0.03[a]
Cheunk et al.	2007	169	0.16[b]
Van de Luijtgaarden et al.	2012	904	0.15[c]

[a] Univariable difference.
[b] Age and gender adjusted difference.
[c] Multivariable adjusted difference.

Associations with aneurysmal and occlusive disease

Multivariable binary logistic regression analysis showed that CIMT (per 1 mm) was associated with occlusive arterial disease (odds ratio 4.5, 95CI%: 2.6-7.7, P<.001, Figure 1). Furthermore, diabetes mellitus (odds ratio 3.0, 95%CI: 2.1-4.5, P<.001) was also associated with occlusive arterial disease, whereas increased age (odds ratio 0.91, 95%CI: 0.89-0.92, P<.001) and male gender (odds ratio 0.25, 95%CI: 0.17-0.37, P<.001) were associated with aneurysmal disease.

DISCUSSION

The results in the current study show that patients with aneurysmal disease have a lower CIMT compared to patients with occlusive arterial disease. This indicates that the atherosclerotic burden is less in patients with aneurysmal disease than in patients with occlusive arterial disease, despite overlapping cardiovascular risk profiles.

The CIMT measurement was developed for easily accessible, non-invasive visualization of early stages of atherosclerosis. Atherosclerotic disease starts with asymptomatic lesions in the arterial wall, including the carotid arteries.[14] Under the influence of cardiovascular risk factors, early atherosclerotic changes progress into severe atherosclerotic changes

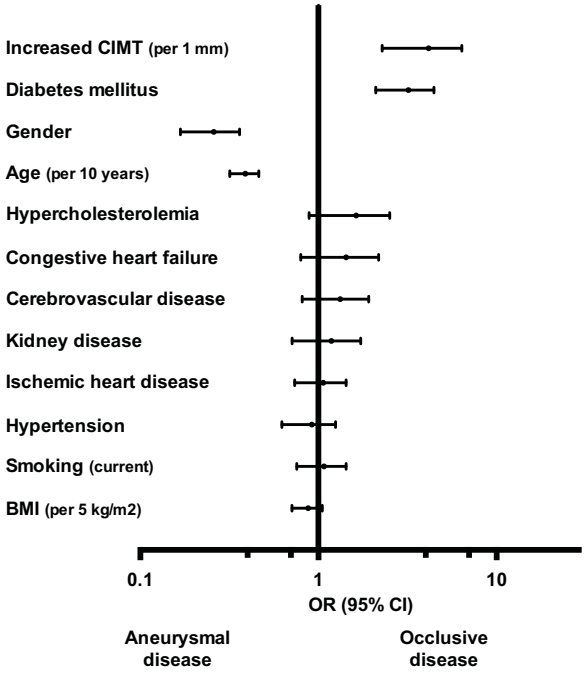

Figure 1 - Multivariable logistic analysis for aneurysmal versus occlusive arterial disease

Adjusted for: age, gender, BMI, congestive heart failure, ischemic heart disease, cerebrovascular disease, kidney disease, diabetes mellitus, hypertension, current smoking, and the use of statins, beta-blockers, renin-angiotensin system (RAAS) inhibitors, diuretics, and antiplatelet drugs.

in the arterial wall. The CIMT measures the thickness of the intimal and medial layers of the carotid artery using ultrasonograpy, which has been shown to correlate well with histological findings of atherosclerosis.[15] The CIMT is considered as a marker for generalized atherosclerosis, which, among others, is based on a study showing the correlation between atherosclerotic lesions in the coronary arteries and increased IMT in the common carotid artery.[13, 16] Increased CIMT is associated with cardiovascular risk factors, including hypertension, smoking, diabetes mellitus, and dyslipidemia.[17] In addition, several studies report the correlation between high CIMT and ischemic events such as coronary events,[18] cerebrovascular events,[19] a combination of coronary and cerebrovascular events,[20] and postoperative cardiovascular events.[12] Aminbaksh et al. concluded that a rise in CIMT of 0.03 mm per year was associated with an overall increased risk of ischemic events.[20] Moreover, Lorenz et al. reported in a meta-analysis that an absolute CIMT difference of 0.1 mm, the future risk for myocardial infarction increases with 10% to 15 %, and the risk for stroke increases with 13% to 18%.[21] Treatment of risk factors, notably hyperlipidemia,

may decrease CIMT and reduce the risk of cardiovascular events.[22, 23] Although most studies assume linearity between CIMT and risk for events, Lorenz et al. found that the relationship was non-linear.[21] Nevertheless, they concluded that linear models fitted well for moderate to high CIMT values.[21] In addition, since there is a lack of uniformity in its definition and the methodology used, no CIMT threshold value for high CIMT has been established.[12, 24] Taken together, these data show a strong association between increased CIMT and the presence and severity of atherosclerotic disease and indicate that measurement of the CIMT is a valuable clinical tool for assessment of subclinical atherosclerosis and prediction of the risk for ischemic events.[24, 25]

The mean CIMT in patients with aneurysmal and occlusive arterial disease was 0.97 and 1.07 mm, respectively, which resembles an absolute difference of about 10%. In the Rotterdam study, which investigated determinants of atherosclerotic disease in the general population, the mean CIMT in healthy people with a mean age of 71±9 years was 0.79 mm.[11] Another study involving a healthy population in the United Kingdom reported an upper limit of 0.81 mm for the CIMT in participants 60 years or older.[26] In contrast to the established relationship between CIMT and atherosclerosis, the results of previous studies on CIMT in patients with aneurysmal disease are conflicting. One study showed a similar increase in CIMT in patients with symptomatic peripheral arterial disease and in patients with AAA as compared to healthy controls (Table 3).[27] Two other studies reported that patients with AAA had a 0.16-0.18 mm lower mean adjusted CIMT than PAD patients.[28, 29] The present study, which is the largest to date, clearly demonstrates a lower CITM in patients with aneurysmal disease than those with occlusive arterial disease, even when corrected for all known factors that might affect the CIMT, including cardiovascular risk factors, comorbidities, and medication. Hence, the results of the study indicate that patients with aneurysmal disease have a lower atherosclerotic burden compared to patients with occlusive arterial disease. Nevertheless, CIMT values in the aneurysm patients were higher when compared to previously reported CIMT values in age-matched general populations, indication that patients with aneurysmal disease were not free of atherosclerosis.

In addition to differences in CIMT, the current study also found differences in several cardiovascular risk factors and comorbidities between aneurysmal and occlusive arterial disease patients in multivariable analysis. In line with previous reports, age and male gender were associated with aneurysmal disease.[30, 31] It is known that with advancing age the incidence of aortic aneurysms increases, and that men are at higher risk of AAA as compared to women. Although the true cause for this matter is still unclear, it seems that genetic susceptibility and risk factor exposure contribute to this phenomenon.[32] Furthermore, also in line with previous reports was the lower prevalence of diabetes mellitus as compared to patients with occlusive arterial disease.[33] Although diabetes is a risk factor for atherosclerosis, it seems protective for aortic aneurysm formation.[34] Exact mechanisms for this effect are still unclear, but therapeutic agents for diabetes might stabilize the

arterial wall, in order to prevent dilatation.[35] No differences in hypercholesterolemia, hypertension, and smoking were observed between the two patient populations.

Remarkably, the number of patients using statins and antiplatelets was significantly lower in patients with aneurysmal disease as compared to patients with occlusive arterial disease. However, given the high cardiovascular risk profile in aortic aneurysm patients, the treatment of these patients should include antiplatelet and statin therapy as part of their cardiovascular risk management.[30]

It is still unclear why some people develop aneurysmal disease whereas others develop occlusive arterial disease, despite similarities in cardiovascular risk profiles. Since not all patients with atherosclerosis develop aortic aneurysms, there is an ongoing discussion whether aneurysmal disease is pathogenetically linked to atherosclerosis or whether the two arterial diseases should be considered as separate entities.[36, 37] Even if classical risk factors for atherosclerosis may influence the evolution and development of aneurysms, the observed difference in CIMT suggests that additional factors are involved in the pathogenesis of aneurysmal disease. About 15% of the AAA patients have a positive family history,[38] which indicates that genetic predisposition plays an important role in aneurysmal disease. However, no major genetic causes for abdominal aortic aneurysms have been identified so far.[39]

Our study has some limitations. Firstly, as stated before, an important difficulty with the CIMT measurement is the lack of uniformity in its definition and the methodology used, which limits quantitative comparisons of absolute CIMT values between studies.[13] In particular, since AAA patients are not free of atherosclerosis, it would be interesting to compare the CIMTs between patients with aneurysmal disease and a propensity score-matched control group. Secondly, patients with PAD might have had higher rates of carotid artery stenosis and, hence, elevated CIMT. However, high CIMT and carotid artery stenosis are not similar in terms of localization, natural history, risk factors, and predictive value for vascular events. Furthermore, in the current study the CIMT was measured in the common carotid artery, whereas carotid artery stenosis is usually located in the internal carotid artery. In addition, the rates of symptomatic cerebrovascular disease (stroke, TIA) were similar in patients with aneurysmal disease and those with PAD, while patients who underwent surgery for carotid artery stenosis were excluded from the study. Thirdly, no systematic screening was performed for the genetic defects causing Marfan, Loeys-Dietz, or vascular Ehlers-Danlos syndrome, patients with specific genetic aneurysm syndromes might have been included in the study. However, the contribution of these syndromes to the study population is probably very small because these syndromes are rare causes for aortic aneurysms. In addition, the mean age of patients with aneurysmal disease is much higher compared to patients with occlusive arterial disease patients and patients with the above-mentioned genetic aneurysm syndromes are generally diagnosed at a younger age.[40]

CONCLUSIONS

The present study shows a lower CIMT in patients with aneurysmal disease than occlusive arterial disease, indicating a lower atherosclerotic burden in patients with aneurysmal disease. Further research is needed to gain insight into the complex transcriptional mechanisms underlying aneurysm development, in particular the diverging processes that lead to dilating or occlusive arterial disease in the presence of common cardiovascular risk factors.

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2

Vitamin D Deficiency May Be an Independent Risk Factor for Arterial Disease

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ABSTRACT

Objectives: The aim of this study was to assess the vitamin D status in patients with occlusive or aneurysmal arterial disease in relation to clinical cardiovascular risk profiles and markers of atherosclerotic disease.

Methods: We included 490 patients with symptomatic peripheral arterial disease (PAD, n=254) or aortic aneurysm (n=236). Cardiovascular risk factors and comorbidities, carotid intima-media thickness (CIMT), ankle-brachial index (ABI), serum high-sensitive C-reactive protein (hs-CRP), and vitamin D were assessed. Patients were categorized into severely (≤25nmol/L) or moderately (26-50nmol/L) vitamin D deficient, vitamin D insufficient (51-75nmol/L), or vitamin D sufficient (>75nmol/L).

Results: Overall, 45% of patients suffered from moderate or severe vitamin D deficiency. The prevalence of vitamin D deficiency was similar in patients with PAD and those with an aortic aneurysm. Low levels of vitamin D were associated with congestive heart failure and cerebrovascular disease. Adjusting for clinical cardiovascular risk factors, multivariable regression analyses showed that low vitamin D status was associated with higher CIMT (P=0.001), lower ABI (P<0.001) and higher hs-CRP (P=0.022).

Conclusions: The current study shows a strong association between low vitamin D status and arterial disease, independent of traditional cardiovascular risk factors and irrespective of the type of vascular disease, i.e. occlusive or aneurysmal disease.

INTRODUCTION

Several large epidemiological studies have concluded that vitamin D deficiency is associated with excess mortality.[1, 2] It is becoming increasingly clear that vitamin D has a much broader range of actions in the human body in addition to its well known effects on calcium homeostasis and bone metabolism. There is accumulating evidence that vitamin D deficiency has important extraskeletal effects, including the cardiovascular system.[3, 4] Several clinical studies have reported a high prevalence of vitamin D deficiency in patients with peripheral arterial disease,[5] coronary artery disease,[6] and stroke,[7] as well as the association of vitamin D deficiency with cardiovascular mortality.[2, 8, 9] Furthermore, low vitamin D status is related to major cardiovascular risk factors, such as hypertension, obesity, and diabetes mellitus.[4, 10, 11]

The aforementioned studies suggest that vitamin D deficiency promotes atherosclerosis.[4, 12] However, it is not known whether this is a direct effect of vitamin D on the arterial wall, and/or the result of a vitamin D deficiency-associated increase in established cardiovascular risk factors. It is also unclear whether the severity of arterial disease is related to the severity of vitamin D deficiency. Furthermore, it is not known whether patients with aneurysmal arterial disease also display vitamin D deficiency.

To answer these questions, we assessed the vitamin D status in a large population of patients with occlusive or aneurysmal arterial disease, and related this to clinical cardiovascular risk profiles as well as to markers for the severity of arterial disease.

MATERIALS AND METHODS

Study population

The study population consisted of patients with peripheral arterial disease (PAD) or aortic aneurysmal disease treated between 2004 and 2011 in the Erasmus University Medical Center in Rotterdam, the Netherlands. Patients with PAD were defined as having symptomatic atherosclerotic lower extremity arterial disease with an ankle-brachial index (ABI) of ≤0.9. Patients with aortic aneurysms were defined as having an aortic diameter >30 mm. Common carotid artery intima-media thickness (CIMT), ankle-brachial index, and high-sensitive C-reactive protein (hs-CRP) were routinely measured in all vascular surgery patients. Patients with routinely measured serum vitamin D levels at the vascular outpatient clinic were included, whereas patients using vitamin D supplementation were excluded from this study. The study complies with the Declaration of Helsinki and was approved by the Institutional Review Board.

Baseline characteristics

A detailed history was obtained from every patient, including traditional risk factors; age, sex, hypertension (defined as a blood pressure ≥140/90 mmHg in non-diabetics, ≥130/80 mmHg in diabetics or use of antihypertensive medication), hypercholesterolemia (defined as a low-density lipoprotein [LDL] cholesterol ≥3.5 mmol/L or use of lipid lowering medication), chronic obstructive pulmonary disease (COPD; defined as a history of COPD or stage ≥1 according to the GOLD classification), diabetes mellitus (defined as a fasting plasma glucose ≥7.0 mmol/L, non-fasting glucose ≥11.1 mmol/L or use of anti-diabetic medication) and smoking status. Furthermore, the atherosclerotic and cardiac risk factors as embedded in the Revised Cardiac Risk (RCR) index were obtained.[13] The RCR index includes congestive heart failure (defined as a history of congestive heart failure), ischaemic heart disease (defined as a history of myocardial infarction, coronary revascularisation or the presence pathologic of Q-waves on the electrocardiogram), cerebrovascular disease (defined as a history of ischaemic/haemorrhagic stroke or transient ischaemic attack), kidney disease (defined as a serum creatinine ≥2.0 mg/dL) and insulin dependent diabetes mellitus. The use of prescription medications was recorded and included statins, beta-blockers, renin-angiotensin system (RAAS) inhibitors and diuretics.

Atherosclerotic markers

The severity of atherosclerotic disease was assessed by measurements of the CIMT, ABI and hs-CRP. The CIMT was measured using the guidelines from the 'Mannheim Carotid Intima-Media Thickness Consensus'.[14,15] Several measurements from the left and the right common carotid artery were made. The highest CIMT value was used for analysis, while measurements of plaques (defined as a focal structure encroaching into the arterial lumen of at least 0.5mm)[14] were excluded from analysis. The ABI was measured at rest using a portable counter-top Doppler 8-MHz vascular probe (Imexdop CT+ Vascular Doppler; Nicolet Vascular, Madison, WI, USA). The ABI was calculated by dividing the higher of the right and left systolic ankle pressures (posterior tibial or dorsal pedal artery) by the highest systolic brachial blood pressure according to the TASC guidelines.[16] Serum hs-CRP was measured using immunochemistry (Beckman Coulter, Woerden, the Netherlands).

Vitamin D measurements

Serum vitamin D was measured in fresh blood samples using a 25-hydroxyvitamin D radioimmunoassay (Diasorin Inc, Stillwater, MN, USA). Within-run coefficient of variation (CV) was 8.6-12.5% and total imprecision CV was 8.2–11.0%. Patients were categorized into 4 groups based on commonly used cut-off values:[17-19] severely (≤25 nmol/L) or moderately (26-50 nmol/L) vitamin D deficient, vitamin D insufficient (51-75 nmol/L), or vitamin D sufficient (>75 nmol/L). To convert nanomolar to nanogram per millilitre one should divide by 2.496.

STATISTICAL ANALYSIS

Dichotomous data are described as counts and percentages. Continuous variables are described as mean±standard deviation (SD), or median and interquartile ranges [IQR] in case of non-Gaussian distribution. Categorical data were compared using chi-square tests. Continuous variables were compared using ANOVA, or using Kruskal-Wallis tests as appropriate. Linear univariable and multivariable regression analyses were performed in separate models using CIMT, ABI, or the natural logarithm of hs-CRP as dependent variable. Vitamin D concentration per 10 nmol/L was used as independent variable and adjustments were made for age, gender, congestive heart failure, ischemic heart disease, cerebrovascular disease, renal function by estimated glomerular filtration rate (eGFR), diabetes mellitus, chronic obstructive pulmonary disease, hypertension, smoking, and type of arterial disease. To address the seasonal fluctuation of vitamin D levels, further adjustments were made for calendar season of vitamin D measurement.

For all tests, a P-value <0.05 (two-sided) was considered significant. All analyses were performed using PASW version 17.0 statistical software (SPSS Inc., Chicago, IL, USA).

RESULTS

A total of 490 patients were included in the study. 254 patients (51.8%) were diagnosed with peripheral arterial disease (PAD) of the lower extremities and 236 patients (48.2%) were diagnosed with a thoracic and/or abdominal aortic aneurysm. The mean age of the population was 67±11 years and the average value of vitamin D concentration was 57±93 nmol/L, as presented in Table 1. A total of 62 patients (12.7%) were severely vitamin D deficient, 160 patients (32.7%) were moderately deficient, 138 patients (28.2%) were vitamin D insufficient, and 130 patients (26.5%) had sufficient vitamin D levels. There were no differences between patients with PAD and those with an aortic aneurysm with regard to the frequencies of vitamin D deficiency (P=.258, Figure 1), or the mean vitamin D concentration (57±31 and 59.2±27 nmol/L, P=.390). Mean ABI in the patients with aneurysmal disease was 0.88 and 47% of these patients had an ABI ≤0.9. No significant differences in vitamin D concentration were found between AAA patients with normal ABI or low ABI (mean 63 nmol/L vs. 55 nmol/L, P=.066). Also, although seasonal variation in vitamin D deficiency was observed in the overall population, no differences between patients with PAD and aneurysms were observed, as presented in Figure 2.

Cardiovascular comorbidities

Patient groups with decreasing vitamin D levels had an increasing prevalence of congestive heart failure (P=.001), cerebrovascular disease (P=.009) and were more frequent current smokers (P=.014), as presented in Table 1. Overall high risk cardiovascular profiles were significantly associated with lower vitamin D levels, as illustrated by a stepwise increase in RCR scores for groups with increasing vitamin D deficiency (P=.004).

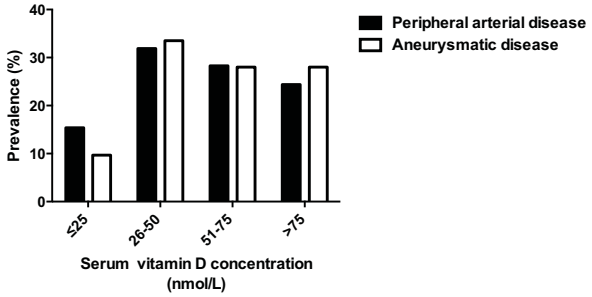

Figure 1 – Prevalence of vitamin D deficiency according to type of arterial disease

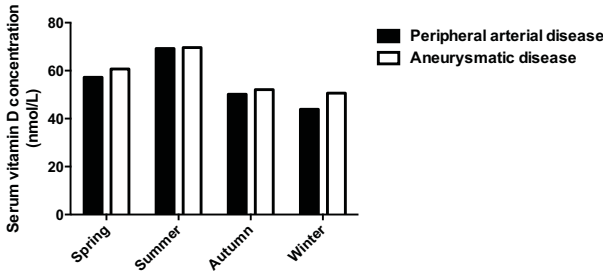

Figure 2 – Seasonal variation in vitamin D deficiency according to type of arterial disease

Atherosclerotic markers

The mean (±SD) CIMT in all patients was 0.97±0.31 mm and a stepwise decrease was observed from 1.06±0.37 mm in patients with severe vitamin D deficiency to 0.90±0.27 in patients with sufficient vitamin D levels (P=.007), as presented in Table 2. The mean ABI was 0.70±0.26 and increased stepwise in each group from 0.56±0.28 in patients with severe vitamin D deficiency to 0.77±0.24 in patients with sufficient vitamin D levels (P<0.001). Furthermore, median hs-CRP in all groups was 4.3 mg/L [IQR: 2.2-7.8 mg/L]. High concentrations of hs-CRP were especially observed in patients with severe vitamin D deficiency with a median of 7.5 mg/L [2.5-12.7 mg/L] (P=.040).

Multivariable linear regression analyses were performed to determine the association between vitamin D concentration and CIMT, ABI and hs-CRP independently of clinical risk factors. Significant associations for vitamin D concentration per 10 nmol/L were observed for CIMT (beta -0.017 mm, 95%CI: -0.027:-0.007, P=.001), ABI (beta 0.015, 95%CI: 0.007:0.022, P<0.001) and hs-CRP (beta -0.047 mg/L, 95%CI: -0.086:-0.007, P=.022) (Table 3).

DISCUSSION

The current study shows a strong association between low vitamin D status and the severity of arterial disease, independent of traditional cardiovascular risk factors and irrespective of the type of vascular disease, i.e. occlusive or aneurysmal disease.

Vitamin D_3 is synthesized in the skin from cholesterol under the action of ultraviolet B light.[3] Furthermore, vitamin D can be ingested as cholecalciferol (vitamin D_3) or ergocalciferol (vitamin D_2). Vitamin D is subsequently converted to 25-hydroxyvitamin D (calcidiol) in the liver or stored in adipose tissue. In the kidneys, 25-hydroxyvitamin D is converted to 1,25-dihydroxyvitamin D (calcitriol), which is the biologically active form of vitamin D.[3] The blood concentration of 25-hydroxyvitamin D reflects the dietary intake of vitamin D_2 or D_3 and the amount of vitamin D_3 produced in the skin, and is considered as the best indicator of vitamin D storage.[17] Since there is still some debate on the best classification of vitamin D status,[17-20] we used a currently proposed vitamin D classification including clinical relevant cut-off values to describe vitamin D status in our patient cohort.

The observed prevalence of vitamin D deficiency (i.e. ≤50 nmol/L) of 45% in patients with arterial disease is comparable to previous reports on vitamin D levels in patients with peripheral arterial disease.[21-24] Since vitamin D deficiency has been identified as an independent risk factor for mortality,[1,2] the question arises if and how vitamin D deficiency is related to the occurrence of cardiovascular events. In line with previous reports,[7,25] we found that vitamin D deficiency is associated with the occurrence of congestive heart failure and cerebrovascular disease in univariable analyses. In addition, as compared to patients with sufficient vitamin D levels, patients with severe vitamin D deficiency had a significantly higher RCR index, a well known predictor of postoperative cardiovascular events in patients undergoing non-cardiac surgery.[13]

Next, we attempted to identify how vitamin D status is related to the severity of arterial disease. We observed a strong association between vitamin D and the atherosclerotic markers of CIMT and ABI. The CIMT and ABI provide information about the progression of atherosclerosis. In previous reports, Flu et al. showed the prognostic value of CIMT and ABI, independent of the RCR index.[26,27] Targher et al. observed a similar association between vitamin D deficiency and CIMT in patients with diabetes mellitus,[28] and Reis et al. reported a significant association between vitamin D deficiency and the internal, rather than the common, carotid intima-media thickness.[29] To our knowledge, only two other studies reported ABI measurements in patients with vitamin D deficiency.[5,30] Although both studies reported mild associations, our study clearly shows the stepwise decrease in ABI per vitamin D deficiency category, and a significant correlation in multivariable linear regression models. Additionally, whereas other studies reported varying results regarding CRP and vitamin D deficiency,[30-32] the current study shows that serum hs-CRP levels are elevated in patients with severe vitamin D deficiency.

Table 1 – Baseline characteristics according to vitamin D status

	Total population	Vitamin D status				P-value for trend
		Severely deficient	Moderately deficient	Insufficient	Sufficient	
		≤25 nmol/L	26-50 nmol/L	51-75 nmol/L	>75 nmol/L	
	n=490	n=62	n=160	n=138	n=130	
Vitamin D level (nmol/L), mean(±SD)	57±93	17±6	39±7	62±7	96±19	-
Baseline characteristics						
Male gender (%)	355(72.4)	42(67.7)	114(71.3)	111(80.4)	88(67.7)	.083
Age (years±SD)	66.8±10.7	64.±11.6	66.9±11.2	67.8±9.6	66.7±10.6	.212
Body mass index (kg/m2), mean(±SD)	26.4±4.4	26.1±5.3	26.4±4.6	26.8±4.2	26.0±4.0	.495
eGFR (ml/min/1,73m2), mean(±SD)	78.32±26.29	86.07±30.75	75.18±28.74	78.46±23.48	78.35±22.97	.053
Type of arterial disease						
Peripheral arterial disease (%)	254(51.8)	39(62.9)	81(50.6)	72(52.1)	62(47.6)	.258
Thoracic and/or abdominal aortic aneurysm (%)	236(48.2)	23(37.1)	79(49.4)	66(47.8)	68(52.3)	
Cardiovascular diseases						
Congestive heart failure (%)	40(8.1)	12(19.3)	16(10.0)	6(4.3)	6(4.6)	.001
Ischemic heart disease (%)	185(37.7)	27(43.5)	69(43.1)	50(36.2)	39(30.0)	.112
Cerebrovascular disease (%)	85(17.3)	13(20.9)	35(21.8)	27(19.5)	10(7.6)	.009
Cardiovascular risk factors						
Kidney disease (≥2.0mg/dl)	46(9.1)	4(6.4)	22(13.7)	11(7.9)	9(6.9)	.103
Diabetes mellitus (%)	100(20.4)	20(32.2)	32(20.0)	25(18.1)	23(17.6)	.103
Hypertension (%)	329(67.1)	41(66.1)	105(65.6)	103(74.6)	80(61.5)	.152
Hypercholesterolemia (%)	455(92.8)	58(93.5)	152(95.0)	126(91.3)	119(91.5)	.573
Smoking – current (%)	209(42.6)	36(58.0)	71(44.3)	62(44.9)	40(30.7)	.014
Smoking – ever (%)	379(77.3)	54(87.0)	120(75.0)	110(79.7)	95(73.0)	.129
COPD (%)	171(34.8)	22(35.4)	59(36.8)	43(31.1)	47(36.1)	.691

Table 1 – Continued

	Total population	Vitamin D status				P-value for trend
		Severely deficient	Moderately deficient	Insufficient	Sufficient	
		≤25 nmol/L	26-50 nmol/L	51-75 nmol/L	>75 nmol/L	
	n=490	n=62	n=160	n=138	n=130	
RCR index						
RCR score, mean(±SD)	1.16±1.01	1.45±1.14	1.27±1.10	1.11±0.84	0.93±0.97	.004
0-1 risk factors (%)	333(67.9)	36(58.0)	100(62.5)	98(71.1)	99(76.1)	
2 risk factors (%)	105(21.4)	14(22.5)	38(23.7)	33(23.9)	20(15.3)	.001
≥3 risk factors (%)	52(10.6)	12(19.3)	22(13.7)	7(5.0)	11(8.4)	
Medication						
Statins (%)	411(83.8)	54(87.0)	139(81.2)	112(81.1)	106(81.5)	.548
Beta-blockers (%)	383(78.1)	50(80.6)	124(77.5)	110(79.7)	99(76.1)	.903
RAAS inhibitors (%)	235(47.9)	32(51.6)	78(48.7)	71(51.4)	54(41.5)	.384
Diuretics (%)	122(24.8)	14(22.5)	44(27.5)	37(26.8)	27(20.7)	.536
Antiplatelets (%)	327(66.7)	49(79.0)	99(61.8)	92(66.6)	87(66.9)	.124

abbreviations: eGFR; estimated glomerular filtration rate, COPD; chronic obstructive pulmonary disease, RCR index; Revised Cardiac Risk index, RAAS inhibitors; renin-angiotensin system inhibitors

Table 2 – Atherosclerotic markers according to vitamin D status

Atherosclerotic markers	Total population	Vitamin D status				P-value for trend
		Severely deficient	Moderately deficient	Insufficient	Sufficient	
		≤25 nmol/L	26-50 nmol/L	51-75 nmol/L	>75 nmol/L	
CIMT (mm) (mean±SD)	0.97±0.31	1.06±0.37	1.01±0.34	0.94±0.27	0.90±0.27	.007
ABI (mean±SD)	0.70±0.26	0.56±0.28	0.68±0.25	0.72±0.26	0.77±0.24	<.001
hs-CRP (mg/L) [IQR]	4.3[2.2-7.8]	7.5[2.5-12.7]	4.0[2.3-7.9]	3.8[1.9-6.8]	4.8[2.2-7.8]	.040

abbreviations: CIMT; common carotid intima-media thickness, ABI; ankle-brachial index, hs-CRP; high-sensitive C-reactive protein

Table 3 – Multivariable linear regression models for associations between vitamin D and atherosclerotic markers

Atherosclerotic markers	n		Beta for vitamin D#	95% CI for Beta	P-value
CIMT	439	Unadjusted	-0.019	-0.029 : -0.009	<.001
		Adjusted*	-0.017	-0.027 : -0.007	.001
ABI	365	Unadjusted	0.017	0.008 : 0.026	<.001
		Adjusted*	0.017	0.008 : 0.026	<.001
hs-CRP	391	Unadjusted	-0.044	-0.082 : -0.005	.027
		Adjusted*	-0.047	-0.086 : -0.007	.022

abbreviations: CIMT; common carotid intima-media thickness, ABI; ankle-brachial index, hs-CRP; high-sensitive C-reactive protein

*adjusted for: age, gender, congestive heart failure, ischaemic heart disease, cerebrovascular disease, renal function using eGFR, diabetes mellitus, chronic obstructive pulmonary disease, hypertension, smoking and calendar season of 25-hydroxyvitamin D measurement.

#Vitamin D per 10 nmol/L.

In contrast to previous studies, we found that vitamin D deficiency was not related to hypertension, obesity, diabetes, and dyslipidemia. Furthermore, the correlation between low vitamin D status and markers of atherosclerotic severity was independent of cardiovascular risk factors.

Interestingly, the association between low vitamin D status and the severity of arterial disease was independent of type of vascular disease. To our knowledge, this relationship between vitamin D status and aneurysm formation has thus far not been reported in humans. Although aortic aneurysms have traditionally been attributed to atherosclerosis, there is increasing epidemiological, biochemical and genetic evidence that aneurysmal arterial disease is different from occlusive atherosclerotic disease, a common denominator being aging of the arterial wall.

Taken together, the data in the current study suggest that the relationship between low vitamin D status and arterial disease is mediated by an independent effect of vitamin D deficiency on the arterial wall. Vitamin D receptors are not exclusively detected in the bone and mineral pathway, but have a wide tissue distribution, including vascular smooth muscle cells and vascular endothelial cells.[17] The diverse physiologic actions of vitamin D on the vascular wall include reduction of smooth muscle cell proliferation,[33] reduction of macrophage secretion of pro-inflammatory cytokines IL-6 and TNF-α, and increased secretion of the anti-inflammatory cytokine IL-10, and therefore reducing the state of vascular inflammation.[34-36] In an atherosclerotic mouse model it has been demonstrated that oral vitamin D_3 reduces the formation of atherosclerotic plaques by the suppression of proatherogenic T lymphocytes.[37] In addition, low circulating levels of vitamin D have been associated with endothelial dysfunction in humans.[38, 39] Furthermore, it has previously been reported that people with vitamin D deficiency have increased vascular calcification, a sign of advanced atherosclerosis,[40, 41] as well as increased aortic stiffness.[42] These vitamin D related effects all promote arterial disease.[4, 12] Experimental studies provide increasing evidence that factors regulating mineral ion homeostasis, such as vitamin D, affect the aging process, including vascular aging.[43]

There are several limitations that need to be considered. Due to the nature of this study it remains uncertain whether the association between low vitamin D status and arterial disease is causal, or whether it is just a bystander. Furthermore, several potentially confounding factors could have influenced our analyses, the most important ones being race, diet, and sunlight exposure. Since our study population consisted mostly of Caucasians, race was not a factor in our analyses. Moreover, as lower vitamin D levels are observed in non-Caucasian populations, the true prevalence of vitamin D deficiency in PAD patients may actually have been underestimated. The influence of low dietary intake, thereby not only reducing vitamin D but also other nutrients, was not taken into account in this study. However, low vitamin D in the European population is mainly caused by low sunlight exposure rather than diet.[17, 44] Therefore, in the multivariable models we corrected for the season of vitamin D measurement to minimize confounding by seasonal variations in sunlight exposure.

CONCLUSIONS

This study demonstrates that low vitamin D status is a risk factor for the severity of arterial disease, independent of traditional cardiovascular risk factors and irrespective of the type of vascular disease, i.e. occlusive or aneurysmal disease. It might be hypothesized that primary and secondary preventive strategies to reduce vascular disease should focus on vitamin D status, in addition to blood pressure reduction, lipid and glucose control, weight loss, and lifestyle changes. A beneficial effect of vitamin D supplementation on blood

pressure reduction has been demonstrated in several clinical trials.[45,46] Although improving vitamin D status might be a promising public health strategy to reduce cardiovascular disease and improve survival,[47,48] there is still much debate about the requirement levels of vitamin D in relation to extra-skeletal outcomes.[20] Further large-scale, randomized clinical trials are needed to test the effects of vitamin D on cardiovascular disease and to further elucidate the biology of vitamin D on the arterial wall.

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3

RNA Expression Profiling of Abdominal Aortic Aneurysmal Disease Identifies Signaling Cross-talk Between TGF-beta and BMP

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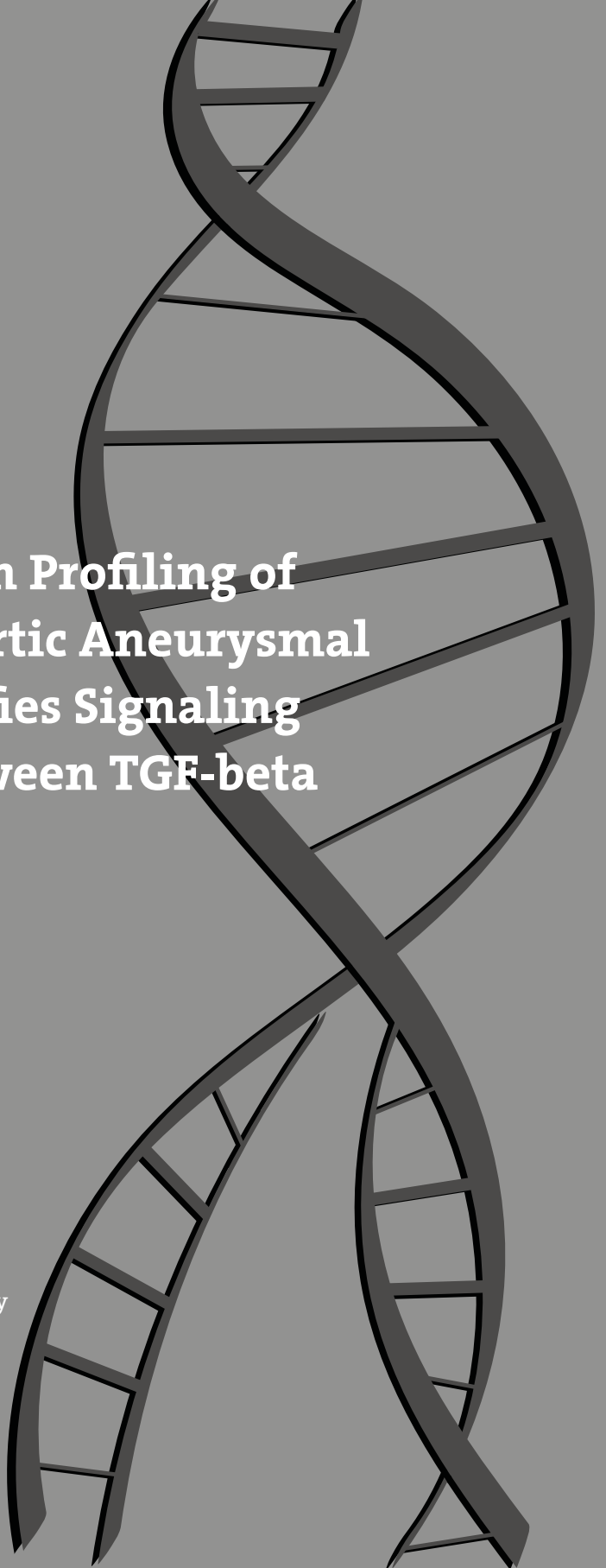

ABSTRACT

An abdominal aortic aneurysm (AAA) is a widening of the aorta below the renal arteries, usually asymptomatic until rupture causes fatal bleeding, and is a major vascular health problem. Abdominal aortic aneurysms are associated with high age, male gender and cardiovascular risk factors such as hypertension and smoking, but the underlying genetic changes remain to be elucidated. In order to develop proper treatment strategies, it is crucial to understand the mechanisms and targets that play a role in AAA. Strikingly, AAA has many if not all risk factors in common with aortic occlusive disease (AOD), yet the outcome is quite opposite; dilation versus occlusion of the aorta. We therefore compared RNA expression profiles of abdominal aortic samples of AAA patients using 'best match control' material of patients with AOD to identify molecular mechanisms that underlie AAA disease. In addition, to identify novel biological mechanisms, pathways and key regulators, we designed an analysis pipeline to select genes based on their level of expression, their potential as blood marker and their possible relevance for aneurysmal disease, resulting in a list of potential targets and markers for further study. The list of significantly changed genes included *COL11A1* (32-fold increase, p=0.00012), *ADIPOQ,* and *LPL* (21-fold increase, p=0.0003) that have previously been associated with AAA, validating our approach. The list also included genes such as *CXCL13, SLC7A5* and *FDC-SP*, for which the connection with aneurysmal disease is novel. IPA analysis revealed an overrepresentation of significantly altered immune related pathways next to pathways previously associated with aneurysmal disease. Our gene expression profiling approach not only identified genes and pathways previously associated with AAA genes, but also revealed that simultaneous inhibition of bone morphogenetic protein and activation of TGF-beta signaling controls aneurysm growth in the abdominal aorta.

INTRODUCTION

Aneurysms are life-threatening arterial diseases identified by the dilatation of a blood vessel with a more than 50% increase of the diameter compared to normal. Aortic aneurysms can arise at different locations, and most common are thoracic and abdominal aortic aneurysms (TAA and AAA). Both these types are associated with high age, male gender and atherosclerosis, as well as with environmental and familial components. Around 5% of TAAs are present in a syndromic form, with early onset, and several responsible genes have been identified so far.[1-3]

Genes that have directly been linked to syndromic forms of TAA encode for transforming growth factor β (TGFβ) components, cytoskeleton proteins, or extracellular matrix (ECM) proteins. Well-known examples are Marfan syndrome (MFS) with a mutation in the extracellular matrix protein Fibrillin-1, and Loeys-Dietz syndrome with mutations in genes including the TGFβ-receptors 1 and 2, and *SMAD3*.[4-6] Histological staining of aortic aneurysm sections of these patients usually show abnormalities in the extracellular matrix, loss of smooth muscle cells and disorganization of elastin and collagen structure.[7] Furthermore, TGFβ-signaling is increased in TAAs of patients and mice, and high serum TGFβ levels correlated directly with aortic root dilation.[8-12] It is unclear if genetic factors affected in TAA also play a role in AAA, though a recent study identified overlapping genetic defects between AAA and familial TAA.[13] The prevalence of AAA is ~8% among men older than 65 years of age and is much higher than for TAA.[14] Yet, in contrast to TAA, for AAA causative genes are hard to identify.

Interestingly, aneurysm formation and arterial occlusive disease (AOD) share a number of important risk factors, such as smoking, hypertension, and older age. In men over 65 years of age, 48% have atherosclerosis in the aorta, of which 9-16% will also develop an aortic aneurysm.[15-17] Based on this common clinical risk profile, aneurysm formation was formerly ascribed to atherosclerosis. Of the many types of cardiovascular diseases, atherosclerosis is most common and contributes to major morbidity and mortality in developed countries. Contributing factors such as dyslipidemia, diabetes and hypertension will result in the manifestation of plaque development, vascular smooth muscle cell (VSMC) proliferation, and extracellular matrix modulation, eventually resulting into obstruction of the blood vessel as seen in AOD. Abdominal aneurysms, with similar risk factors and alike pathologic processes as AOD, shows another form of extracellular matrix modulation and a different role of VSMCs. The process of matrix modulation results in enzymatic degradation of the elastin laminae causing disruption of arterial wall integrity, therefore weakening and dilatation of the aortic wall, resulting in an aneurysm.[2] Pathologically, aneurysm formation is characterized by destruction of elastin and collagen in the media and adventitia of the arterial wall, loss of medial smooth muscle cells, and transmural infiltration of lymphocytes and macrophages. However,

despite many similarities in risk factors for AAA and AOD, the two diseases have opposite outcomes, i.e. dilatation versus stenosis/occlusion.

Targeted ultrasound screening of high risk cases may allow reduction of AAA related mortality and early diagnosis therein is crucial. Thus, a clinical risk prediction model will aid in earlier, and more efficient, identification of persons at risk. Identifying the underlying processes and genes that differentiate these two diseases will be a first step towards such a risk prediction model. In this study we therefore investigated the transcriptional profiles and molecular processes that differentiate AAA from AOD.

MATERIALS AND METHODS

Tissue analysis

Patient cohort Micro-array

Aortic tissue was derived from patients undergoing elective open surgical reconstruction of the infrarenal abdominal aorta for either abdominal aortic aneurysm (AAA) or aortoiliac occlusive disease (AOD) in the Erasmus University Medical Center between 2008 and 2012. The study complies with the Helsinki declaration on research ethics. Aortic biopsies were obtained by protocol approved by the institutional Medical Ethics Committee (MEC-2012-387, MEC 2013-265, MEC-2014-057).

Aortic biopsies

Full thickness aortic tissue samples for RNA expression profiling in AAA patients were collected from the infrarenal anterior aneurysm wall in AAA patients. Full thickness aortic tissue samples in AOD patients were obtained from the infrarenal anterior aortic wall at the site of the proximal anastomosis of the prosthetic graft. Tissue samples were snap frozen in liquid nitrogen directly after harvesting and stored at -80°C until RNA isolation.

RNA isolation and Microarray hybridization

Total RNA including miRNAs were isolated using the miRNeasy Mini Kit (Qiagen, Hilden, Germany). Tissues were disrupted with a 5mm steal bead by a disruption program of 2 times 20Hz in the TissueLyser II (Qiagen, Hilden, Germany). RNA quality was checked with the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Samples with a high quality RNA Integrity number (RIN) and with a 28S/18S ratio of >0.9 were used for hybridization. Microarray hybridization and scanning were performed at SkylineDiagnostics (Skyline Diagnostics, Rotterdam, The Netherlands). In short, 625 ng RNA was processed to generate cRNA. Fragmented and biotinylated cRNA was subsequently hybridized on Affymetrix U133 plus 2.0 microarrays (Affymetrix Inc, Santa Clara, CA, USA) and these microarrays were scanned with an Affymetrix Genechip System 3000Dx v.2 microarray scanner (Affymetrix Inc, Santa Clara, CA, USA).

RNA expression analysis

The CEL files generated by the Affymetrix Genechip System 3000Dx v.2 microarray scanner were subsequently imported into Partek Genomics Suite version 6.4 (Partek Inc, St Louis, MO, USA). Quantile normalization and background correction was applied to the raw intensity values of all samples via GC Robust Multichip Analysis. As the data was processed in 3 hybridization batches, hybridization batch effect correction was applied. To visualize the correlation between the samples, principal component analysis and unsupervised hierarchical clustering were used. For the comparison of AAA with AOD samples, 2-sample T-test statistics were applied to calculate the fold changes with associated p-values.

Microarray data processing

During data processing within Partek Genomics Suite 6.4, all microarray CEL files were assessed for passing of quality control (QC) thresholds. We started the analysis with 14 AAA samples and 7 AOD samples. One AAA samples failed QC due to bad hybridization and this sample was removed from the analysis. During unsupervised clustering, all AAA samples grouped together and all AOD samples also grouped together. One AAA sample grouped together with the AOD samples. Since the two groups clustered into 2 clear groups we suspected a potential sample misidentification and therefore this AAA sample was removed from the analysis.

IPA analysis

A set of differential expressed genes was uploaded into Ingenuity/Qiagen IPA (Qiagen, Redwood City, CA, USA) and a core analysis was performed on 1047 significant expressed genes (-2<=FC=>2 and p-value=<0.05), as part of the core analysis we looked at functions, pathways and upstream regulator. First the log2Ratio (fold change) and p-value data generated from the 2-sample test analysis in Partek Genomics Suite 6.4, was uploaded into IPA. For the Ingenuity Pathway Analysis (IPA), significance thresholds of log2Ratio=1 (this equals -2<=FC=>2) and p-value=<0.05 were applied for the comparison of the AAA vs AOD groups. During upload of the data into IPA, the probe level data was mapped to the gene level and averaged based on the median Fold Change values. For the upstream analysis the z-score significance thresholds were set to -1.8=<z-score>=1.8 and p-value =<0.01.

Selection of genes

A list of 50 genes that could serve as potential markers for AAA was generated by applying the following potential prioritization protocol, which was designed to identify best possible markers. The two parts of the IPA core analysis that contributed to this

prioritization schedule were the list of significantly upregulated genes scored by highest fold change and p-value together with the list of significant Upstream Regulators (Figure 2). These are genes that are not necessarily themselves differentially regulated, but are identified based on the prediction to regulate a significant (or substantial) set of genes present within the gene expression dataset being analyzed. The final selection list consists of 30 genes; 15 based on selection of the most significantly upregulated genes (left selection procedure in Figure 2) and 15 genes based on the most significant Upstream regulators (right selection procedure in Figure 2).

Steps to prioritize the 15 most significantly upregulated genes were as follows (1) The normalized raw expression values were divided into 3 categories: Low (<80), Medium (80-800) and High (>800). Only genes with High or Medium expression levels were considered as we reasoned it will be technically difficult to detect a gene with low expression values. (2) Only genes that showed an increase in expression levels in the AAA samples relative to the AOD samples were selected since detection of increased expression (presence) is more robust than detection of decreased expression (absence). (3) Genes that at the protein level are expressed on the cell membrane or that are secreted extracellularly were selected as we reasoned that this would improve the ability to detect a potential marker in blood. (4) All genes were marked that were part of our in-house developed Vascular Gene Set. The Vascular Gene Set (4209 genes) is a list of genes with relevance to vascular tissue development, maintenance and disease, including aortic aneurysms, that are selected based on HGMD and OMIM information, GO terms, KEGG pathways, Ingenuity IPA pathways, GWAS studies and literature (Supplemental Table I). (5) As a last step we also marked the genes that were identified as significant upstream regulators. Here we reasoned that prioritization via two independent analyses gives increased overall confidence in the proper selection.

Table 1 – A t-test (continuous data) or Fisher's exact test (categorical data) was applied for the analysis between groups. All statistical analyses were performed using Graphpad Software (Graphpad Software inc, La Jolla, CA, USA). All statistical tests were two-sided and P<0.05 was considered statistically significant.

Characteristic	AAA (n=12)	AOD (n=7)	P-value
Male gender – n (%)	11 (92)	2 (29)	.0095
Age – (y, mean ± SD)	68 ± 6.7	56 ± 5.7	.001
Diabetes mellitus – n (%)	0 (0)	1 (14)	.3684
Ischemic heart disease – n (%)	2 (17)	1 (14)	1
Renal insufficiency – n (%)	4 (33)	1 (14)	.6027
Hypertension – n (%)	9 (75)	5 (71)	1
Dyslipidemia – n (%)	9 (75)	6 (86)	1
Current smoking – n (%)	6 (50)	4 (57)	1
Ever smoking – n (%)	4 (33)	3 (43)	1

Steps to prioritize the 15 most significantly upstream regulators: (1) Upstream regulators were prioritized based on the highest Upstream Regulator z-score with a minimal p-value of 0.01. (2) All genes that were part the Vascular Gene Set were marked. (3) Upstream regulators that were identified as being significantly upregulated at the mRNA level in our dataset, above the threshold of log2Ratio=1(fold change=2) were also marked. Here we reasoned that prioritization via two independent analysis gives increased overall confidence in the proper selection.

QPCR analysis

Expression data of *COL11A, Adiponectin, CXCL13, SLC7A5* and *FDC-SP* were analyzed in diseased aortic tissue. Total RNA was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad, Veenendaal, The Netherlands). cDNA samples were subjected to 40 cycles real-time PCR analysis using SYBR Green qPCR Master Mix 2x (Bio-Rad, Veenendaal, Netherlands) and primers; Actin-β 5'-CTCCCTGGAGAAGAGCTACG-3', 5'-GAAGGAAGGCTGGAAGAGTG-3'; Hypoxanthine-guanine phosphoribosyltransferase (HPRT) 5'-TGACACTGGCAAAACAATGCA-3', 5'-GGTCCTTTTCACCAGCAAGCT-3', *COL3A1* 5'-ACAATAGCACAGACGGAGGC-3', 5'-GGATTTGGCTCATTTGTCCCAG-3', *Apiponectin* 5'-GTGATGGCAGAGATGGCACC-3', 5'-ACTCCGGTTTCACCGATGTC-3', *CXCL13* 5'-CGAATTCAAATCTTGCCCCGT-3', 5'-ACTTGTTCTTCTTCCAGACTATGA-3', *SLC7A5* 5'-TCATCATCCGGCCTTCATCG-3', 5'-AGCAGCAGCACGCAGAG-3', and *FDC-SP* 5'-GGCTGTTGGTTTCCCAGTCTC-3', 5'-TGGTGGAAGTGGGCGAAATG-3'. Gene expression was calculated using actin-β and HPRT as housekeeping genes and the comparative Ct method (ΔΔCt) was used for relative quantification of gene expression.

Vascular surgery aortic tissue sample collection

The study population consisted of a cohort of vascular surgery patients consecutively operated at the Erasmus Medical Center in Rotterdam. Patients undergoing elective open or endovascular surgery for aortic aneurysm repair, peripheral arterial disease or carotid artery disease, were included in the study. Patients were classified as aneurysmal disease (AA) or arterial occlusive disease (peripheral arterial disease or carotid artery disease). The study complies with the Helsinki declaration on research ethics and was approved by the institutional Review Board of the Erasmus Medical Center (MEC 2011-510).

Clinical characteristics vascular surgery patient cohort

The clinical characteristics of the index AAA patients were obtained from medical files and included gender, age at diagnosis, age at surgery, body mass index (BMI), as well as the cardiovascular comorbidities and risk factors. Cardiovascular comorbidities included congestive heart failure, ischemic heart disease (history of myocardial infarction, angina pectoris, coronary revascularisation or pathologic Q-waves on the electrocardiogram),

and cerebrovascular disease (history of ischemic/hemorrhagic stroke or transient ischemic attack). Cardiovascular risk factors included kidney disease (serum creatinine ≥2.0 mg/dL), diabetes mellitus (fasting plasma glucose ≥7.0 mmol/L, non-fasting glucose ≥11.1 mmol/L or use of anti-diabetic medication), and hypertension (blood pressure ≥140/90 mmHg in non-diabetics, ≥130/80 mmHg in diabetics or use of antihypertensive medication). Smoking status was obtained and included current smoking and ever smoking (ie, patients who are currently smoking OR patients with a history of smoking). Prescription medications were recorded and included the use of statins, beta-blockers, renin-angiotensin system inhibitors, diuretics, and antiplatelets.

Lipoprotein and inflammatory parameters

Serum levels of triglycerides, high-density lipoprotein, low-density lipoprotein and high-sensitivity C-reactive protein (hs-CRP) were determined as described.[18] Patients with an hs-CRP higher than 10 mmol/L were excluded from analysis due to the chance of active inflammation status.[19]

Statistical analysis

Dichotomous data are presented as numbers and percentages. Continuous variables are presented as mean ± standard deviation or median and interquartile range (IQR) when not normally distributed. Categorical data were analysed with Fisher's exact test or chi-square test and continuous variables with t-test, ANOVA or Kruskal-Wallis test. Linear univariable and multivariable regression analyses were performed to evaluate the difference in lipoprotein and inflammatory markers (triglycerides, high-density lipoprotein, low-density lipoprotein and hs-CRP) between patients with aortic aneurysm and those with arterial occlusive disease. Multivariable analyses were adjusted for age, gender, body mass index, congestive heart failure, ischemic heart disease, cerebrovascular disease, kidney disease, diabetes mellitus, hypertension, current smoking, and the use of statins, beta-blockers, renin-angiotensin system inhibitors, diuretics and antiplatelets. Covariates were chosen on the basis of biological plausibility.

For all tests, a p-value <0.05 (two-sided) was considered significant. Analyses were performed using Graphpad Software (Graphpad Software inc, La Jolla, CA, USA) or SPSS statistics (version 21.0; IBM Inc, Chicago, IL, USA)

RESULTS

AAA and AOD patient and sample characteristics

Based on high quality RNA RIN values, 14 AAA and 7 AOD samples were selected for microarray hybridization. During the quality control of the microarray data, 2 AAA

samples were removed from the analysis: one due to bad microarray hybridization and the other as it was an outlier in the principal component analysis (PCA) and the non-supervised hierarchical clustering. The final selection of patient samples therefore included 12 AAA samples and 7 AOD samples. Patient characteristics are depicted in Table 1 accordingly. The baseline characteristics showed a difference in age and gender, though as expected, no significant differences were found in cardiovascular risk factors such as diabetes mellitus, ischemic heart disease, renal insufficiency, hypertension, dyslipidemia and smoking status.

Gender difference exclusion

Both age and gender are important risk factors for AAA. In our dataset, 11 out of 12 AAA patients were male and 5 out of 7 AOD patients were female. Due to the overlap of gender with disease phenotype, our analysis could also potentially identify differences between males and females. To identify genes that are differentially expressed between male and female aortic samples we obtained microarray expression data from another study investigating AAA.[20] We downloaded the expression data from GEO (GSE 7084) and performed a 2 sample t-test on the male and female sample groups within the control group only. This dataset consisted of an Affymetrix array based analysis and an Illumina array based analysis (for both array based analysis: 2 females vs. 4 males). We identified genes as significantly differentially expressed in the Affymetrix analysis with p-value<0.05 and FC cut off of >3.5 whereas in the Illumina analysis we applied p value<0.05 and FC > 2.5. With these stringent settings we identified 137 gender specific genes. As in the present study, we were specifically interested in the genes that differentiate aneurysmal disease from occlusive disease irrespective of gender, these 137 gender specific genes were removed from our AAA vs AOD analysis. For example, we show in Table 2 a top selection of 50 upregulated genes with 10 gender specific genes marked (see M symbol in column). For our AAA specific gene selection, all marked 'gender-specific' genes were excluded. In addition we performed an IPA core analysis on the dataset with and without the gender specific genes (1077 and 1047 ready molecules, respectively). Both analyses showed very similar results regarding functions, pathways and upstream regulators, suggesting that the differences between AAA and AOD state are the predominant state difference in this dataset (data not shown).

Non supervised hierarchical clustering and Principal Component Analysis

Non-supervised hierarchical clustering was performed on the genome wide microarray gene expression data of the 19 samples (Figure 1A). This analysis showed a clear separation of the AAA and AOD groups, and thus can be considered a validation of clear microarray gene expression differences between the two groups. In addition, Principal Component Analysis (PCA) was performed on the samples and again a clear separation of the AAA and

Table 2 – Top upregulated genes in AAA vs AOD with the gender specific genes marked (M) that were excluded for further analysis.

Gene Symbol	Entrez Gene Name	Fold Change	p-value	Gender specific
RPS4Y1	ribosomal protein S4, Y-linked 1	48,386	0,00238	M
CXCL13	chemokine (C-X-C motif) ligand 13	32,269	0,000112	
DDX3Y	DEAD (Asp-Glu-Ala-Asp) box helicase 3, Y-linked	30,326	0,00208	M
COL11A1	collagen, type XI, alpha 1	27,046	5,29E-05	
SAA2	serum amyloid A2	24,96	3,95E-07	
PLIN1	perilipin 1	23,989	9,49E-05	M
ADIPOQ	adiponectin, C1Q and collagen domain containing	21,454	0,000301	
FDCSP	follicular dendritic cell secreted protein	21,38	0,000101	
PTX3	pentraxin 3, long	19,063	1,10E-05	M
POU2AF1	POU class 2 associating factor 1	18,842	0,000479	
MS4A1	membrane-spanning 4-domains, subfamily A, member 1	18,414	0,000261	
KDM5D	lysine (K)-specific demethylase 5D	17,854	0,00275	M
MZB1	marginal zone B and B1 cell-specific protein	17,072	0,00126	
SLC7A5	solute carrier family 7 (amino acid transporter light chain, L system), member 5	15,716	5,37E-07	
LEP	leptin	14,288	1,94E-06	
MARCO	macrophage receptor with collagenous structure	13,563	0,000412	
LPL	lipoprotein lipase	12,984	3,51E-05	
IL1RN	interleukin 1 receptor antagonist	12,873	0,00116	
IGLL5	immunoglobulin lambda-like polypeptide 1	12,848	0,000973	
CR2	complement component (3d/Epstein Barr virus) receptor 2	12,123	0,00113	
KIAA1199	KIAA1199	12,122	0,0016	
TREM1	triggering receptor expressed on myeloid cells 1	11,851	0,000433	
P2RX5	purinergic receptor P2X, ligand-gated ion channel, 5	11,706	8,53E-05	
EIF1AY	eukaryotic translation initiation factor 1A, Y-linked	11,554	0,00304	M
SPAG4	sperm associated antigen 4	11,463	0,00106	M
HMOX1	heme oxygenase (decycling) 1	10,932	5,47E-05	
IGLJ3	immunoglobulin lambda joining 3	10,776	0,00729	
IGH	immunoglobulin heavy locus	10,257	0,000424	
ISG20	interferon stimulated exonuclease gene 20kDa	10,238	1,31E-05	
CCL18	chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	10,164	0,000151	
CD79A	CD79a molecule, immunoglobulin-associated alpha	10,064	0,000197	
FNDC1	fibronectin type III domain containing 1	10,028	0,00028	
IL8	interleukin 8	9,89	0,00235	M
TIMD4	T-cell immunoglobulin and mucin domain containing 4	9,859	0,00259	
PIM2	pim-2 oncogene	9,838	0,000235	

Table 2 – Continued

Gene Symbol	Entrez Gene Name	Fold Change	p-value	Gender specific
CXCL5	chemokine (C-X-C motif) ligand 5	9,659	0,000276	
FCRL5	Fc receptor-like 5	9,596	0,00249	
CXCL3	chemokine (C-X-C motif) ligand 3	9,526	3,06E-06	
MIAT	myocardial infarction associated transcript (non-protein coding)	9,359	0,00011	
GZMB	granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	9,211	0,000277	
IGHM	immunoglobulin heavy constant mu	8,93	0,0051	
AQP9	aquaporin 9	8,908	0,00445	
COMP	cartilage oligomeric matrix protein	8,739	0,00511	
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	8,677	1,07E-05	M
PAX5	paired box 5	8,41	0,000776	
IGK	immunoglobulin kappa locus	8,403	0,00257	
USP9Y	ubiquitin specific peptidase 9, Y-linked	8,296	0,00493	M
SYTL1	synaptotagmin-like 1	8,235	4,38E-06	
C15orf48	chromosome 15 open reading frame 48	8,225	0,0064	
DPH1	diphthamide biosynthesis 1	8,218	0,00019	

AOD samples was observed (Figure 1B).

Selection procedure of top upregulated genes reveals 'known' and novel 'marker genes' for AAA

Top upregulated genes were selected based on fold change and p-value (Figure 2), and categorized by location and type. In addition, we checked the presence of filtered genes in our Vascular Gene Set, which is an enriched gene set consisting of genes expressed in vascular tissues and/or having a role in vascular related pathways and functions.

The top 10 upregulated genes with highest fold changes are depicted in Table 3. In the columns of this table, fold change, p-value, location, and presence in the Vascular Gene Set are depicted. A literature search of these top 10 upregulated genes was performed where we screened for relevance in AAA or atherosclerotic disease, which is summarized in Table 3. Collagen-alpha1(XI) (*COL11A1*) appears highly relevant for AAA based on its location in the ECM and its previous association with aneurysmal disease.[21] Also Adiponectin (*ADIPOQ*) seems relevant, as *ADIPOQ* is elevated in Kawasaki patients (aneurysms in coronary artery).[22] Both associations show that our filtering is able to identify potential or known AAA-relevant genes. Furthermore, many highly upregulated genes are associated with the immune system, for instance CXC motif chemokine 13 (*CXCL13*),

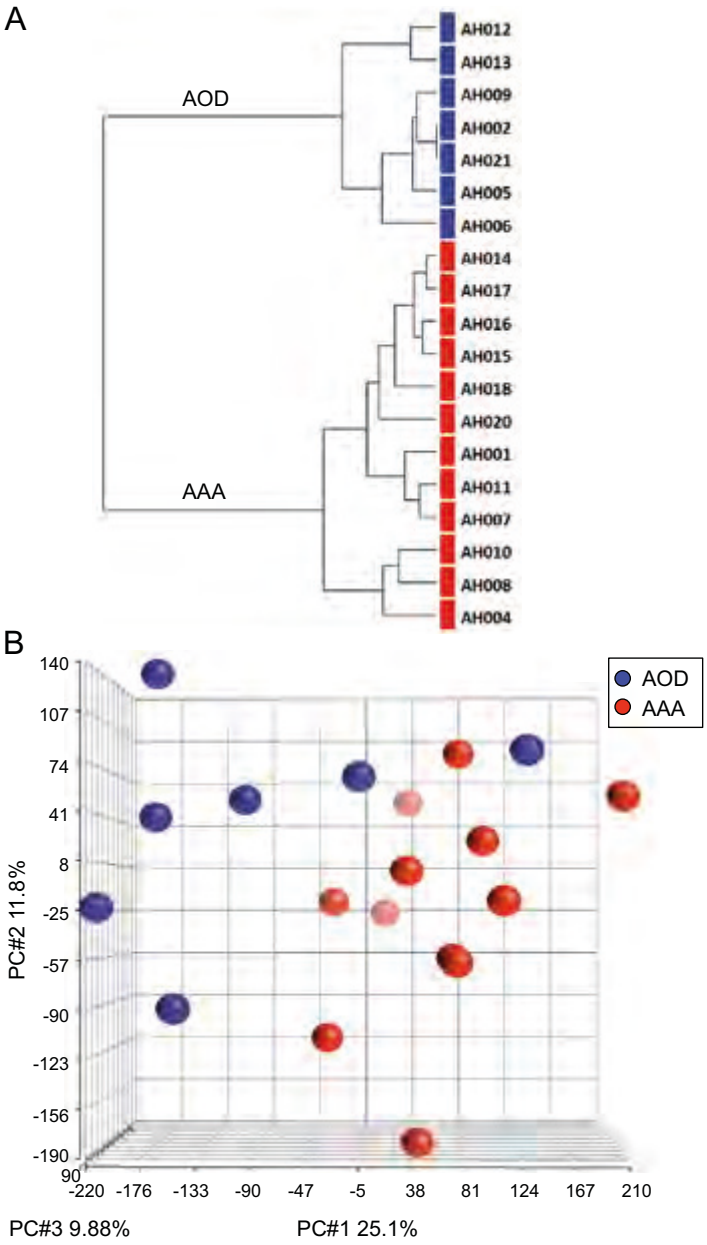

Figure 1 – (A) Non-supervised hierarchical clustering dendrogram of AAA and AOD samples. (B) Principal Component Analysis plot of AAA and AOD samples. In red the AAA patient samples, in blue the AOD patient samples. On the x, y, and z axis: PC#1 25.1%, PC#2 11.8%, PC#3 9.88%, respectively.

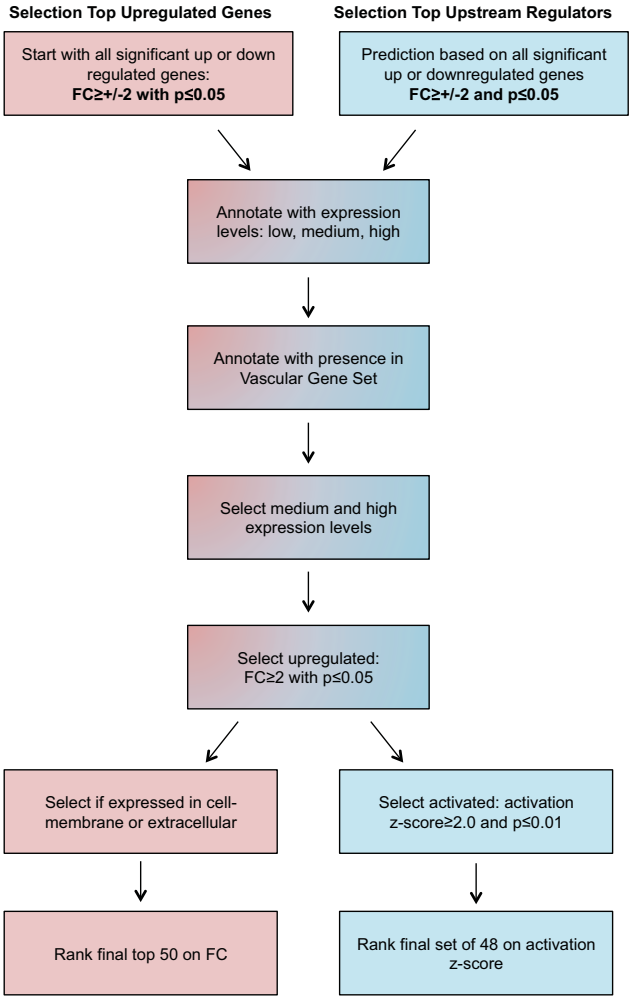

Figure 2 – Selection procedure flowchart of the top upregulated genes (left) and top upstream regulators (right).

Table 3 – Top 10 genes up-regulated genes in AAA vs AOD.

Gene	Function and relation to AAA or atherosclerosis	FC	p-value	Location	Vascular Gene Set
CXCL13 C-X-C motif chemokine 13	Selective chemotactic for B cells (B-1 and B-2 subsets), by interacting with chemokine receptor CXCR5. Control of B cell organization within follicles of lymphoid tissues. High levels of CXCL13 are found in aneurysm and in atherosclerotic lesions [34-36].	32.26	.000112	Extracellular space	YES
COL11A1 Collagen alpha-1(XI) chain	Adds structure and strength to connective tissues supporting muscles, joints, organs, and skin. Col11a1 protein levels are upregulated in TAA and AAA tissue [21, 37, 38]	27.05	5.3E-05	Extracellular space	YES
SAA2 Serum amyloid A protein	Production primarily in liver, circulates in low levels in the blood. Although its function is not fully understood, serum amyloid A appears to play a role in the immune system. Different biomarker studies have shown association of SAA with atherosclerotic disease. Patients with atherosclerotic disease show increased levels of Amyloid A protein. [39-42]	24.96	4.0E-07	Extracellular space	NO
ADIPOQ Adiponectin	Involved in the control of fat metabolism and insulin sensitivity, with direct anti-diabetic, anti-atherogenic and anti-inflammatory activities. Stimulates AMPK phosphorylation and activation in liver and skeletal muscle, enhancing glucose utilization and fatty-acid combustion. Negatively regulates TNF-alpha expression in various tissues such as liver and macrophages. Inhibits endothelial NFκB signaling through a cAMP-dependent pathway. Adiponectin is dysregulated in aneurysm and atherosclerotic disease. [43-45]	21.45	.000301	Extracellular space	YES
FDCSP follicular dendritic cell-secreted protein	FDCSP bind to the surface of B-lymphoma cells. Functions as a secreted mediator acting upon B-cells. No direct associations of FDCSP with atherosclerotic disease or AAA are described in literature. [46, 47]	21.38	.000101	Extracellular space	NO
POU2AF1 POU domain class 2-associating factor 1	Transcriptional coactivator that specifically associates with either OCT1 or OCT2. It boosts the OCT1 mediated promoter activity and to a lesser extent that of OCT2. Essential for the response of B-cells to antigens and required for the formation of germinal centers. Little is known about this factor in AAA, though in carotid plaque formation analysis it is shown that POU2AF1 is upregulated, which is related to the immune and inflammatory processes linked to atherosclerosis. [48, 49]	18.84	.000479	Nucleus	NO

Table 3 – Continued

Gene	Function and relation to AAA or atherosclerosis	FC	p-value	Location	Vascular Gene Set
MS4A1 membrane-spanning 4A / CD20	B-lymphocyte surface molecule which plays a role in the development and differentiation of B-cells into plasma cells. B-lymphocytes with MS4A1 expressed are found in aneurysm and atherosclerotic. [50-53]	18.41	.000261	Plasma membrane	YES
MZB1 Marginal zone B and B1 cell-specific protein	Associates with immunoglobulin M (IgM) heavy and light chains and promotes IgM assembly and secretion. Acts as a hormone-regulated adipokine/ proinflammatory cytokine implicated in causing chronic inflammation, affecting cellular expansion and blunting insulin response in adipocytes. No direct association of MZB1 with atherosclerotic disease or AAA are described in literature. [54]	17.07	.00126	Extracellular space	NO
SLC7A5 Solute carrier family 7 member 5	Encodes for a protein called y+L amino acid transporter 1 (y+LAT-1). Involved in transport of amino acids, namely lysine, arginine, and ornithine. The y+LAT-1 protein forms one part (the light subunit) of a complex called the heterodimeric cationic amino acid transporter, responsible for binding to the amino acids that are transported. There is no direct association of SLC7A5 with atherosclerotic disease or AAA described in the literature. [55]	15.72	5.4E-07	Plasma membrane	YES
LEP Leptin	Hormone involved in the regulation of body weight. As fat accumulates in cells, more leptin is produced, indicating that fat stores are increasing. Increased leptin levels are associated with atherosclerotic disease. Furthermore, in an AAA animal model mRNA and protein levels of leptin were found to be upregulated in aneurysmatic tissue.[56-60]	14.29	1.9E-06	Extracellular space	YES

follicular dendritic cell secreted protein (*FDC-SP*), POU domain class 2-associating factor 1 (*POU2AF1*), membrane-spanning 4A (*MS4A1* or *CD20*), and marginal zone B and B1 cell-specific protein (*MZB1*). Upregulation of these genes indicates that our gene expression profiling approach identifies the prominence of inflammation genes in AAA. In our literature research many of these genes showed no direct link with aneurysmal disease, therefore these genes could be 'novel' for aneurysmal disease.

A subset of 5 potential 'marker genes' were selected from Table 3 to be verified by QPCR, as additional check for the micro-array results. Selection criteria were; increased fold change, extracellular location and an association with aneurysmal disease, resulting in selection of *CXCL13, COL11A1* and *ADIPOQ*. Additionally, *FDC-SP* and Solute carrier family 7 member 5 (*SLC7A5*) were selected as they had not previously been associated

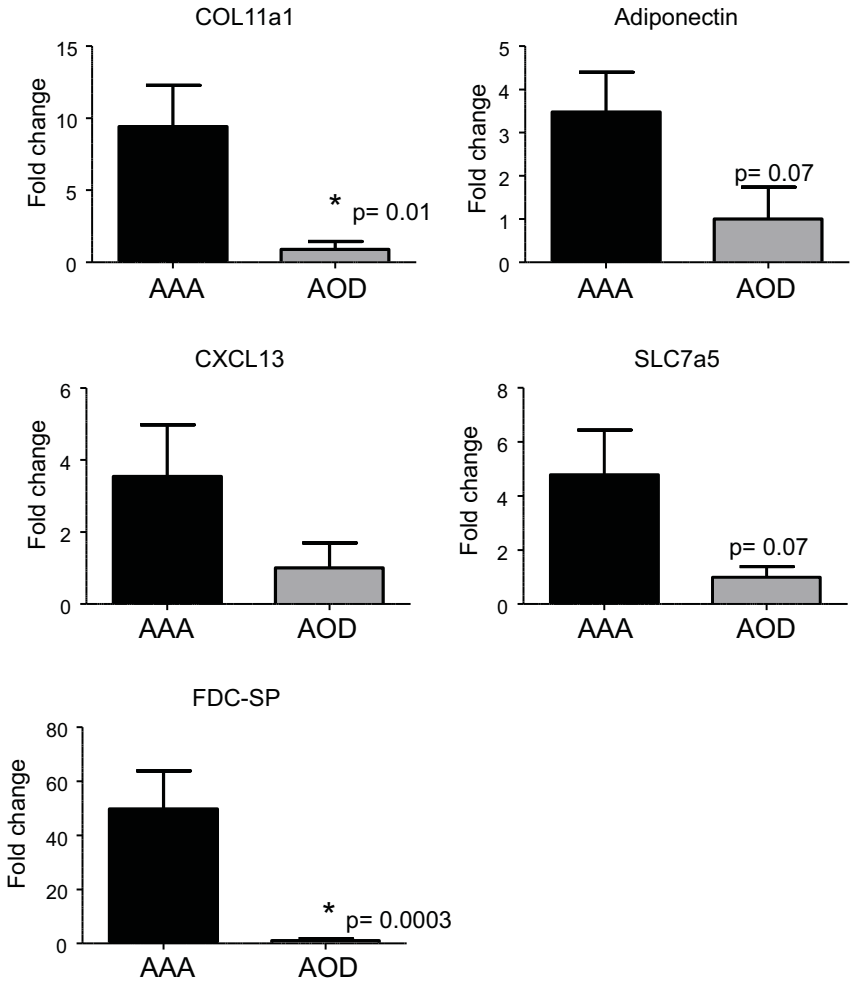

Figure 3 – Significantly regulated genes from the top 10 selection (Table 4), verified by QPCR. Plotted are the fold changes of *COL11A1*, *ADIPOQ*, *CXCL13*, *SLC7A5* and *FDC-SP* gene (AAA vs AOD n=5). *p<0.05 vs AAA.

with aneurysmal disease. QPCR data shows that *COL11A1, ADIPOQ, CXCL13, SLC7A5* and *FDC-SP* are upregulated in AAA compared to AOD (Figure 3), which corresponds to the micro-array data, although only *COL11A1* and *FDC-SP* were significantly upregulated. The other genes were upregulated, but not significantly, probably due to small availability of samples.

Selection of top upstream regulators indicating potential key regulators in AAA

The parallel selection procedure to identify novel genes in AAA was performed by

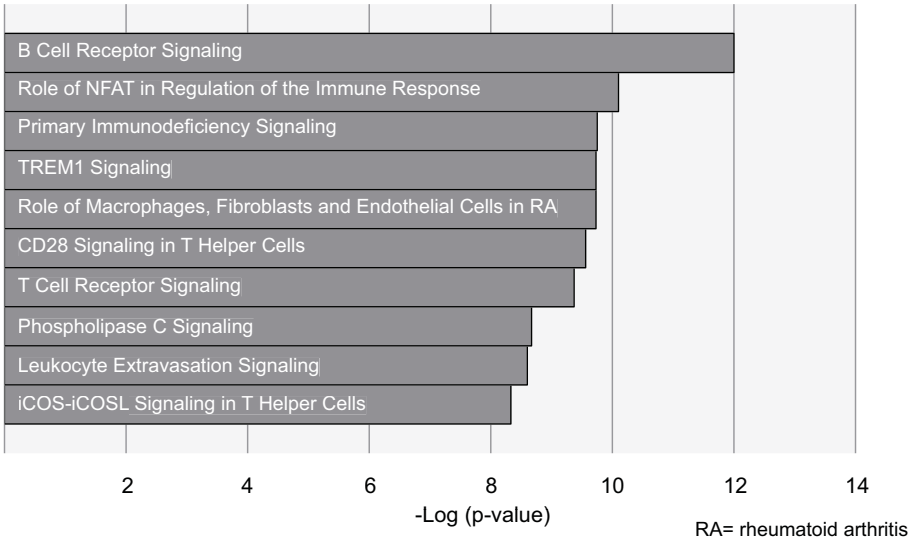

Figure 4 – Top 10 IPA list of upregulated pathways in AAA disease. The –log(p) value depicted on the x-axis represents significance of the depicted pathways.

prioritizing our data with upstream regulator information available within IPA (Figure 2). The upstream regulator analysis is based on the idea that the activation state of a known upstream regulator can be determined by assessing the expression fold changes of all of its downstream targets and then using a z-score based algorithm to test if there is a good correlation between the hypothetical regulatory state of the upstream regulator and the regulatory state of all of its known downstream targets. The data was prioritized by highest upstream regulator z-score, with a minimal p-value of 0.01, and a threshold of log2Ratio=1, resulting in a gene list selected on the basis of upstream regulators. In Table 4 we show 46 genes which are upregulated with a cut-off z-score of >2, together with their respective fold changes in the gene expression dataset. This list of genes could indicate novel markers and key regulators involved in AAA disease.

Pathway selection by Ingenuity Pathway Analysis shows a clear inflammatory component

Functional analysis, pathway analysis and upstream regulator analysis was performed with the data set of 1047 ready genes. In Figure 4 we show a top 10 IPA list of pathways which are significantly altered in AAA disease. Interestingly these top 10 pathways are all of an inflammatory nature, indicating the immune system as an important component in the differences between AAA and AOD phenotype. Therefore, the immune system and its associated markers would be interesting to further investigate in these patient groups. However our analysis was not sufficient to pinpoint one inflammation pathway

Table 4 – List of up-regulated genes prioritized by upstream regulator selection (z-score; p<0.01) in AAA vs AOD.

Upstream Regulator	Activation z-score	p-value of overlap	FC	Molecule Type	Predicted Activation State	Target molecules in dataset
IL1B	7,3	9E-27	6,7	cytokine	Activated	ABCG2,ACTA2,ADAM8,ADM,AIF1,AMPD3,ANGPT1,ANGPTL4,APOB,APOE,ARC,ARG1,BCL2A1,BCL3,BGN,BIRC3,BMP4,CCL3,CCL5,CCR1,CCR5,CCR6,CCR7,CCRL2,CD14,CD4,CD44,CD83,CD86,CEBPB,CEBPD,CFLAR,CHI3L1,COL10A1,CREM,CSF2RB,CSF3,CTSB,CTSS,CTSZ,CX3CL1,CXCL1,CXCL2,CXCL3,CXCL5,CXCR4,CYBA,CYBB,CYSLTR1,CYTIP,DAB2,DDIT4,DUSP5,EDN1,ENPP1,ERBB2,ESR1,F2RL1,FABP5,FAM129A,FCGR2B,FGF2,FOSL1,FST,G0S2,GAD1,GADD45B,GBP1,GCH1,GLA,GM2A,HAS1,HEY2,HGF,HIF1A,HK2,HMGA1,HMOX1,HSD11B1,IBSP,ICAM1,IER3,IGFBP5,IGFBP6,IL10,IL10RA,IL16,IL18,IL18R1,IL18RAP,IL1B,IL1R2,IL1RN,IL33,IL6,IL6R,IL8,IRAK1,IRF1,IRF7,ISG20,ITGAM,LCP1,LEP,LIF,MCL1,MMP1,MMP12,MMP3,MMP9,MYEF2,MYH11,NAMPT,NFIL3,NR4A3,OCLN,OLR1,OSM,PCDH7,PDE4B,PIM1,PLAT,PLAU,PRKCD,PTGS1,PTGS2,PTP4A1,PTX3,RAC2,REL,RUNX2,S100A8,S100A9,SAA2,SCUBE2,SDC1,SERPINB9,SERPINE1,SLAMF1,SLC12A1,SLC14A1,SLC1A3,SLC20A1,SOD2,SPP1,SRGN,STAT4,STMN2,TAC1,TACR1,THBS1,THRSP,THY1,TLR2,TLR3,TLR8,TMEM176B,TNFAIP3,TNFRSF11A,TNFRSF1B,TREM1,TREM2,TYMP,UAP1,UGCG,VDR,VEGFA,XYLT1,ZC3H12A
CEBPA	4,9	1E-20	3,1	transcription regulator	Activated	ACSL1,ADCY7,ADH1B,ADIPOQ,AGT,ALOX5AP,ANPEP,APOB,ARG1,ARL4C,BCL2A1,BTG1,C3AR1,CCR1,CD14,CD19,CD3G,CEBPA,CEBPB,CEBPD,CHI3L1,COL10A1,CSF1R,CSF3,CSF3R,CXCR4,DDX21,DGAT2,EFNB2,EMCN,FABP4,FASN,FCAR,FHL1,G0S2,GABPB1,GAS1,GATA6,GBP1,GCH1,GLRX,HCAR3,HGF,HMOX1,HSD11B1,ICAM1,IER3,IL10,IL1RN,IL6,IL6R,IL8,ITGAL,ITGAM,ITGAX,LCK,LEP,LPL,LST1,LTF,MALT1,MNDA,NFATC2,NFIL3,OLR1,PAX5,PCK1,PFN2,PGD,PLIN2,PPP1R3C,PTAFR,PTGS1,PTGS2,PTPN3,PTPRC,PTPRE,PTX3,RGS2,RUNX2,RUNX3,S100A8,S100A9,SCD,SEMA3E,SERPINE1,SMPDL3A,SOD2,SPP1,TAC1,TBXAS1,THRB,TRIB1,VDR,VLDLR
IL6	4,6	3E-19	4,0	cytokine	Activated	ABCA1,ABCG2,ACP5,AGT,ANPEP,APOB,APOE,ARG1,ARL4C,BATF,BCL2L11,BCL3,BGN,C5AR1,CCL5,CCR1,CCR5,CCR6,CCR7,CD14,CD163,CD209,CD36,CD48,CD53,CD68,CD79A,CD83,CD86,CDKN2B,CEBPA,CEBPB,CEBPD,CFLAR,CLU,CSF2RB,CSF3R,CXCL1,CXCL13,CXCL2,CXCL3,CXCL5,CXCR4,CYBB,CYTIP,EZH2,GADD45B,GLRX,GSTA4,GZMB,HGF,HIF1A,HLA-DQA1,HMOX1,ICAM1,ICAM3,ICOS,IGFBP5,IGFBP6,IGHM,IGJ,IL10,IL1RN,IL6,IL6R,IL7R,IL8,IRF1,IRF4,ITGAM,JAK1,JAK2,KIAA0101,KLRB1,KRT14,LEFTY2,LEP,LIF,LPL,LRG1,LRP6,LTF,LY86,MCL1,MERTK,MMP1,MMP12,MMP3,MMP9,MRVI1,MSR1,NAMPT,NCF2,PIM1,PLAT,PLAU,PRF1,PROK2,PTGS2,PTPRC,PTTG1,RAB27A,RNASE6,RRM2,S100A9,SAA2,SEMA4A,SERPINA1,SERPINE1,SGK1,SLC14A1,SLC7A7,SNX10,SOD2,SPP1,SRA1,STAT4,TAC1,TBXAS1,THBS1,THRSP,TLR1,TLR10,TLR2,TLR3,TLR8,TNFRSF11A,TNFRSF17,TNFRSF1B,TOP2A,VEGFA,VLDLR,XBP1

Table 4 – Continued

Upstream Regulator	Activation z-score	p-value of overlap	FC	Molecule Type	Predicted Activation State	Target molecules in dataset
IL18	4,4	4E-11	2,6	cytokine	Activated	ADIPOQ,CCL3,CCL5,CCR5,CCR7,CD44,CD69,CD83,CD86,CFLAR,CXCL16,CXCL3,GADD45B,GZMB,HAVCR2,ICAM1,IL10,IL12RB1,IL18,IL18R1,IL1B,IL6,IL8,INPP5D,IRF1,ITGAM,KLRC4-KLRK1/KLRK1,MMP1,MMP3,MMP9,PRF1,PTGS2,SELL,SPP1,TACR1,TXK,VEGFA
IRF7	4,3	2E-03	2,6	transcription regulator	Activated	CCL5,CCRL2,CD69,CTLA4,FAM26F,GBP1,GBP5,IRF1,IRF7,IRF8,ISG20,ITGAM,ITGAX,JAK2,MCL1,MX2,NAMPT,PELI1,PLAC8,PMAIP1,S100A8,TLR8,TMBIM6,TNFAIP8,ZBP1,ZC3HAV1
TLR2	4,1	9E-09	4,3	transmembrane receptor	Activated	ARG1,CCL5,CCR1,CCR5,CD69,CD86,CEBPB,CEBPD,CSF3,CXCL2,CXCL3,CYLD,GRIN2A,GZMB,HLA-DQA1,HMOX1,ICAM1,IL10,IL18,IL1B,IL1RN,IL6,IL8,IRAK1,IRF1,ITGA4,LEP,MMP1,MMP9,PTGS2,TREM1,VDR,XBP1
CEBPB	4,0	3E-12	2,4	transcription regulator	Activated	ACTA2,ADIPOQ,AGT,ALDH1A1,ALOX5AP,APOB,ARG1,BCL2A1,BLNK,CCL3,CCL5,CCR5,CD14,CDKN2B,CEBPA,CEBPB,CEBPD,CIRBP,COL10A1,CSF1R,CSF3,CSF3R,CXCL2,CXCL3,CXCL5,DAB2,DGAT2,EFNB2,EMCN,FABP4,FBLN1,FCAR,FHL1,GAS1,HGF,HSD11B1,ICAM1,IER3,IGKC,IL10,IL11RA,IL1B,IL1RN,IL6,IL8,ITGAL,LCP2,LEP,LYN,MBP,MGP,MMP1,MMP3,MSR1,NFATC2,NFKBID,PCK1,PLAUR,PRKCD,PTGS1,PTGS2,RAC2,RUNX2,SAA2,SAT1,SCD,SEMA3E,SERPINA1,SERPINE1,SGK1,SPP1,TAC1,TLR8,TMEM176B,TRIB3,UPP1,VDR,VLDLR,XIST
CCL5	3,9	2E-08	2,4	cytokine	Activated	C5AR1,CCL3,CCL5,CCR1,CCR5,CCRL2,CD163,CD44,CXCL2,CXCL3,EMP1,F2RL1,HMGA1,IL1B,IL6,IL8,MMP19,MMP9,NAMPT,OLR1,PLAUR,PNP,PPIF,SGK1
OSM	3,8	3E-13	6,1	cytokine	Activated	ABCA1,ABCG1,ADAM17,ADH5,AMPD3,AQP9,ARG1,ARHGEF12,ARL4C,BHLHE40,BTC,CALB2,CCL5,CEBPA,CEBPD,CH25H,CHD1,CPM,CSF3,CTSL,CXADR,CXCL1,CXCL13,CXCL2,CXCL3,CXCL5,CYP4F3,DNAJC3,DSC2,ECM2,FGF2,FOXC1,GAB1,GBP1,GLUL,GRIN2A,HGF,HIF1A,HK2,HMOX1,HOXA9,HSD11B1,ICAM1,IGFBP6,IL10,IL18,IL1B,IL1R2,IL33,IL6,IL6R,IL8,IRAK1,IRF1,IRF7,ISG20,ITGAL,JAG1,LIF,LRRFIP1,MAP2,MARCKS,MICA,MLLT11,MMP1,MMP3,MMP9,MYEF2,MYH10,NAMPT,NEDD4,NELL2,NOTCH3,NUAK1,OSM,P2RY10,PDPN,PFKFB3,PLAU,PRDM1,PTP4A1,PTPN21,S100A12,S100A8,S100A9,S100P,SEL1L,SERPINA1,SERPINE1,SLC16A3,SLC16A6,SOST,STK4,STX11,TLR2,TLR3,TMBIM6,TNC,TNFRSF11A,TOP2A,TPM1,TYMP,UAP1,VDR,VEGFA,ZBTB43,ZC3HAV1
XBP1	3,8	5E-04	3,3	transcription regulator	Activated	COL10A1,CXCL2,DERL1,DNAJB9,DNAJC3,EDEM1,ERO1LB,ESR1,ETS1,FASN,FKBP11,FKBP7,HMOX1,HSPA13,ICAM1,IL6,IL8,IRF4,NCF1,PDIA4,POU2AF1,PRDM1,RUNX2,SDF2L1,SEC11C,SEC24D,SEC61A1,SERPINA1,SPCS3,SRPRB,SSR4,STARD5,TXNDC11,TXNDC5

Table 4 – Continued

Upstream Regulator	Activation z-score	p-value of overlap	FC	Molecule Type	Predicted Activation State	Target molecules in dataset
SELPLG	3,7	5E-07	3,5	other	Activated	BCL2A1,CCL3,CXCL2,CXCR4,HCAR3,HCK,IL10,IL1B,IL1R2,IL8,ITGAM,PLAUR,PRKCD,SERPINB9
STAT4	3,6	3E-06	3,1	transcription regulator	Activated	ACADL,ACAP1,ARMCX1,BCL2L11,BCL3,CCR5,CXCL2,CXCL3,ERO1L,FCER1G,FYB,GRTP1,IER3,IL10,IL10RA,IL12RB1,IL18R1,IL18RAP,IL6,IRF1,IRF4,ISG20,ITGA7,KDM6B,LRRFIP1,MAP3K1,MGARP,PCGF5,PDK1,PLAC8,PRDM16,RASL12,RGCC,SAT1,SELENBP1,SELPLG,SERPINE1,SLC2A3,SMPDL3A,STC2,TPD52,VEGFA,VLDLR
CD2	3,6	8E-05	2,9	transmembrane receptor	Activated	CCR7,CD4,CD44,CD48,CD86,CD8A,HLA-DPA1,ICAM1,IL10,ITGAL,PTPRC,SELL,STAT4
CD44	3,6	2E-09	2,5	enzyme	Activated	ADAM8,ARHGEF12,BCAM,BGN,BIRC3,CCL5,CCR5,CCR7,CD36,CD44,CD69,CD8A,CIDEC,CLEC7A,CX3CL1,CXADR,ERBB2,FASN,IL10,IL1B,IL1R2,IL1RN,IL6,ITGA4,ITGAX,LIMS2,LTBP1,MCL1,MMP12,MMP3,MMP9,NPNT,PLAU,SELL,SMAD1,SPP1,THBS1,THY1,TLR8,TNFRSF11A,TPM2,WNT2
TYROBP	3,4	1E-06	2,6	transmembrane receptor	Activated	CCL3,CCR7,CD69,CD83,CD86,FCGR2B,ICAM1,IL6,IL8,ITGAX,NOD2,TYROBP
POU2AF1	3,3	9E-11	18,8	transcription regulator	Activated	CCND3,CCR5,CD79A,CD79B,IDH2,IGH,IGHA1,IGHG1,IGHM,IGK,KCNN4,LCK,MS4A6A,PAX5,PRDM1,RBP1,SDS,SPIB,SPP1
TREM1	3,3	2E-15	11,9	transmembrane receptor	Activated	ABL2,AREG/AREGB,ATP1B1,CCL18,CCL3,CCL5,CCRL2,CD14,CD86,CDKN2B,CEBPB,CKS2,CRTAM,CXCL1,CXCL2,CXCL3,CXCL5,DUSP14,DUSP4,EDN1,FOSL1,GADD45B,GCLM,GLA,HAS1,HS3ST3B1,IL10,IL1B,IL6,IL6R,IL8,IRF1,ITGAX,KANK1,LAMP3,LIF,LPL,LY9,MAFF,MCOLN2,MLF1IP,MMP1,MMP19,NOD2,NRIP3,PIM2,PLAC8,PLCXD1,PTGS2,RGS1,SCG5,SFMBT2,SLAMF7,SLC1A3,SPP1,TARP,THBS1,TLR2,TNFSF15
PLAU	3,3	7E-06	4,2	peptidase	Activated	ABCG1,ARG1,C5AR1,CCL5,CCR5,CXCL3,HGF,ICAM1,IL1B,IL6,MMP1,MMP12,MMP9,PLAU,PLAUR,S100A8,S100A9,SERPINE1
ETS1	3,3	2E-15	2,9	transcription regulator	Activated	ANPEP,ARL4C,ATP2A3,BCL11A,BMP4,CD14,CD27,CD79A,CD79B,CRTAM,CSF1R,ERBB2,ETS1,FCGR2A,FOXD1,GZMB,GZMK,HCST,HGF,HMOX1,HPSE,HSPA6,HSPB8,ICAM1,IL10,IL2RB,INSIG1,ITGB2,ITK,JAK1,KLRC4-KLRK1/KLRK1,LAIR1,LCK,LTB,MCL1,MMP1,MMP3,MMP9,MSR1,NCF1,NFIL3,NPR1,PLAU,PRF1,RUNX2,RUNX3,SELL,SERPINE1,SLAMF6,SPP1,SRGN,TBXAS1,TGFA,THY1,TRPC1,VEGFA,WAS,ZAP70,ZEB1
NFATC2	3,3	2E-06	2,5	transcription regulator	Activated	ABCA1,ACP5,BATF,CD3G,CFLAR,CTLA4,CXCL3,DAB2,E2F5,EDN1,ICOS,IKZF1,IL10,IL18,IRF1,IRF4,IRF7,ISG20,MERTK,NFATC1,PELI1,PLAT,PLK2,PPP3R1,PTGS2,PTPN1,REL,RGS1,RGS2,RILPL1,TLR3,TNFSF8

Table 4 – Continued

Upstream Regulator	Activation z-score	p-value of overlap	FC	Molecule Type	Predicted Activation State	Target molecules in dataset
VEGFA	3,3	5E-06	2,1	growth factor	Activated	ACSL1,ADH5,ANPEP,BCL2A1,BTK,CCRL2,CD34,CTSB,CTSS,CXCR4,DUSP4,DUSP5,EFNB2,ETS1,FABP4,FGF2,GBP1,GRIA2,HMOX1,ICAM1,IGFBP5,IL6,IL8,INPP5D,MCL1,MEOX2,MMP1,MMP12,MMP9,NME1,OCLN,PIM1,PLAT,PLAU,PTGS1,PTGS2,RUNX2,SCO2,SERPINE1,SNCG,SOD2,THBS1,VEGFA
MAP3K1	3,2	6E-04	2,0	kinase	Activated	BIRC3,CSTA,HMOX1,IL8,MMP3,PGR,PLAU,PLAUR,PTGS2,SERPINE1,THBS1,TNC,TOP2A,TPH1
C5AR1	3,1	8E-07	4,8	G-protein coupled receptor	Activated	C5AR1,CD28,CD86,CSF3,CXCL2,FCER1G,FCGR2A,FCGR2B,IL1B,IL6,IL8,SERPINE1
IL6R	3,1	1E-08	2,9	transmembrane receptor	Activated	CCL3,CCL5,CD36,CXCL2,CXCL3,CXCL5,ICAM1,ICAM3,IGFBP5,IL10,IL6,IL8,IRF1,MCL1,MMP3,MMP9,NAMPT,PTGS2,TAC1,TNFRSF11A,VEGFA
IL17RA	3,1	1E-06	2,2	transmembrane receptor	Activated	CCR1,CSF3,CSF3R,CXCL2,CXCL3,CXCR2,IL1B,IL6,IL8,MMP3,S100A8,S100A9
PLAUR	3,0	1E-03	7,1	transmembrane receptor	Activated	C5AR1,CCL5,CTSB,CXCL3,CYBB,ITGAM,MMP3,MMP9,PLAU,PLAUR
CD14	3,0	1E-04	3,0	transmembrane receptor	Activated	BCL2A1,CCL3,CCL5,CXCL2,CXCR2,IL10,IL10RA,IL1B,IL6,IL8,PTGS2,TLR2,TNFAIP3
ICAM1	2,9	9E-06	2,4	transmembrane receptor	Activated	ACTA2,CCL5,CD69,CD86,CXCL2,CXCL3,ICAM1,IL1B,IL6,ITGA4,ITGAL,MMP9,VEGFA
SAMSN1	2,8	4E-05	4,7	other	Activated	BATF,CXCL2,DAB2,EDN1,IL18,IL6,IRF1,IRF7,ISG20,MARCO,MERTK,PELI1,PLAT,PTGS2,RGCC,RILPL1,SDC1,TLR3,TNFSF8,XBP1,ZC3H12A
NAMPT	2,8	2E-06	3,5	cytokine	Activated	CXCL1,CXCL2,IL6,IL8,MMP1,MMP3,MMP9,NAMPT,NELL2,NPY1R,TMSB15A
SPP1	2,7	5E-09	7,1	cytokine	Activated	ABCG2,ACP5,ADIPOQ,ANGPT1,BCL2L11,CCL18,CCL3,CCL5,CD44,CXCL1,CXCL2,CXCL3,CXCL5,HMOX1,ICAM1,IL10,IL1B,IL6,IL8,INSIG1,JAG1,MMP1,MMP9,PLAU,RUNX2,SPP1
PRKCB	2,7	5E-03	5,5	kinase	Activated	CD83,CD86,FASN,ICAM1,IL10,IL6,MMP9,PRKCB,PTGS2,SERPINE1,SOD2
BTK	2,7	1E-04	3,3	kinase	Activated	BCL2A1,BTK,CCND3,CD2,CD86,CXCL3,FABP5,HMOX1,IGLL1/IGLL5,IL10,IL18,IL6,IL8,ITGAX,NFATC1
PTGS2	2,7	2E-08	2,6	enzyme	Activated	ANGPT1,AQP1,AREG/AREGB,BIRC3,CCL5,CCR7,CD44,CD68,CLU,CTSD,CXCL3,CXCL5,CXCR2,CXCR4,EDNRA,ERBB2,EZR,ICAM1,IL10,IL1B,IL6,IL8,ITGAL,LEP,MCL1,MMP9,MSR1,NOP2,PTGER2,PTGS1,PTGS2,RUNX2,SELL,SOST,VEGFA
CEBPD	2,7	3E-07	2,0	transcription regulator	Activated	ALOX5AP,BCL2A1,CD14,CEBPB,COL10A1,CSF1R,CSF3R,CXCL1,CXCL3,FABP4,HGF,IGFBP5,IL10,IL1B,IL6,IL8,ITGAM,ITGAX,MBP,MMP3,PTAFR,PTGS2,TLR8,VLDLR

Table 4 – Continued

Upstream Regulator	Activation z-score	p-value of overlap	FC	Molecule Type	Predicted Activation State	Target molecules in dataset
LEP	2,6	1E-07	14,3	growth factor	Activated	ACADL,ADAM8,ADIPOQ,ANGPTL4,AQP3,AQP9,AR,BCL2A1,CCL5,CD14,CD36,CD68,CEBPB,CPT1B,CSF3,CTSL,CYBA,CYBB,EDN1,ERBB2,ESR1,FABP4,FASN,GPAM,HEY2,IBSP,ICAM1,IL10,IL1B,IL1R2,IL1RN,IL6,ITGAM,JAK2,KISS1R,LEP,LIF,LIPA,LPL,MBP,MCL1,MMP1,MMP12,MMP19,MMP3,NAMPT,NCF1,NCF2,NPR1,NPR2,NPY1R,NTS,PCK1,PGR,PLAT,PLIN1,PLIN2,PLN,PRF1,PTGS2,SCD,SERPINE1,SOD2,SPP1,THBS1,THRSP,UCP2,VEGFA
REL	2,5	8E-05	3,6	transcription regulator	Activated	BCL2A1,BCL3,CD86,CR2,CREM,ESR1,GADD45B,ICAM1,IER3,IGHG1,IGHM,IGK,IL18,IL6,IL8,IRF4,IRF5,MMP9,NFKBID,PMAIP1,REL,SLC25A27,SOD2,TNFAIP3,TNFRSF10C
TGIF1	2,4	7E-04	2,9	transcription regulator	Activated	CXCL1,CXCL2,CXCL3,IL1B,IL6,IL8
CYBB	2,4	3E-03	2,6	enzyme	Activated	CCL5,CXCL3,CYBA,CYBB,ICAM1,IL1B,IL6,TNFRSF11A
IRF8	2,3	4E-10	3,5	transcription regulator	Activated	CCL5,CCR6,CCR7,CD4,CD83,CD86,CEBPA,CSF1R,CSF3R,CTSS,CXCL16,CYBB,DAB2,GBP1,ICAM1,IL17RA,IL18,IL1B,IL6,IRF8,ITGAM,JAK1,LIF,MMP9,MSR1,PCDH7,PRDM1,TLR3,TYROBP
IRF1	2,3	5E-03	2,9	transcription regulator	Activated	ADAM8,CCL5,CTSS,CXCL16,CYBB,DST,IL10,IL12RB1,IL17RA,IL18,IL1B,IL6,IL8,IRF1,IRF4,IRF5,IRF7,JAK2,LTB,MMP9,PCDH7,PTGS2,SELL,TLR3
CSF3	2,2	1E-21	5,8	cytokine	Activated	ARG1,ARHGDIA,BATF,BIRC3,C3AR1,CAPG,CCL3,CCL5,CCND3,CCR7,CD14,CD300LF,CD44,CEBPA,CEBPB,CSF3R,CTLA4,CTSD,CXCL5,CXCR4,CYBB,EDN1,ETS1,FPR1,GADD45B,GPR183,GZMB,HGF,HLA-DPA1,HLA-DQA1,HMOX1,HOXA7,HOXB7,ICAM1,IL10,IL1RN,IL6,IL8,ITGAM,ITGB2,JAK3,LTB,LTF,MMP9,NFATC1,NFIL3,PIM1,PRKCB,PROK2,PYHIN1,RAB27A,RGS1,S100P,SELL,TFRC,TLR2,TLR8,TNFAIP3,TNFRSF1B,VEGFA,YPEL3
PTPRE	2,2	8E-03	2,6	phosphatase	Activated	CXCL2,CXCL3,IL18,MARCO,SLA
INHBB	2,2	7E-04	2,3	growth factor	Activated	FST,HGF,MMP3,SERPINE1,THBS1,TNC
CD86	2,1	4E-06	3,0	transmembrane receptor	Activated	CCL3,CD28,CD69,CD86,CTLA4,ICAM1,ICOS,IL10,IL6,POU2F2,TNFAIP3,TNFRSF11A,XBP1
DOCK8	2,0	3E-04	2,9	other	Activated	DAB2,EDN1,IL18,IL6,IRF1,IRF7,ISG20,MERTK,PELI1,PLAT,PTGS2,REL,RGS1,RILPL1,TLR3,TNFSF8

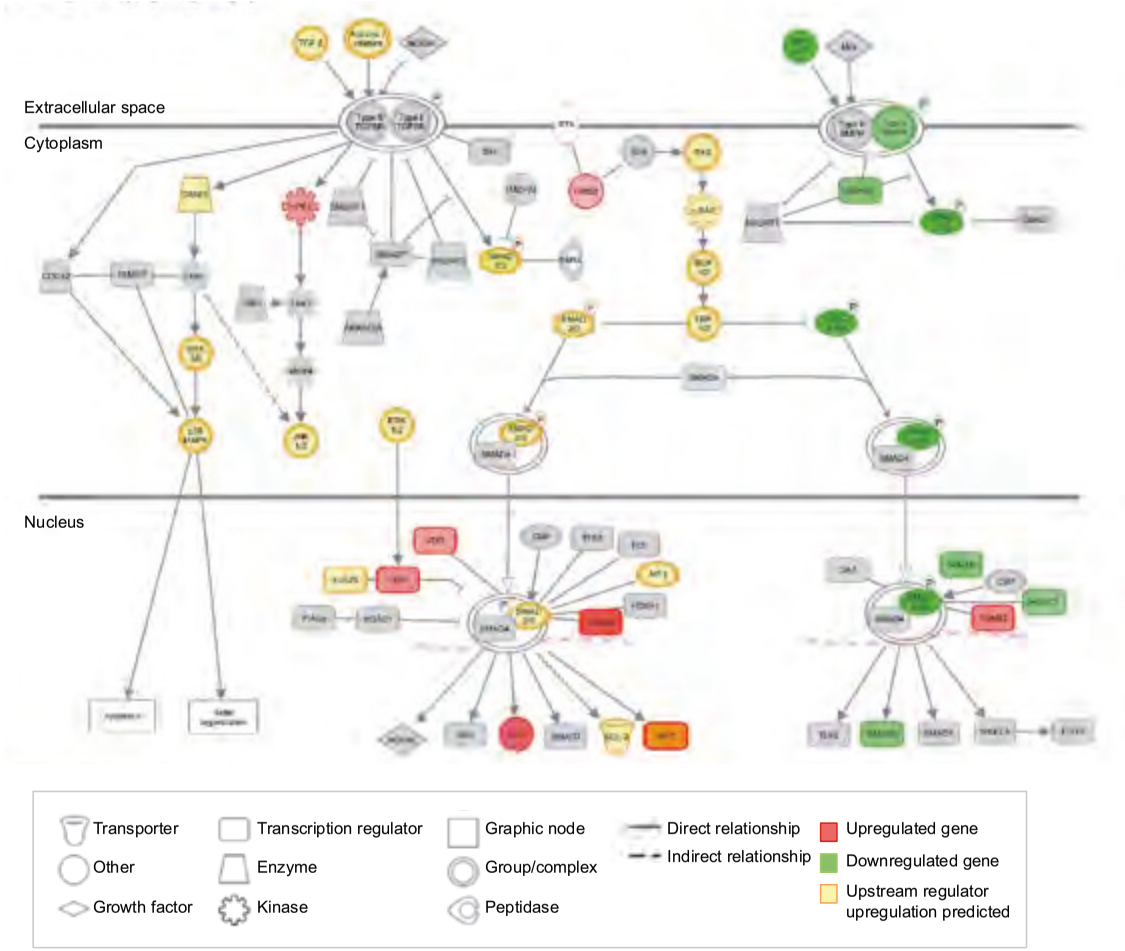

Figure 5 – TGFβ signaling pathway with mediators in the TGFβ pathway and BMP pathway are depicted, adapted from IPA. Upregulated genes in red, downregulated genes in green, and upstream regulators which are predicted to be upregulated in yellow.

which exclusively differentiates AAA disease from AOD. More likely, we need to look for a combination of different significantly altered immune pathways, together providing an 'immune signature' that is different for AAA compared to AOD. This could subsequently further be explored in blood of AAA and AOD patients.

Inflammation changes in a large vascular surgery patient cohort with aneurysmal or arterial occlusive disease

We analyzed 1393 cardiovascular patients for indications of inflammation changes. The population consisted of patients with either aortic aneurysm (n=614), peripheral arterial disease (n=491) or carotid artery disease (n=288). Endovascular procedures were performed in 598 patients (43%). The mean age of the population was 68 ±10 years and the majority of patients were men (75%). The clinical characteristics of the patients were as follows; 614 patients (44%) were classified as aortic aneurysms and 779 patients (56%) as arterial occlusive disease. The patient and baseline characteristics are depicted in

Table 5 – Clinical characteristics of patients with aneurysmal or arterial occlusive disease. Abbreviations: RAAS inhibitors; renin-angiotensin system inhibitors.

	Total	Aneurysmal disease	Occlusive disease	P-value
	n=1393	n=614	n=779	
Baseline characteristics				
Male gender (%)	1046 (75.1)	525 (85.5)	521 (66.9)	<.001
Age (years±SD)	68.1 ±10.1	71.3 ±7.8	65.6 ±11.0	<.001
Body mass index (kg/m2), mean(±SD)	26.1 ±4.1	26.1 ±3.9	26.2 ±4.3	.540
Cardiovascular comorbidities (%)				
Congestive heart failure	155 (11.1)	66 (10.7)	89 (11.4)	.692
Ischemic heart disease	578 (41.5)	272 (44.3)	306 (39.3)	.059
Cerebrovascular disease	455 (32.7)	89 (14.5)	366 (47.0)	<.001
Cardiovascular risk factors (%)				
Kidney disease (≥2.0mg/dl)	200 (14.4)	94 (15.3)	106 (13.6)	.368
Diabetes mellitus	328 (23.5)	103 (16.8)	225 (28.9)	<.001
Hypertension	932 (66.9)	408 (66.4)	524 (67.2)	.761
Hypercholesterolemia	1240 (89.0)	534 (87.0)	706 (90.6)	.030
Smoking – ever	1086 (78.0)	473 (77.0)	613 (78.7)	.459
Smoking – current	576 (41.7)	236 (39.0)	338 (43.9)	.068
Medication (%)				
Statins (%)	1079 (77.4)	446 (72.6)	633 (81.2)	<.001
Beta-blockers (%)	1123 (80.6)	531 (86.4)	592 (75.9)	<.001
RAAS inhibitors (%)	640 (45.9)	271 (44.1)	369 (47.3)	.247
Diuretics (%)	349 (25.0)	138 (22.4)	211 (27.0)	.052
Antiplatelets (%)	934 (67.0)	353 (57.4)	581 (74.5)	<.001

Table 5. A significant difference was observed between aneurysmal and occlusive disease patients in age (71 versus 66 years, respectively) and male gender (86% versus 67%, respectively), as was likewise present in our small patient group used for gene expression profiling, showing the representative nature of this database for the general population, and the samples used in this study. In Table 6 it is shown that the inflammatory marker hs-CRP was slightly, though significantly higher in patients with AAA compared to occlusive disease (4.07 mg/L versus 3.06 mg/L). Although this is a rather small difference and cannot be used to distinguish aneurysmal versus occlusive disease in a clinical setting, an adult population with a hs-CRP of >3.0 mg/L have a 2 fold increased relative risk for cardiovascular disease as compared with patients with a hs-CRP<1.0 mg/L.[19] Multivariable regression analysis showed an unadjusted difference for hs-CRP (β -0.51, 95% CI: -0.78 : -0.25, P<.001), as shown in Table 7. Importantly hs-CRP, remained significant in multivariable analysis after adjustment for potential confounding factors (β -0.55, 95% CI: -0.99 : -0.11, P=.015).

Taken together, these differences in gene expression as well as in the broader patient population study hint towards differences in the immune system of AAA and AOD patients, which might explain at least part of the different arterial outcomes. Therefore, it would be interesting to perform immunoprofiling studies on the blood of these two patient groups.

The TGFβ pathway is significantly regulated at both gene and upstream regulator level

Although IPA analysis showed many significantly altered inflammation pathways, also other interesting pathways were significantly altered, amongst which the TGFβ signaling pathway. As the TGFβ pathway is also an important factor in the development of TAA, we next examined this pathway more closely. In Figure 5 we show the TGFβ signaling and the bone morphogenetic protein (BMP) pathway, as derived from IPA, with all genes and upstream regulators that are significantly altered. As shown, many genes and upstream regulators from our dataset are upregulated in the TGFβ pathway, e.g. the known factors *TGFβ, ERK1/ERK2, SMAD2/SMAD3* and *PAI-1*. Notably, *IRF7* is not only upregulated at the mRNA level but also predicted to be upregulated at the upstream regulator level. Interestingly, many genes in the BMP signaling pathway were significantly downregulated, which implies that the pathway itself is inhibited in AAA disease compared to AOD. Moreover, genes involved in the pERK pathway are predicted to be upregulated. This pathway has been previously associated with (thoracic) aneurysmal disease, and it is interesting to note that it can regulate both the TGFβ as well as the BMP signaling pathway, which warrants further investigation.

Table 6 – Inflammatory markers of patients with aneurysmal or arterial occlusive disease.

Inflammatory marker	Number	Total	Aneurysm	Occlusive	P-value
triglyceride (mmol/L)	1307	1.61 [1.16-2.27]	1.58 [1.13-2.19]	1.63 [1.18-2.34]	.053
high-density lipoprotein (mmol/L)	1314	1.20 [0.97-1.46]	1.18 [0.97-1.42]	0.97[1.20-1.48]	.275
low-density lipoprotein (mmol/L)	1297	2.72 [2.04-3.47]	2.83 [2.13-3.53]	2.59 [1.95-3.41]	.003
hs-CRP (mg/l) [IQR]	872	3.53 [1.58-5.73]	4.07 [2.21-6.39]	3.06 [1.52-5.22]	<.0001

Abbreviations: high sensitivity C-reactive protein. Data presented in median with inter quartile range.

Table 7– Multivariable logistic regression models for inflammatory markers in patients with aneurysmal or arterial occlusive disease.

Inflammatory marker		β	95% CI for β	P-value
triglyceride	unadjusted	0.18	0.06 : 0.13	.005
	adjusted*	0.12	0.01 : 0.25	.079
high-density lipoprotein	unadjusted	0.03	-0.03 : 0.08	.334
	adjusted*	0.00	-0.06 : 0.06	.913
low-density lipoprotein	unadjusted	-0.14	-0.26 : -.21	.021
	adjusted*	-0.08	-0.22 : 0.51	.226
hs-CRP	unadjusted	-0.71	-1.07 : -.35	<.0001
	adjusted*	-0.55	-0.99 : -.11	.015

Abbreviations: CI, confidence interval; hs-CRP, high sensitive C-reactive protein. *adjusted for: age, gender, body mass index, congestive heart failure, ischeamic heart disease, cerebrovascular disease, kidney disease, diabetes mellitus, hypertension, current smoking, and the use of statins, beta-blockers, renin-angiotensin system inhibitors, diuretics and antiplatelets.

DISCUSSION

In this study we investigated the genetic factors and molecular processes that differentiate abdominal aortic aneurysm from arterial occlusive disease, despite overlapping characteristics between both diseases. By comparing the gene expression profiles of both diseases we show important pathway differences, in particular differences in upregulation of distinct inflammation pathways, but also differences in two previously identified TAA-related pathways; TGFβ and BMP signaling.

Non-hierarchal clustering and Principal Component Analysis of the data showed two distinct datasets of genes which are up- or downregulated in AAA compared to AOD. Clinical characteristics of the 19 patients included in our microarray dataset were analyzed and we observed no differences in the cardiovascular risk factors, indicating that indeed these factors do not explain the observed phenotypic differences between AAA and AOD. Additionally, the subsequent database study of 1393 patients likewise showed

no differences in cardiovascular risk factors, strengthening our findings. Smoking, gender, obesity, age, hypertension, and dyslipidemia are associated with an increased risk for AAA, whereas diabetes is associated with a reduced risk.[23] We observed in our cohort also a significant lower prevalence of diabetes in the AAA compared to the AOD group. In line with this observation, it has been described that diabetes seems to be protective when it comes to AAA formation and growth.[24, 25]

In both the micro-array and database study we show a gender and age difference between AAA and occlusive disease patients with a male dominance in AAA compared to occlusive disease patients. This observation has been described earlier as it is known that the incidence of AAA disease rises rapidly after the age of 55 years in men.[14] Therefore, these datasets reflect the actual AAA and occlusive disease patient population. We used a dataset of gender specific genes to correct our data for sex differences, as gender could be an influencing factor for several upregulated genes. However, comparison of the gender-dependent and gender-independent datasets revealed only minor differences. We performed an IPA core analysis on the dataset with and without the gender specific genes and both analyses showed very similar results regarding functions, pathways and upstream regulators, suggesting that the differences between AAA and AOD are the predominant determinant in this dataset. To select AAA-specific genes irrespective of gender, we used the list of gender-independent significantly regulated genes, for further IPA analysis of AAA disease.

From the list with significantly upregulated genes we selected a top 10 of potential markers, based on their expression level, significance and presence in vascular tissue, and performed literature research to identify possible connections of these genes to AAA or AOD. Of these 10 genes, 4 showed an association with aneurysmal disease, showing that our selection procedure indeed can reveal aneurysm relevant markers. At the same time, the other 6 genes showed no previously known association, making them potential novel markers for AAA disease. We performed an additional validation step of 5 upregulated genes by QPCR. As determined by microarray analysis, all these genes showed upregulation, however, probably due to small sample size only *COL11A1* and *FDC-SP* were significantly upregulated. At this point upregulation of potential AAA markers at the transcriptional level should be further verified in blood of AAA and control patients, for which an independent AAA patient cohort is needed.

The IPA analysis showed an overrepresentation of significantly regulated immune-specific pathways for AAA disease. Moreover, analysis of hs-CRP levels in an additional patient cohort of 1393 patients showed slightly increased hs-CRP levels in AAA compared to occlusive disease patients, both in the unadjusted and adjusted data analysis. Although in this larger cohort we show increased inflammation based on hs-CRP, data of other known inflammation markers were not available. However, the significance of increased hs-CRP in already established aneurysms is unknown, as inflammation is a multifactorial

process. Similar to what we find, other studies reported the role of the immune-related genes and pathways in AOD and AAA disease.[26, 27] Though, we used additional IPA analysis and upstream regulator information to go in depth of distinct pathways to distinguish both pathogenic mechanisms. Together the changes in distinct inflammation pathways derived from our gene expression analysis, as well as the finding that hs-CRP levels differ significantly between AAA and occlusive disease patients, imply that a more thorough analysis of immune factors in the blood for these two patient groups would be a very relevant next step.

Dysregulation of the TGFβ and BMP signaling pathway, previously described for TAA patients,[11] was also shown for AAA patients in our IPA analysis. While we find most components of the TGFβ signaling pathway were significantly upregulated, most components of the BMP-pathway were downregulated in AAA compared to AOD. Moreover, many upstream regulators involved in the TGFβ pathway were predicted to be upregulated in our analysis. Interestingly, TGFβ signaling was mostly reported to be upregulated in TAA, and intervention therapy aimed at reducing TGFβ is able to reduce aneurysmal growth. In addition, blockade of TGFβ-signaling by TGFβ-neutralizing antibody (Nab) showed beneficial effects in MFS rodent models. In contrast, TGFβ-Nab administration exacerbated the pathology of aneurysms in angiotensin-II induced AAA mice models.[28, 29] But in AAA (dys)regulation of the TGFβ signaling pathway is not clear yet. For example, a small study in 12 AAA and 6 control biopsies showed downregulation of TβRII subtype mRNA.[30] Yet, about 20-30% of AAA patients later in life also develop a TAA.[31, 32] Vice versa, many TAA patients have aneurysms at multiple sites, including the abdominal part.[33] Therefore similar mechanisms might be at work in both AAA and TAA patient groups. In this respect it is very interesting that our data show that the TGFβ signaling pathway might be dysregulated in AAA aorta samples, with predictions that the pathway is upregulated. At the same time, the closely associated BMP pathway is predicted to be downregulated. These data could indicate that an imbalance between TGFβ and BMP signaling causes part of the AAA phenotype. This might also explain the different findings described above on TGFβ signaling pathway involvement in AAA versus TAA. It would therefore be interesting to further investigate factors involved in both the TGFβ and BMP signaling pathways in tissue or serum samples from AAA patients. In particular, measuring the TGFβ ligands 1-3 in the serum could be of great importance, in parallel to measurements of TGFβR subtype mRNA levels.

CONCLUSIONS

Our data show that gene expression profiling is an important tool to distinguish AAA from AOD, clinical entities that share the same risk factors but show completely different disease progression, as we revealed that simultaneous inhibition of BMP and activation of TGFβ signaling plays a role in abdominal aortic aneurysms. Besides, these profiles are important in the identification of novel genes, markers and processes that can shed light on the molecular mechanisms underlying abdominal aneurysm formation.

Limitation of the study

One limitation of this micro-array study is the small number of samples in the AAA and AOD groups, however previously other studies showed novel differences of upregulated genes and pathways in comparison analysis of similar small patient groups.[26,27] In addition, we did identify novel markers and pathways with significant p-values, that separate AAA disease from aortic occlusive disease. One reason for these small sample groups is that it is becoming increasingly difficult to obtain the abdominal aortic tissue as nowadays AAA disease and occlusive patients are generally operated by endovascular procedures. Another limitation of the study is that one disease (AAA) is compared with another disease (AOD) without a 'healthy' control group as part of this comparison. Because of this limitation, genes that are considered upregulated in AAA in this study, could be higher expressed in AAA as compared to a healthy individuals or they could be genes that are downregulated in AOD as compared to a healthy individual. Nonetheless, this study clearly demonstrates that interesting biological interpretations can be made from this comparison as TGF-beta signaling is identified both as a significant differentially regulated pathway and several of its components are identified as significant upstream regulators of the AAA versus AOD differentially expressed dataset.

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Supplemental Table 1 – The Vascular Gene Set, constructed from HGMD, OMIM, relevant GO terms, relevant KEGG pathways, relevant Ingenuity IPA pathways, GWAS studies and the literature.

KEGG pathways
Vascular Smooth Muscle Contraction hsa04270
Tight Junction hsa04530
ECM receptor interaction hsa04512
TGFβ signaling hsa04350
notch signaling hsa04330
Focal Adhesion hsa04510
Adherens junctions hsa04520
Fat Dig and Absorption hsa04975
Renin-Angiotensin-System hsa04614
GO-Terms
Vasculogenesis GO 0001570
Vasculature Development 0001944
Relaxation of smooth muscle GO 0060087
Cardiovascular system development GO:0072358
Vascular smooth muscle contraction GO:0014829
Reg. of vascular permeability GO 0002528
Regulation of vascular smooth muscle contraction GO:0003056
Cardiac vascular smooth muscle cell differentiation GO:0060947
macrophage derived foam cell differentiation GO:0010742
Negative regulation of macrophage derived foam cell differentiation GO 0010745
positive regulation of macrophage derived foam cell differentiation GO 0010744
regulation of macrophage derived foam cell diff GO 0010743
IPA functions and pathways
Adherens Junction IPA
Cardiovascular IPA
Extracellular matrix IPA
Fat Digestion and Absorption IPA
Foam Cell IPA
Focal Adhesion IPA
Notch Signaling IPA
Renin Angiotensin IPA
TGFβ Signaling IPA
Tight Junction IPA
vascular smooth muscle IPA
Vasculature Development IPA
Vasculogenesis IPA
Vascular Permeability IPA
AAA GWAS gene
aneurysm HGMD genes
aneurysm OMIM
aneurysm custom

4

Extracellular Matrix Defects in Aneurysmal Fibulin-4 mice Predispose to Lung Emphysema

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ABSTRACT

Background: In this study we set out to investigate the clinically observed relationship between chronic obstructive pulmonary disease (COPD) and aortic aneurysms. We tested the hypothesis that an inherited deficiency of connective tissue might play a role in the combined development of pulmonary emphysema and vascular disease.

Methods: We first determined the prevalence of chronic obstructive pulmonary disease in a clinical cohort of aortic aneurysms patients and arterial occlusive disease patients. Subsequently, we used a combined approach comprising pathological, functional, molecular imaging, immunological and gene expression analysis to reveal the sequence of events that culminates in pulmonary emphysema in aneurysmal Fibulin-4 deficient (Fibulin-4^{R}) mice.

Results: Here we show that COPD is significantly more prevalent in aneurysm patients compared to arterial occlusive disease patients, independent of smoking, other clinical risk factors and inflammation. In addition, we demonstrate that aneurysmal Fibulin-$4^{R/R}$ mice display severe developmental lung emphysema, whereas Fibulin-$4^{+/R}$ mice acquire alveolar breakdown with age and upon infectious stress. This vicious circle is further exacerbated by the diminished antiprotease capacity of the lungs and ultimately results in the development of pulmonary emphysema.

Conclusions: Our experimental data identify genetic susceptibility to extracellular matrix degradation and secondary inflammation as the common mechanisms in both COPD and aneurysm formation.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is worldwide one of the major causes of morbidity and mortality.[1] In addition to chronic airflow obstruction due to airway inflammation and alveolar destruction, COPD is associated with extrapulmonary manifestations, including cardiovascular diseases.[2-5] These comorbid conditions contribute to the overall disability of patients and complicate the management of COPD.

Aortic aneurysm (AA) is one of the cardiovascular diseases related to COPD.[6, 7] The nature of this relationship is currently unknown. Patients with COPD, AA, and/or atherosclerosis share a number of risk factors, including age, hypertension, and tobacco smoking.[8, 9] Resemblances in risk profiles between these conditions, most notably smoking, may account for the relation between AA and COPD. Furthermore, a systemic inflammatory response has been suggested as a common denominator.[10]

The association between COPD and AA prompted us to investigate the prevalence of COPD in a large cohort of patients with aneurysmal or arterial occlusive disease (AOD) in relation to their clinical risk profiles. Here, we found that COPD is much more prevalent in patients with AA compared to those with AOD, irrespective of common clinical risk factors. Since AA and COPD are associated with destruction of the extracellular matrix (ECM),[11-13] we hypothesized that a primary ECM defect may provide a common ground for the combined development of COPD and aneurysm formation. We previously demonstrated that mice with reduced expression of the ECM glycoprotein Fibulin-4 exhibit ECM degradation in the aortic wall and AA formation.[14, 15] We here investigated the role of Fibulin-4 deficiency in the development of lung emphysema.

MATERIAL AND METHODS

Clinical study

Patients

Consecutive patients undergoing elective open or endovascular surgery for aortic aneurysm, peripheral arterial disease, or carotid artery disease between 2002 and 2011 in the Erasmus MC, Rotterdam, were included. Patients with an aortic aneurysm (AA) were classified as aneurysmal disease. Patients with atherosclerotic peripheral arterial or carotid artery disease were classified as arterial occlusive disease (AOD). Patients treated with combined AA and symptomatic AOD, and patients with a genetic aneurysm syndrome like Marfan, Loeys-Dietz or vascular Ehlers-Danlos syndrome were excluded. The study complies with the declaration of Helsinki and was approved by the Institutional Review Board of the Erasmus Medical Center (permit number MEC-2011-510) in accordance with national and international guidelines. Our institutional review board waived the need for written informed consent from the participants since the data was obtained for clinical

purpose, there was no intervention and there was a retrospective study design. Patient data were de-identified prior to analysis.

Clinical characteristics

Medical history was obtained from every patient, including the cardiovascular risk factors age, gender, body mass index (BMI), smoking status, hypertension (blood pressure ≥140/90 mmHg in non-diabetics, ≥130/80 mmHg in diabetics, or use of antihypertensive medication), hypercholesterolemia (low-density lipoprotein [LDL] cholesterol ≥3.5 mmol/L or use of lipid lowering medication), diabetes mellitus (fasting plasma glucose ≥7.0 mmol/L, non-fasting glucose ≥11.1 mmol/L, or use of anti-diabetic medication), and kidney disease (serum creatinine ≥2.0 mg/dl). Cardiovascular comorbidities were recorded, including congestive heart failure (defined as history of congestive heart failure), ischemic heart disease (defined as a history of angina pectoris, myocardial infarction, coronary revascularization, or presence of pathologic Q-waves on the electrocardiogram), cerebrovascular disease (defined as a history of ischemic/hemorrhagic stroke or transient ischemic attack). Prescription medications were recorded and included the use of statins, beta-blockers, renin-angiotensin system inhibitors, diuretics, and antiplatelet drugs. Serum concentrations of the inflammatory biomarker high-sensitivity C-reactive protein (hs-CRP) were measured using immunochemistry (Beckman Coulter, Woerden, the Netherlands).

Chronic obstructive pulmonary disease

The diagnosis and classification of COPD was made using spirometry, which was part of the routine preoperative workup and was obtained in 92% of COPD patients. COPD was defined as the presence of a forced expiratory volume in one second (FEV1) to forced vital capacity (FVC) ratio (FEV1/FVC) <0.70. In the presence of a FEV1/FVC ratio of <0.70, mild COPD was defined as a FEV1 >80% of the predicted FEV1 (GOLDI), moderate COPD was defined as a FEV1 of 50-80% of the predicted FEV1 (GOLDII), and severe COPD was defined as a FEV1 <50% of the predicted FEV1 (GOLDIII/IV).[16] Patients without spirometry were classified based on the presence of pulmonary symptoms (i.e. cough, dyspnea, sputum) and the use of pulmonary medication.

Statistical analysis

Dichotomous data are presented as numbers and percentages. Continuous variables are presented as mean ± standard deviation or median and IQR when not normally distributed. Categorical data were analyzed with chi-square tests and continuous variables with ANOVA or Kruskal-Wallis tests. Multivariable binary logistic regression analysis was used to calculate odds for having COPD between AA and AOD. Adjustments were made for age, gender, BMI, congestive heart failure, ischemic heart disease, cerebrovascular

disease, kidney disease, diabetes mellitus, hypertension, hypercholesterolemia, smoking, statins, beta-blockers, renin-angiotensin system inhibitors, diuretics, antiplatelets, and hs-CRP. Furthermore, we performed a propensity score to adjust for the possibility of receiving a pulmonary function test prior to surgery. Covariates were chosen on the bases of biological plausibility.

For all tests, a p-value <0.05 (two-sided) was considered significant. All analyses were performed using IBM SPSS Statistics version 20.0 (SPSS Inc., Chicago, IL, USA).

Experimental study

Animals

Fibulin-4 animals were generated as previously described.[14] All mice used were bred in a C57BI/6J background and were kept in individually ventilated cages to keep animals consistently micro-flora and disease free. To avoid stress-related vascular injury, mice were earmarked and genotyped 4 weeks after birth. Mice used were either newborn or adult (110 ± 10 days). Adult mice were challenged by a single intratracheal injection with either 80 µl ultra-pure, sterile Lipopolysaccharide (LPS) 1 mg/ml from *E. coli* Serotype R515 (Alexis Corporation Switzerland) or 80 µl PBS (Lonza). Animals were housed at the Animal Resource Centre (Erasmus University Medical Center), which operates in compliance with the "Animal Welfare Act" of the Dutch government, using the "Guide for the Care and Use of Laboratory Animals" as its standard. As required by Dutch law, formal permission to generate and use genetically modified animals was obtained from the responsible local and national authorities. An independent Animal Ethics Committee of the Erasmus Medical Center (Stichting DEC Consult) approved these studies (permit number 139-10-12 and 139-12-02), in accordance with national and international guidelines.

Quantitative real time PCR

RNA was isolated using the RNeasy minikit from Qiagen according to the provided protocol and synthesized to cDNA with the RevertAid H Minus First Strand cDNA Synthesis Kit according to the provided instructions. Quantitative Real-Time PCR was performed using Maxima SYBR Green qPCR Master Mix 2x (Fermentas) also according to the provided protocol. Reactions were performed in triplicates per gene for each sample. The primers used for Fibulin-4, Gapdh and Hprt (Invitrogen) are indicated in Supplemental Table 7. Product specificity was determined by melting curve analysis and gel electrophoresis. The average Ct values of the triple reactions were calculated for each gene according to cell type. The relative gene expression level was calculated by the following formula for each gene:

$$\text{Relative gene expression level} = 2^{(\text{Ct control} - \text{Ct sample})\ \text{gene}} / 2^{(\text{Ct control} - \text{Ct sample})\ \text{housekeeping gene}}$$

The levels of fold-change for each gene were calculated by dividing the relative gene

expression levels in Fibulin-$4^{+/R}$ or Fibulin-$4^{R/R}$ lungs to the relative gene expression levels in wild type lungs.

Whole body plethysmography

Conscious mice were placed in a single-chamber, whole body, plethysmograph (Emka Technologies, Paris, France) as described previously.[17] After an adaptation period of 9 minutes (acclimatization), Peak Inspiration Flow (PIF) and Peak Expiration Flow (PEF) were measured in 6 time blocks of 3 minutes. Differences in PIF and PEF indicate differences in inspiration and expiration strength.

Lung morphometry

A random selection of images of HE stained alveoli were obtained with the Leica DFC280DFC480 (Aristoplan) with a magnification of 10x. Large airways and vessels were generally avoided. Next, alveolar airspace size quantification was performed according to the fully automated D_2 method as described in Jacob RE et. al, where it was compared to the semi-automated mean linear intercept measurements, and turned out to be more sensitive and specific for subtle airspace enlargement expected to be found in mild or early stage emphysema.[18] All images were converted to grayscale before performing the analysis. Fuzzy-c-means clustering with simultaneous correction of potential luminance inhomogeneity was applied to each image for pre-segmenting it into two classes: the foreground and the background. The final segmentation was obtained by the graph-cut method with the energies given by the class membership functions calculated on the previous step. The resulting foreground was split into separate compartments corresponding to the connected components belonging to this class; see Supplemental Figure 1 and the accompanying legend for an illustration. Vector of the compartment sizes obtained in such a way was converted from pixels to micrometers. For each of the vectors we calculated the D_2 measure, an index based on the equivalent diameters of airspaces and by incorporating higher moment factors from the airspace diameter distributions, where enlarged airspaces are weighed more heavily. This measure is useful to detect early or mild emphysema. The compartments whose sizes were less than 138 μm were disregarded according to the threshold previously reported in.[18]

Histological analysis and immunohistochemistry

For the lung morphometry procedure, mice were euthanized with a lethal dose of pentobarbital (60 mg/ml, 0.1-1.5 ml per mouse according to weight). Lung lobes were excised and the left lobe was pressure fixed through the bronchi at a pressure of 25 cm H_20 with 4% paraformaldehyde (PFA), and fixed overnight at 4°C before paraffin embedding. Lungs from newborn mice were immersion fixed. The 5-μm sections were prepared from the paraffin embedded lungs and put on Superfrost Ultra plus slides (Menzel-Glaser). For

the morphometric analysis paraffin sections of the lungs were stained with Haematoxylin-Eosin (HE).

For histological analysis 100-day-old female mice were dissected. Mice were euthanized by CO_2-inhalation. After opening thorax and abdomen, mice were fixed by perfusion fixation through the left ventricle, with PBS and 4% paraformaldehyde (PFA). Organ weights were determined and macroscopic abnormalities noted. Organs and tissues were fixed in 4% PFA. Lungs and aortas were dehydrated through the histokinette processor (Microm), and paraffin embedded, after which 5-µm sections were prepared. Lungs and aortas were stained with HE for general pathology and Resorcin-Fuchsin (Elastin von Gieson) for elastin structure. For immunohistochemical analyses, sections were emerged in 3% H_2O_2 in PBS to inhibit endogenous peroxidase. Antigen retrieval was performed by boiling slides in 10 mM citrate buffer, pH 6.0, at 600 W for 15 minutes in a microwave for TTF-1 and CC10 staining, 100 mM Tris 10 mM EDTA buffer, pH 9.0, at 300 W for 20 minutes for pSmad-2 staining, or with pronase treatment for α-SMA. Slides were first blocked in 5% Bovine Serum Albumin (BSA) in PBS and 0.5% Tween (and 5% Protifar in PBS and 0.025% Triton X-100 for pSmad-2), and incubated with the primary antibodies overnight at 4 °C; TTF-1 (1:250 mouse monoclonal Ab-1 Clone 8G7G3/1 Thermo Fisher Scientific), CC10 (1:100 goat Ab (T-18): sc-9772 Santa Cruz Biotechnology), Anti-Human Smooth Muscle Actin (1:250 mouse, clone 1A4 Biogenex Laboratories Inc.), and pSmad-2 (1:100 monoclonal Rabbit anti-pSmad2 (S465|467 (138D4) Cell Signaling). The next day slides were incubated with Horse Radish Peroxidase (HRP) labelled secondary antibodies (1:100 DAKO) and avidin-biotinylated secondary antibodies (Vectastain Universal Elite ABC kit Vector Laboratories) for pSmad-2. DAB chromogen (DAKO Liquid Dab substrate-chromogen system) was used as substrate and slides were counterstained with haematoxylin.

Immunohistochemical stainings for inflammatory cells were performed in a half-automatic stainer (Sequenza, Amsterdam, the Netherlands). Acetone-fixed slides were blocked in diluted normal goat serum (CLB, Amsterdam, the Netherlands) and stained against mouse CD3 (1:10 rat monoclonal antibodies KT3 AbD Serotec) and against mouse CD11c (1:20 hamster antibodies N418 Ebioscience). Primary antibodies were revealed by incubation with diluted appropriate secondary antibodies coupled to alkaline phosphatase for 30 min. Slides were subsequently incubated with New Fuchsin substrate for alkaline phosphatase conjugates. Finally, the sections were counterstained with Gills triple-strength haematoxylin and mounted in VectaMount (Brunschwig, Amsterdam).

Micro-array hybridizations

RNA was isolated using the RNeasy minikit from Qiagen with the provided protocol and delivered to the department of Biomics, Erasmus MC. Synthesis of double stranded cDNA and biotin labelled cRNA was performed according to the instructions of the manufacturer

(Affymetrix). Fragmented cRNA was hybridized to Mouse Genome 430 V2.0 arrays, using a hybridization Oven 640 (Affymetrix), washed and subsequently scanned on a GeneChip Scanner 3000 (Affymetrix). To examine the quality of the various arrays, several bioinformatic R packages (including affyQCreport and affyPLM) were run starting from the raw CEL data files. All created plots, including RNA degradation, RLE and NUSE plots indicated a high quality of all samples and an overall comparability, except for one sample (Fibulin-$4^{R/R}$ newborn lung), which was excluded from further analysis. Raw intensity values of all samples were normalized by robust multichip analysis normalization (background correction and quantile normalization) using Partek version 6.4 (Partek Inc., St. Louis, MO). The normalized data file was transposed and imported into OmniViz version 6.0.1 (Biowisdom, Ltd., Cambridge, UK) for further analysis. For each probe set, the geometric mean of the hybridization intensities of all samples was calculated. The level of expression of each probe set was determined relative to this geometric mean and ^{2}log transformed. The geometric mean of the hybridization signal of all samples was used to ascribe equal weight to gene expression levels with similar relative distances to the geometric mean. Differentially expressed genes were identified using ANOVA (Partek) and SAM (Omniviz). The cut-off value for significantly expressed genes was FDR 10% for adult Fibulin-$4^{R/R}$ lungs compared to Fibulin-$4^{+/+}$ lungs. Functional and network analysis was done using Ingenuity Pathway Analysis (IPA; Ingenuity® Systems, www.ingenuity.com, Mountain View, CA). Ingenuity pathway analysis is a web-based software application that enables to analyze and integrate data derived from gene expression microarrays into biological networks and pathways. All Ingenuity products leverage the Ingenuity Knowledge Base, which houses biological and chemical relationships extracted from the scientific literature.

Significantly expressed genes from the adult Fibulin-$4^{R/R}$ to Fibulin-$4^{+/+}$ lungs comparison were compared to COPD-associated genes in Ingenuity and a list of literature based genes associated with COPD. CEL files from GEO dataset GSE8581 were obtained and were analyzed following the above described procedures. The GEO dataset GSE8581 consisted of 15 COPD cases, with a predicted FEV1<70% and FEV1/FVC<0.7, and 18 control cases, with a predicted FEV1>80% and FEV1/FVC>0.7. Subjects were undergoing surgical resection of a suspected lung tumor and tissue for this dataset was derived from histologically normal lung tissue distant from the tumor margin.[19] Cut-off values for significantly expressed genes were FDR 30% and 1.5-fold. Comparison to significantly expressed genes from the adult Fibulin-$4^{R/R}$ to Fibulin-$4^{+/+}$ lungs comparison was done using IPA.

Preparation of cell suspensions, flow cytometry and ELISA

Broncho Alveolar Lavage (BAL) was performed with 3 times 1 ml of Ca^{2+}- and Mg^{2+}-free PBS, containing 10 mM EDTA. Furthermore, lungs were enzymatically digested using

collagenase type III (Worthington) for 1 hour at 37° C, followed by washing and filtering. Cell suspensions were stained with antibodies specific for F4/80-Fitc, MHC class II-PE, CD11c-PeTexasRed, CD3-PECy5, CD19-APCCy7, CD25-APC and GR-1-PECy7 (Becton Dickinson or eBiosciences). Nonspecific binding to Fc-receptors was blocked by incubation with 2.4G2 antibodies, and DAPI (Invitrogen) was used as life/dead marker. Acquisitions were performed on an LSRII flow cytometer (Becton Dickinson) and data were analyzed by FlowJo (Treestar, Costa Mesa, CA) software. Supernatants of BAL fluid were stored for ELISA. BAL fluid cytokines were measured by commercially available specific ELISA systems for IL-6, KC, MCP-1, TARC, IL-10, IL-12, IL-1β, TNF-α, IFN-gamma and IL-17 according to the manufacturers' instructions. In a separate set of experiments, flow cytometric analyses of BAL samples and pulmonary cell suspensions were performed 18 or 72 hours after a single intratracheal injection with either sterile lipopolysaccharide (LPS) 1 mg/ml in PBS or PBS alone in adult (100-days-old) Fibulin-$4^{+/R}$ and Fibulin-$4^{+/+}$ mice.

Western blot analysis

Western blot analysis was performed as described before.[20] In short, equal amounts of lung tissue homogenates (40 ug) were separated under reducing conditions on 10% SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Whatman, Germany) and blocked with 5% milk. After washing, membranes were incubated with rabbit anti-phosphorylated Smad2 (Cell Signaling Technologies, USA) and rabbit anti-phosphorylated Smad3, kindly provided by Dr. E. Leof, Mayo Clinic, Rochester, MN, USA followed by HRP labelled secondary antibodies (GE Healthcare) and detection with a chemiluminiscent substrate (Pierce). Afterwards membranes were stripped and reprobed with anti Smad2/3 antibodies (BD biosciences) , β-actin (Sigma) or GAPDH (Millipore) as a loading control.

Fluorescence imaging

We used vascular fluorescent mediated tomography (FMT) imaging with near-infrared fluorescent protease activatable probes as previously described.[15, 21] Open chest FMT imaging of fibulin-4 mice was performed using an FMT 2500 system (Perkin Elmer Inc.) at 680- and 750-nm excitation and emission wavelengths, respectively, at five hours after tail vein injection of 4 nmol of Neutrophil Elastase 680 FAST and 2 nmol of MMPsense 750 FAST (Perkin Elmer Inc.). Mice with open chests were fixed into the portable animal imaging cassette that lightly compressed the mouse between optically translucent windows. The FMT 2500 quantitative tomography software was then used to calculate 3D fluorochrome concentration distribution of Neutrophil Elastase 680 FAST and MMPsense 750 FAST.

After open chest fluorescence imaging, complete lungs were harvested and fluorescence was quantified using the FMT 2500 or Odyssey imaging systems (LI-COR Inc.). Near-infrared images were obtained in the 680- and 700-nm channels, respectively.

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis for lung morphometry was performed using the Kolmogorov-Smirnov test. The Kruskal-Wallis one-way ANOVA was used to determine any significant differences between groups. The nonparametric Mann-Whitney U-test was performed to analyze the specific sample pairs for significant differences.

A p-value of <0.05 was considered to indicate a significant difference between groups. All analyses were performed using IBM SPSS Statistics version 20.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Clinical study

Patient characteristics

We included 1393 patients; 614 patients (44%) were diagnosed with AA and 779 patients (56%) with AOD. The majority of AA patients were treated for an abdominal aortic aneurysm (AAA). None of the patients in this series were treated for an aneurysm of the ascending aorta; 62/614 (10%) of patients were treated for an aneurysm of the descending thoracic aorta (TAA). Clinical characteristics of AA and AOD patients are presented in Table 1.

Patients with AA were on average older and more frequently of male gender. Patients with AOD had higher rates of diabetes, hypercholesterolemia, and cerebrovascular disease. In addition, there were differences in medication use between the two groups: statins and antiplatelet drugs were more commonly used by patients with AOD, whereas beta-blockers were more often used by patients with AA. Importantly, smoking rates were similar in the two patient groups. A differentiation in clinical characteristics between AAA and TAA patients is presented in Supplemental Table 1.

Association between COPD and AA

COPD was more common in AA patients as compared to AOD patients (42% vs. 26%, p<0.001, Figure 1A). COPD rates did not differ between TAA and AAA patients (Supplemental Table 2). Univariate logistic regression analysis showed a significant association between COPD and AA (odds ratio 2.08, 95%CI: 1.66-2.61, p<0.001; Supplemental Table 3). Since patients with COPD, AA, and AOD shared a number of cardiovascular risk factors, we subsequently performed a multivariable regression analysis. Even after adjustment for potentially confounding factors the association between COPD and AA remained significant (odds ratio 1.56, 95%CI: 1.16-2.10, p=0.003; Supplemental Table 3).

Table 1 – Clinical characteristics of patients with aortic aneurysm (AA) or arterial occlusive disease (AOD).

	AA	AOD	P-value
	n=614	n=779	
Baseline characteristics			
Male gender (%)	525 (85.5)	521 (66.9)	<.001
Age (years ± SD)	71.4 ± 7.8	65.6 ± 11.0	<.001
Body mass index (kg/m2, mean ± SD)	26.1 ± 3.9	26.2 ± 4.3	.540
Cardiovascular comorbidities (%)			
Congestive heart failure	66 (10.7)	89 (11.4)	.692
Ischemic heart disease	272 (44.3)	306 (39.3)	.059
Cerebrovascular disease	89 (14.5)	366 (47.0)	<.001
Cardiovascular risk factors (%)			
Kidney disease	94 (15.3)	106 (13.6)	.368
Diabetes mellitus	103 (16.8)	225 (28.9)	<.001
Hypertension	408 (66.4)	524 (67.2)	.761
Hypercholesterolemia	534 (87.0)	706 (90.6)	.030
Smoking – current	236 (38.4)	338 (43.3)	.068
Smoking – ever	473 (77.0)	613 (78.7)	.459
Medication (%)			
Statins	446 (72.6)	633 (81.2)	<.001
Beta-blockers	531 (86.4)	592 (75.9)	<.001
Renin-angiotensin system inhibitors	271 (44.1)	369 (47.3)	.247
Diuretics	138 (22.4)	211 (27.0)	.052
Antiplatelets	353 (57.4)	581 (74.5)	<.001

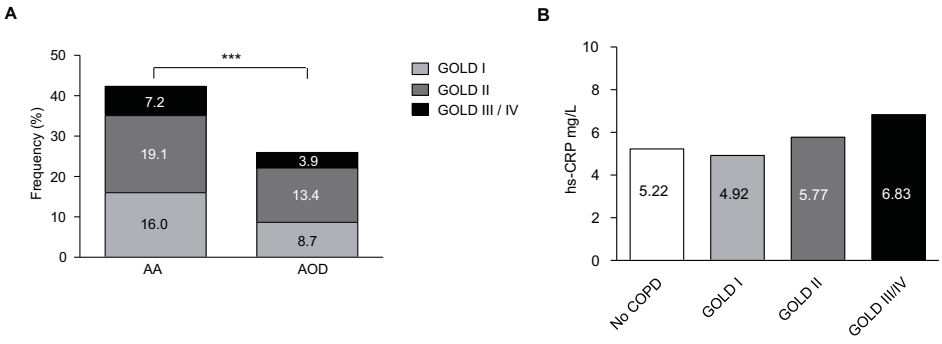


Figure 1 – Prevalence and severity of COPD in patients with an aortic aneurysm (AA) or arterial occlusive disease (AOD).

(A) The prevalence of COPD in all GOLD classes was higher in AA (n=614) compared to AOD patients (n=779, *** p<.001). (B) Serum high-sensitivity CRP levels according to severity of COPD in patients with AA or AOD. There was no significant difference between patients with and without COPD (p for trend = .123).

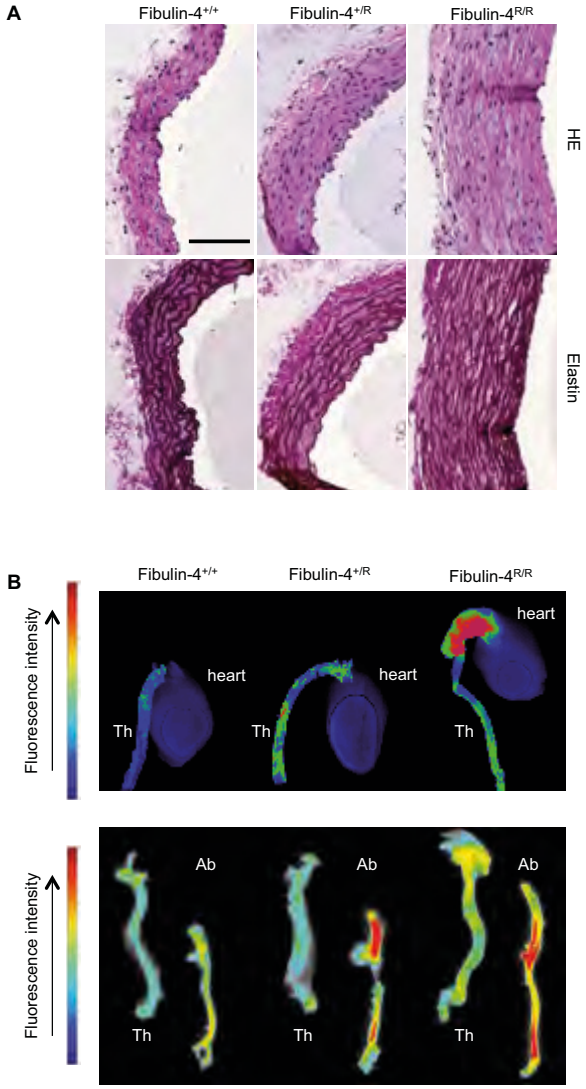


Figure 2 – Degenerative arterial wall changes and increased MMP activity in Fibulin-4 animals

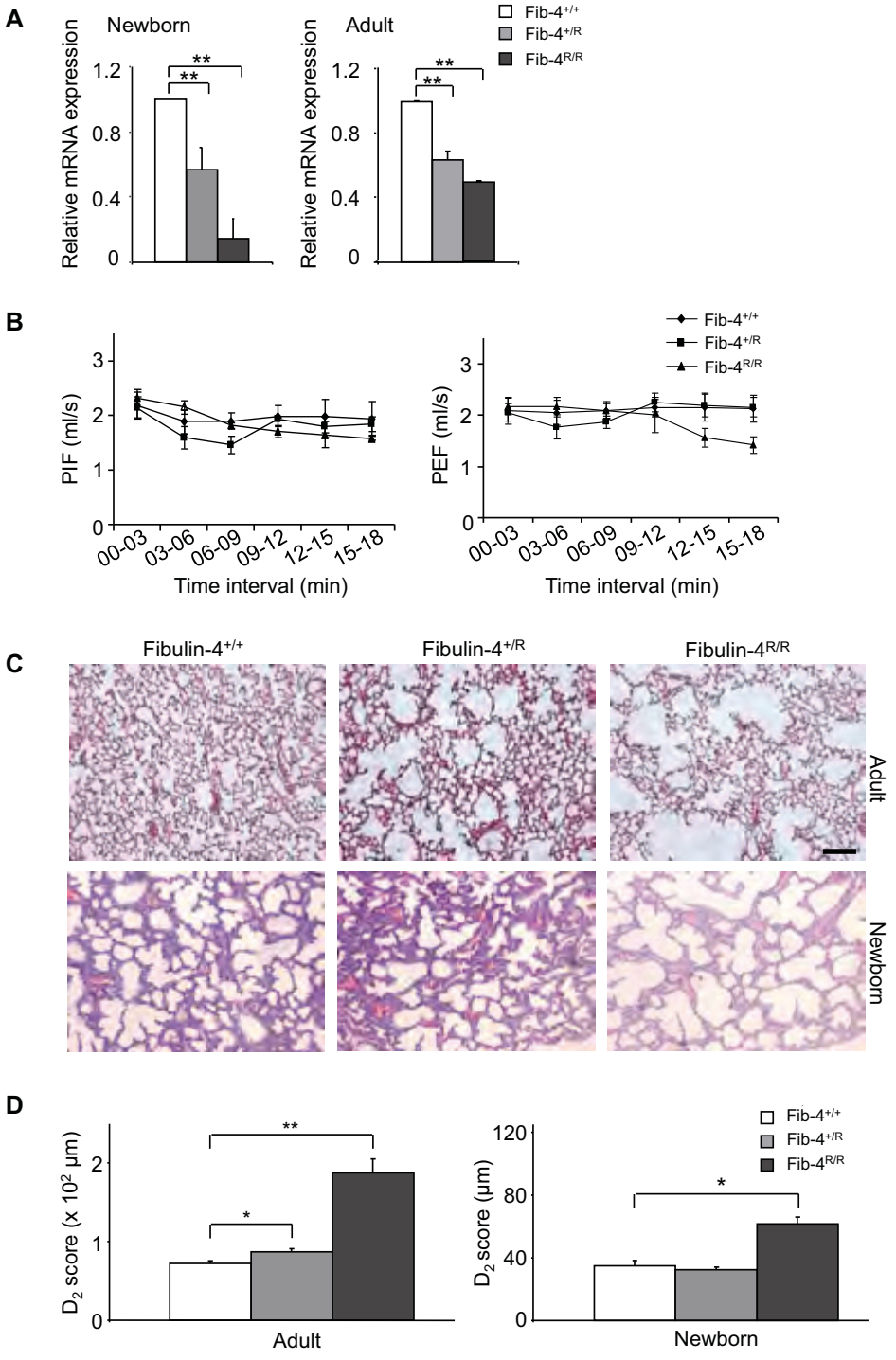
(A) Comparison of the architecture of the aortic wall in Fibulin-$4^{+/+}$, Fibulin-$4^{+/R}$, and Fibulin-$4^{R/R}$ mice. Top: Haematoxylin- eosin (HE) staining of cross-sections from 120 day-old mice. Bottom: Aberrations in elastic laminae in Fibulin-$4^{R/R}$ mice, consisting of a fragmented and disorganized appearance of elastin in the medial layers of the aorta. In heterozygous Fibulin-$4^{+/R}$ mice, interrupted elastin layers and areas in the aortic wall with a granular appearance of elastic laminae are present, albeit milder, indicating a gene dosage effect. Scale bar indicates 100 µm (B) Ex-vivo analysis shows a gradual increase in active MMPs as detected with the activatable NIRF probe MMPSense 680. Within the aneurysmal lesions in the thoracic aorta (Th), as well as in the abdominal aorta (Ab) of Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ mice, a graded increase in MMP activity is detected.

As inflammation is involved in aneurysm development, atherosclerosis, and COPD, we measured serum levels of the systemic inflammatory biomarker. The median serum hs-CRP concentration was higher in patients with AA compared to AOD (5.9 [IQR 2.9-12.5] vs 4.8 mg/L [IQR 2.1-11.1], p=.02). However, there were no differences in hs-CRP levels between arterial disease patients with COPD and those without COPD (median 5.4 vs 5.2 mg/L, p=.776; Figure 1B). These data strongly support the association between AA and COPD in patients independently of smoking and other cardiovascular risk factors.

Experimental study

Extracellular matrix remodeling in aortas of Fibulin-4 deficient mice

Complete disruption of Fibulin-4 is incompatible with life as targeted disruption of Fibulin-4 abolishes elastogenesis and causes perinatal lethality in mice.22 We previously generated a viable mouse model for Fibulin-4 using a hypomorphic Fibulin-4 allele (Fibulin4R) which results in reduced expression by transcriptional interference through placement of a TKneo targeting construct in a downstream gene (Mus81).14 While Mus81 knockout mice, from which the selectable marker was removed, were born at expected Mendelian frequencies and were indistinguishable from wild type littermates in terms of development, growth, immune function and fertility,23 our Fibulin-4 hypomorphic mice displayed a 2-fold lower expression of Fibulin-4 in heterozygous Fibulin-4+/R aortas and a 4-fold downregulation in homozygous Fibulin-4R/R aortas, resulting in ECM defects and vascular abnormalities, including aortic aneurysms in the aorta ascendens.14 Indeed, comparison of haematoxylin-eosin (HE) and elastin stained aortas of 3-month old Fibulin-4+/+, Fibulin-4+/R and Fibulin-4R/R mice showed severe thickening and decellularization of the medial layer of the ascending aortic wall in Fibulin-4R/R mice and fragmented and disorganized elastin laminae resulting in 2-3 fold dilated ascending aortic aneurysms in all homozygous knockdown animals analyzed (Figure 2A). While Fibulin-4+/R aortas are not dilated, careful histological comparison showed an increased medial thickness, signs of elastin breakage and increased deposition of amorphous cell material between the elastin layers compared to wild type animals (Figure 2A). The downregulation of Fibulin-4 leads to elastin abnormalities in the ascending aorta accompanied by extensive remodeling of the ECM presumably through activation of matrixmetalloproteinases (MMPs). The increased activity of MMPs can be visualized with MMPSense 680, an MMP-specific activatable near-infrared (NIRF) probe developed for in vivo imaging. Fibulin-4+/R and Fibulin-4R/R mice injected with this protease sensing probe show a gradual increase in NIRF signal in the thoracic area, indicative of aneurysm formation. Here, we injected this MMP activatable NIRF probe and sacrificed the mice after 24 hrs after which the hearts with aortas were excised. When we compared the ex vivo fluorescence in the aortic arch and descending aorta using the Odyssey imaging


A
Newborn
Adult
Relative mRNA expression
1.2
0.8
0.4
0
**
**
Fib-4+/+
Fib-4+/R
Fib-4R/R
B
PIF (ml/s)
PEF (ml/s)
3
2
1
0
00-03
03-06
06-09
09-12
12-15
15-18
Time interval (min)
C
Fibulin-4+/+
Fibulin-4+/R
Fibulin-4R/R
Adult
Newborn
D
D2 score (x 10² µm)
D2 score (µm)
120
80
40
*
Adult
Newborn

system, we measured a gradual increase of MMPSense 680 activation in Fibulin-4+/R and Fibulin-4R/R compared to wild type Fibulin-4+/+ mice (Figure 2B, upper row). Since this was consistent with the gradual elastin degradation we noticed histologically, we then used these protease sensing probes to analyze the abdominal part of the aorta, below the diaphragm. Ex-vivo analysis confirmed the in vivo observed gradual increase in MMPs (MMPSense 680) within the aneurysmal lesions in the thoracic aorta (Th) and showed as well a gradual increased activity in the abdominal aorta (Ab) in both Fibulin-4+/R and Fibulin-4R/R mice. Thus, decreased expression of fibulin-4 not only leads to MMP activation in the thoracic part of the aorta, but equally affects the abdominal aorta, predisposing the complete aorta for arterial disease. Since we find extracellular matrix remodeling activity in both thoracic and abdominal aorta, we conclude that the Fibulin-4+/R and Fibulin-4R/R mouse models mimics different stages of both TAA and AAA.

Alveolar airspace enlargement in Fibulin-4 deficient mice

In the clinical study, we observed a significant association between COPD and AA. To investigate whether ECM abnormalities may provide a common ground for aneurysm formation and COPD we subsequently analyzed whether fibulin-4 deficiency also predisposes for lung abnormalities in these Fibulin-4 hypomorfic mice.

To this end, we first tested whether the transcriptional downregulation of Fibulin-4 also occurs in the lungs of these mutant mice. Expression levels of Fibulin-4 mRNA in newborn and adult lungs of Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ mice were indeed significantly lower compared to Fibulin-$4^{+/+}$ mice (Figure 3A). Next, we examined whether Fibulin-4 animals display lung emphysema. Assessment of respiratory performance by whole-body

Figure 3 – Enlarged alveolar airspaces in lungs of Fibulin-4 knockdown mice.

(A) Expression levels of Fibulin-4 in lungs isolated from newborn (n=4, n=4, n=3) and adult (n=4, n=4, n=4) Fibulin-$4^{+/+}$, Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ mice relative to Fibulin-$4^{+/+}$ lungs (** $p<0.01$). (B) Mean peak inspiratory flow (PIF) and peak expiratory flow (PEF) values for Fibulin-$4^{+/+}$ (n=4), Fibulin-$4^{+/R}$ (n=4) and Fibulin-$4^{R/R}$ mice (observed for n=4, but two animals died during the procedure) at 3-minute intervals. After a 9 minute adaptation period (the first three time intervals), PIF follows similar trends in Fibulin-$4^{+/+}$, Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ mice, while Fibulin-$4^{R/R}$ mice show a decrease in PEF. (C) HE stained sections of formalin fixed lungs of male mice. Enlarged alveolar airspaces are observed in Fibulin-$4^{+/R}$ (middle, n=3) and Fibulin-$4^{R/R}$ lungs (right, n=3), with the latter being more pronounced, compared to Fibulin-$4^{+/+}$ (n=3). Enlarged alveolar airspaces are already present in Fibulin-$4^{R/R}$ newborn lungs (n=3), while lungs of Fibulin-$4^{+/R}$ littermates (n=5) show no difference compared to Fibulin-$4^{+/+}$ lungs (n=4). Scale bar 100 μm. Magnification 10x. (D) D_2 quantification (see methods and Supplemental Figure S1 for further explanation) of the alveolar airspaces revealed a significant difference between adult Fibulin-$4^{+/+}$ and Fibulin-$4^{+/R}$ (* $p<0.05$) and between adult Fibulin-$4^{+/+}$ and Fibulin-$4^{R/R}$ lungs (** $p<0.01$) as well as between newborn Fibulin-$4^{+/+}$ and Fibulin-$4^{R/R}$ lungs (* $p<0.05$).

plethysmography showed similar breathing frequencies and Peak Inspiratory Flows (PIF) in adult Fibulin-4 deficient mice and Fibulin-$4^{+/+}$ littermates, whereas the Peak Expiratory Flow (PEF) tended to decrease over time in Fibulin-$4^{R/R}$ mice (Figure 3B). Significance could not be determined since 2 out of 4 Fibulin-$4^{R/R}$ mice died during the course of the experiment.

Downregulation of Fibulin-4 was accompanied by alveolar airspace enlargement in adult Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ lungs (Figure 3C). In newborn mice, reduced pulmonary Fibulin-4 expression levels coincided with clear alveolar airspace enlargements in Fibulin-$4^{R/R}$ lungs, but not in Fibulin-$4^{+/R}$ lungs (Figures 3C and 3D, and accompanying Supplemental Figure 1A and B). Importantly, analysis of the aortas of the adult mice used for lung analysis showed a gradual thickening of the medial layers of the aorta (Supplemental Figure 1C). Elastin staining showed sites of complete fragmentation and disarray of the elastin layers in Fibulin-$4^{R/R}$ aortas and increased deposition of amorphous material between the elastin layers in Fibulin-$4^{+/R}$ animals (Supplemental Figure 1D), indicating that a similar gene-doses decrease in Fibulin-4 expression affects both lungs and the aortic wall in these mice. Immunohistochemistry on lung tissue with antibodies specific for certain lung cell markers, including thyroid transcription factor 1 (TTF-1), Clara-cell-specific protein (CC-10), and α-smooth muscle actin (α-SMA) demonstrated no differences in the presence and relative distribution of the major cell types in the lungs of Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ mice (Supplemental Figure 2), which may exclude altered airway-cell differentiation.

These results show that in addition to aortic abnormalities, a decrease in Fibulin-4 expression leads to gene dose-dependent alterations in the lung. While the emphysematous changes in the lungs of newborn Fibulin-$4^{R/R}$ mice suggest a developmental defect, Fibulin-$4^{+/R}$ mice acquired the COPD phenotype with age.

Transcriptome analysis of Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ lungs

In order to get an idea of the underlying processes involved, we performed gene expression analysis on mRNA isolated from Fibulin-$4^{+/+}$, Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ lung tissue, both newborn as well as adult. Comparison of RNA expression between newborn Fibulin-$4^{+/+}$, Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ lungs with Significance Analysis of Microarrays revealed a limited set of differentially regulated genes.[24] Comparison of RNA expression between adult Fibulin-$4^{+/+}$ and Fibulin-$4^{R/R}$ lungs using SAM (FDR 10%) revealed 374 deregulated genes (both up- and downregulated), whereas no deregulated genes were found between Fibulin-$4^{+/+}$ and Fibulin-$4^{+/R}$ lungs.

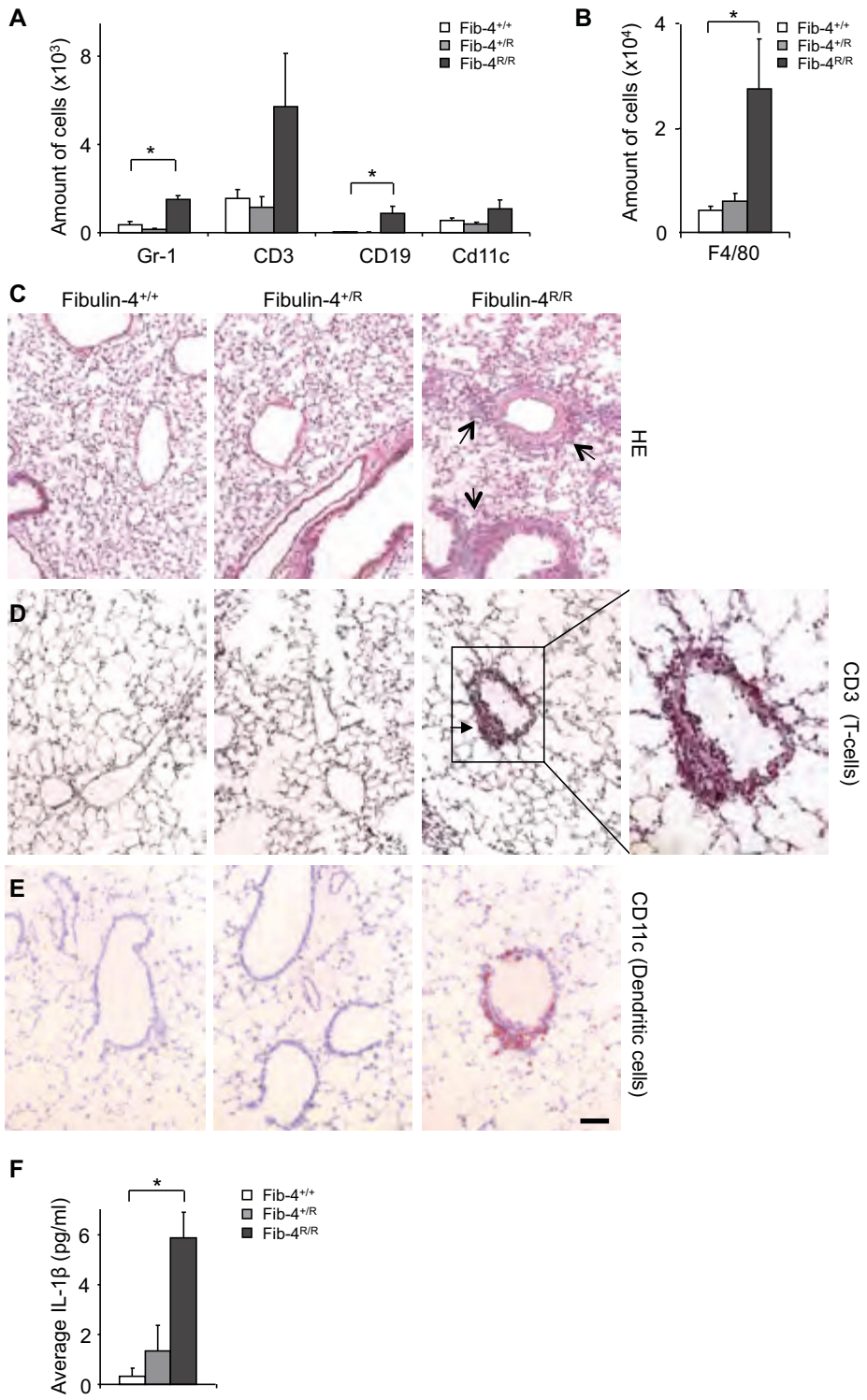
Table 2 - The most significantly up-regulated genes in adult Fibulin-$4^{R/R}$ lungs. Genes are indicated with their ratios compared to Fibulin-$4^{+/+}$ lungs and the process involved.

Top up-regulated genes		
Genes	**Ratio**	**Function**
Arg1	3.68	Urea cycle
Slpi	3.37	Inhibitor serine proteases
Ms4a4b	2.38	T-cell regulation
Wisp2	2.26	Inhibits proliferation of vascular smooth muscle cells
Prkcb	2.03	B-cell activation, apoptosis
Emr4	1.98	Mediate between myeloid- and B-cells
Cd300a	1.92	Leukocyte cell surface proteins
Gzma	1.93	Cytotoxic T-cell and natural killer cell specific serine proteases
Klra4	1.91	Natural killer cell receptor
Nkg7	1.90	Natural killer cell granule protein
Ctsw	1.84	Regulation of T-cell cytolytic activity
Wisp1	1.83	Matrix remodeling
Lrat	1.64	Retinoid cycle
Plac8	1.81	Defense response to bacterium
Ccl5	1.78	Chemotactic cytokine and plays active role in recruiting leukocytes
Tspan32	1.73	Tumor suppressing fragment
Cyp51a1	1.70	Production of sterols
Rbm3	1.68	Temperature induced
Bcl2	1.66	Apoptosis regulator
Mef2c	1.65	Transcription factor important for vascular development

Of the 20 most significantly up-regulated genes in adult Fibulin-$4^{R/R}$ lungs, 50% were involved in inflammation processes (Table 2 and Supplemental Table 4). Network analysis with Ingenuity pathway analysis (IPA) on the 374 deregulated probes revealed many significantly changed pathways involved in the immune system (Supplemental Table 5). This suggests that the severe airspace enlargement in adult Fibulin-$4^{R/R}$ lungs coincides with overexpression of genes involved in inflammatory processes.

Spontaneous inflammation in adult Fibulin-$4^{R/R}$ lungs

To investigate whether indeed the immune system shows significant alterations in the lungs of Fibulin-$4^{R/R}$ animals, we did flow cytometric analysis of broncho-alveolar lavage (BAL) samples. This analysis showed more inflammatory cells, in particular granulocytes (Gr-1+) and B-cells (CD19+), in lungs of adult Fibulin-$4^{R/R}$ compared to Fibulin-$4^{+/+}$ and Fibulin-$4^{+/R}$ mice (Figure 4A). Cell suspensions of Fibulin-$4^{R/R}$ lungs contained significantly more macrophages (F4/80+) compared to Fibulin-$4^{+/+}$ and Fibulin-$4^{+/R}$ lungs (Figure 4B), and


A
Amount of cells (x10³)
8
4
0
*
*
Gr-1
CD3
CD19
Cd11c
Fib-4+/+
Fib-4+/R
Fib-4R/R
B
Amount of cells (x10⁴)
4
2
0
*
F4/80
Fib-4+/+
Fib-4+/R
Fib-4R/R
C
Fibulin-4+/+
Fibulin-4+/R
Fibulin-4R/R
HE
D
CD3 (T-cells)
E
CD11c (Dendritic cells)
F
Average IL-1β (pg/ml)
6
4
2
0
*
Fib-4+/+
Fib-4+/R
Fib-4R/R

tended to contain more T-cells (CD3+) and dendritic cells (CD11c+). Immunohistochemical analysis showed focal infiltrations of inflammatory cells around veins and bronchi in adult Fibulin-$4^{R/R}$ lungs (Figure 4C), mainly consisting of T-cells and dendritic cells. Cytokine analysis of BAL samples showed significantly higher levels of IL-1β in Fibulin-$4^{R/R}$ but not in Fibulin-$4^{+/R}$ as compared to Fibulin-$4^{+/+}$ mice (Figure 4D) Interestingly, IL-1b is a pro-inflammatory cytokine which is mainly produced by activated macrophages and which is increased in patients with COPD.[25, 26] These data indicate that the severe airspace enlargement observed in lungs of adult Fibulin-$4^{R/R}$ mice was accompanied by up-regulation of inflammatory pathways, whereas the milder lung abnormalities in Fibulin-$4^{+/R}$ animals were not associated with an explicit inflammation process.

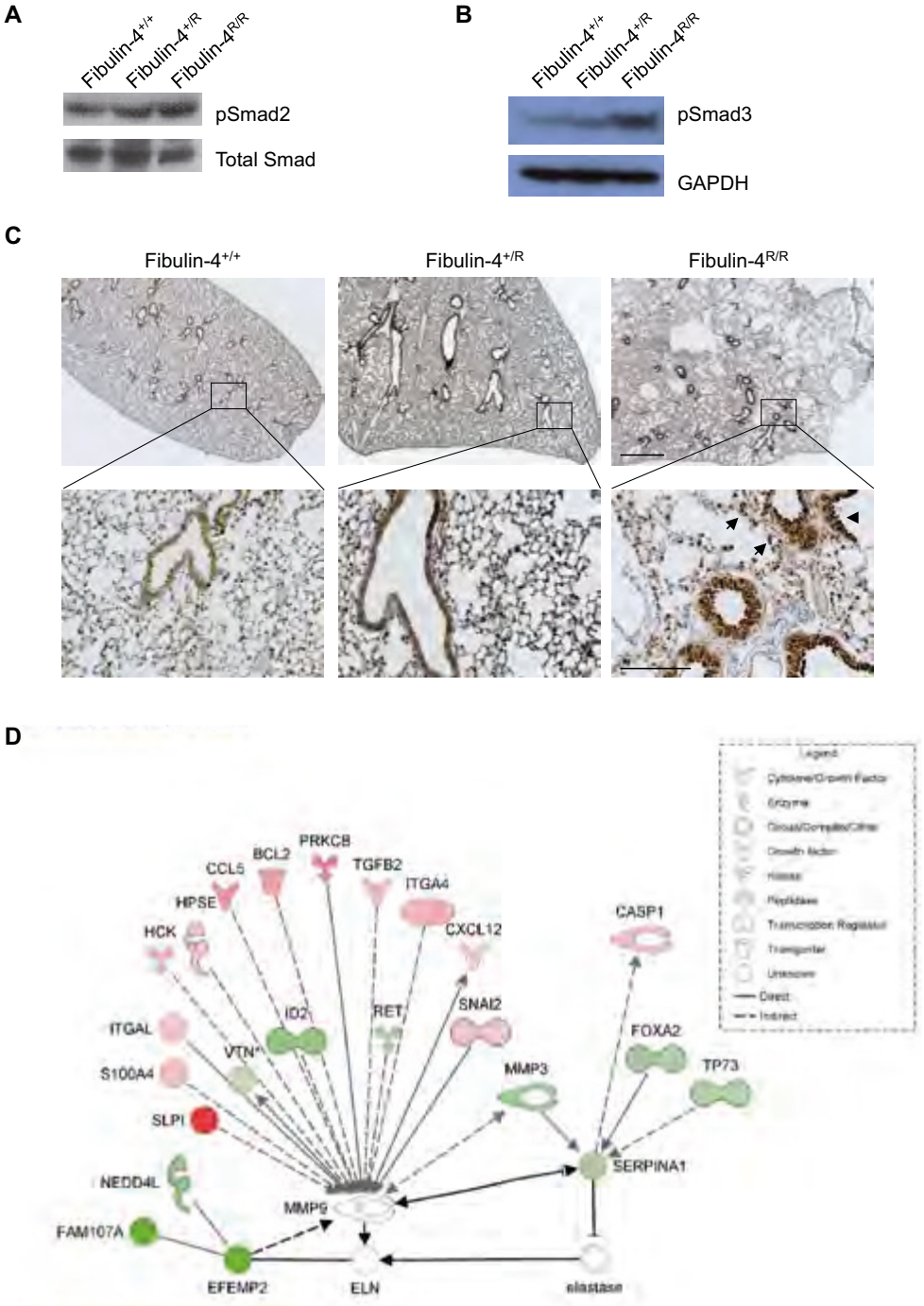
Disturbed TGF-ß signaling in Fibulin-4 deficient lungs

Since degradation of the vascular wall in aortic aneurysms is related to disturbances in the TGF-β signaling pathway,[14, 27] we next investigated the role of TGF-β signaling in alveolar wall degradation in Fibulin-4 deficient mice. Although the gene expression analysis in lung mRNA samples only gave rise to a limited set of deregulated genes in newborn Fibulin-$4^{R/R}$ animals, it did reveal downregulation of the Pias4 gene in Fibulin-$4^{R/R}$ compared to Fibulin-$4^{+/+}$ lungs (1.2-fold, $p<0.05$, Supplemental Table 6). In adult Fibulin-$4^{R/R}$ lungs we identified up-regulation of TGF-β2 and downregulation of the type 2b activin A receptor. In adult Fibulin-$4^{+/R}$ lungs the 'SMAD specific E3 ubiquitin protein ligase 1' (Smurf1) gene was significantly downregulated compared to Fibulin-$4^{+/+}$ lungs.

To check at the protein level whether changes in the TGF-β system occurred, we performed immunoblot analysis for phosphorylation of Smad2 (pSmad2), an intracellular mediator of the TGF-β pathway. These blots showed a gradual increase in pSmad2 in adult Fibulin-4 deficient lungs, indicating increased TGF-β activity (Figure 5A). In Fibulin-$4^{+/R}$ lungs we observed a 1.32-fold change for pSmad2 relative to total Smad and a 1.23-fold change relative to actin when compared to Fibulin-$4^{+/+}$ lungs. In Fibulin-$4^{R/R}$ lungs we observed a 1.67-fold and 1.5-fold change, respectively (Figure 5A and data not

Figure 4 – Increased inflammation in Fibulin-$4^{R/R}$ lungs compared to Fibulin-$4^{+/+}$ and Fibulin-$4^{+/R}$ lungs.

(A) Flow cytometric analysis revealed more Gr1+ granulocytes and CD19+ B-cells in BAL samples from Fibulin-$4^{R/R}$ (n=4) compared to Fibulin-$4^{+/+}$ mice (n=4, * $p<.05$) and (B) increased numbers of F4/80 macrophages in Fibulin-$4^{R/R}$ lungs (n=4, * $p<.05$). (C) HE stained sections from adult (n=4, n=4, n=4) Fibulin-$4^{+/+}$, Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ lungs showing focal infiltrations around vessels and airways in Fibulin-$4^{R/R}$ lungs (black arrows). (D) Staining for T-cells (CD3+) and (E) dendritic cells (CD11c+) points to the presence of inflammatory cells within the focal infiltrations. Magnification 20x. Scale bar 50 μm. (F) ELISA analysis showing increased IL-1β levels in Fibulin-$4^{R/R}$ lungs (n=4, * $p<.05$).


A
Fibulin-4+/+
Fibulin-4+/R
Fibulin-4R/R
pSmad2
Total Smad
B
Fibulin-4+/+
Fibulin-4+/R
Fibulin-4R/R
pSmad3
GAPDH
C
Fibulin-4+/+
Fibulin-4+/R
Fibulin-4R/R
D
PRKCB
BCL2
CCL5
TGFB2
ITGA4
HPSE
CXCL12
HCK
CASP1
ID2
RET
SNAI2
ITGAL
FOXA2
VTN
MMP3
TP73
S100A4
SLPI
SERPINA1
NEDD4L
MMP9
FAM107A
EFEMP2
ELN
elastase

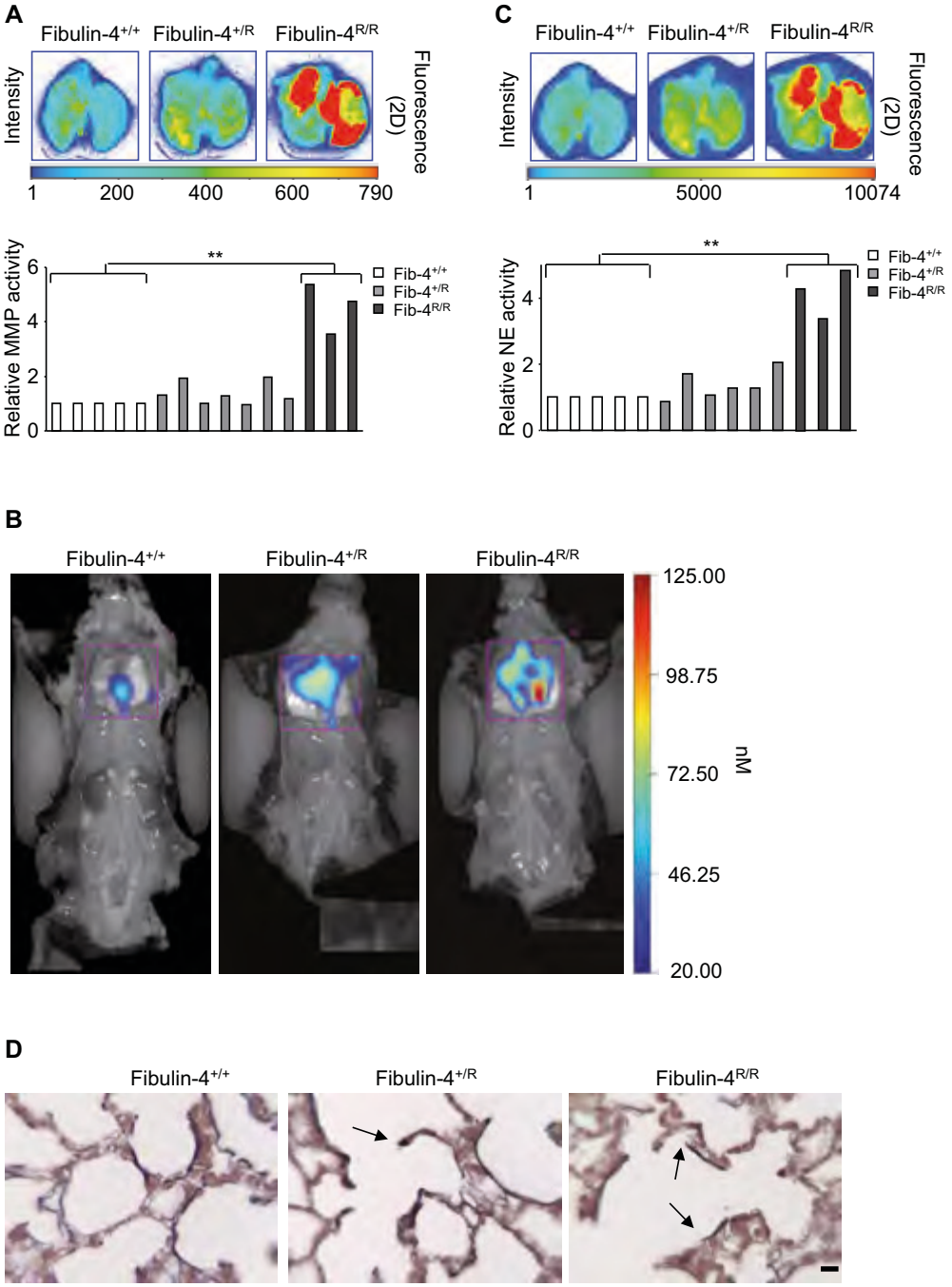
shown). Accumulation of phosphorylated smad2 is a general read out of activity for the TGF-β signaling pathway. Smad4 binding to phosphorylated Smad2 is necessary for translocation to the nucleus Subsequently the smad2/3/4 complex can then bind to the DNA after which transcription is initiated. We therefore also determined phosphorylation of pSmad3 in protein extracts of the lungs of Fibulin-$4^{+/+}$, Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ mice and find a gradual increase of pSmad3 in the mutant animals confirming the activation of the central TGF-β transcription factor Smad2/3/4 (Figure 5B). Immunohistochemistry confirmed increased pSmad2 expression in Clara cells lining the bronchioles of the lungs as well as in inflammatory cell infiltrates (Figure 5C). Together these data show that TGF-β activity is mildly increased in adult Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ lungs, which may contribute to the breakdown of alveolar walls in adult Fibulin-4 deficient mice. This upregulation of TGF-β signaling is reminiscent of the upregulation that has been observed in the aortas of both mouse models of aortic aneurysms as well as in patients.

Overlapping downregulation of *SERPINA1* in lungs of Fibulin-4 deficient mice and COPD patients

To investigate a potential common underlying mechanism of the observed lung emphysema phenotype in our Fibulin-4 animals and that in COPD patients, we compared our mouse dataset to gene lists related to COPD that we derived from IPA and gene expression datasets from lung emphysema patients. A search in IPA with the search term 'chronic obstructive pulmonary disease' gave 248 records, which we refer to as 'COPD-related genes'. Next, gene expression data from the comparison between Fibulin-$4^{+/+}$ and Fibulin-$4^{R/R}$ lungs (374 genes) were compared to this list of COPD-related genes derived from IPA, where we found an overlap of 6 genes: *PDE3B* (1.28 ↑), *HCK* (1.55 ↑), *PRF1* (1.47 ↑), *SERPINA1* (1.38 ↓), *FGFR3* (1.28 ↓), and *EFEMP2* (i.e. Fibulin-4, 3.57 ↓).

Figure 5 – Increased TGF-β signaling in Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ lungs.

Immunoblot analysis of pSmad2 in lung homogenates shows an increase in the amount of pSmad2 (A) and pSmad3 (B) in Fibulin-$4^{+/R}$ (n=3) and Fibulin-$4^{R/R}$ (n=3) lungs, compared to the total amount of Smad, and to their Fibulin-$4^{+/+}$ control (n=3). (C) Increased pSmad2 staining of inflammatory and endogenous cells on Fibulin-$4^{+/R}$ (n=3) and Fibulin-$4^{R/R}$ (n=3) lung sections. Magnification 2.5x (scale bar 1 mm) upper panel and 20x (scale bar 200 μm) lower panel. (D) Ingenuity pathway explorer showed *MMP9* as the shortest connection between Fibulin-4 and *SERPINA1*. *SERPINA1* inhibits neutrophil elastase, which affects elastin. *MMP9* itself was not deregulated in Fibulin-$4^{R/R}$ lungs (n=4), but could be connected to 16 deregulated genes in Fibulin-$4^{R/R}$ compared to Fibulin-$4^{+/+}$ lungs (n=4, red, up-regulated; green, downregulated), suggestion altered *MMP9* activity. Black arrows indicate the connection between Fibulin-4 (*EFEMP2*), *MMP9*, *SERPINA1*, elastase and *ELN*. Grey arrows indicate the connection of these genes with deregulated genes between Fibulin-$4^{+/+}$ and Fibulin-$4^{R/R}$ lungs.


A
Fibulin-4+/+
Fibulin-4+/R
Fibulin-4R/R
Intensity
Fluorescence (2D)
1
200
400
600
790
Relative MMP activity
**
Fib-4+/+
Fib-4+/R
Fib-4R/R
C
Fibulin-4+/+
Fibulin-4+/R
Fibulin-4R/R
Intensity
Fluorescence (2D)
1
5000
10074
Relative NE activity
**
Fib-4+/+
Fib-4+/R
Fib-4R/R
B
Fibulin-4+/+
Fibulin-4+/R
Fibulin-4R/R
125.00
98.75
72.50
46.25
20.00
nM
D
Fibulin-4+/+
Fibulin-4+/R
Fibulin-4R/R

In a second analysis, we compared the 374 deregulated mouse genes to a list of 125 deregulated genes from the comparison of GEO dataset GSE8581 (1.5-fold, FDR 30%), consisting of 15 COPD cases (predicted FEV1<70%, FEV1/FVC<0.7) and 18 control cases (predicted FEV1>80%, FEV1/FVC>0.7). This comparison showed an overlap of ITPKC (1.28 ↓), KIAA1377 (1.45 ↓), and *SERPINA1* (1.38 ↓). Remarkably, these two independent methods both identified *SERPINA1* as an overlapping downregulated gene. *SERPINA1* encodes for the serine protease inhibitor α-1 antitrypsin, whose targets include elastase. Interestingly, deficiency in α-1 antitrypsin in patients is associated with lung emphysema.[28, 29]

In the mouse lung mRNA gene expression analysis *SERPINA1* was significantly downregulated in both Fibulin-$4^{R/R}$ lungs (1.38-fold, $p<0.01$) as well as in Fibulin-$4^{+/R}$ lungs (1.59-fold, $p<0.01$) compared to Fibulin-$4^{+/+}$ lungs. We used Path explorer in IPA that identifies pathways between differentially expressed genes, in order to determine the relation between Fibulin-4 and *SERPINA1*. By calculating the shortest path between Fibulin-4 (*EFEMP2*) and *SERPINA1*, an indirect connection of Fibulin-4 to *MMP9* and a direct connection of *MMP9* to *SERPINA1* was revealed, as indicated by the black arrows in Figure 5D.[14, 30, 31] Surprisingly, by connecting Fibulin-4, *MMP9*and *SERPINA1* (both direct and indirect) to the significantly deregulated genes identified in the SAM comparison between Fibulin-$4^{+/+}$ and Fibulin-$4^{R/R}$ lungs (grey arrows), we found an interaction between 16 of those significantly deregulated genes and our dataset with *MMP9* (Figure 5C). Importantly, MMPs are proteins involved in remodeling of the ECM, and play a role in aneurysm formation. As we hypothesized that ECM defects provide the link between the relation between AA and COPD, MMPs could very well be part of the underlying mechanism. Since our gene expression analysis did not show a deregulation of *MMP9* at the mRNA level, yet the pathway analysis pointed towards involvement of *MMP9*, we next investigated *MMP9* protein activity in Fibulin-$4^{R/R}$ and Fibulin-$4^{+/R}$ lungs.

Figure 6 – Higher MMP and NE activity in Fibulin-4+/R and Fibulin-4R/R lungs.

In (A) and (C) *ex vivo* imaging of excised lungs using Odyssey shows increased activity of MMP and NE respectively in Fibulin-$4^{+/R}$ (n=7) and Fibulin-$4^{R/R}$ lungs (observed for n=5, but two animals died during the procedure) as compared to Fibulin-$4^{+/+}$ lungs (n=5), with a significant upregulation for Fibulin-$4^{R/R}$ lungs (** $p<0.01$). (B) Open-chest registration of NE activity with Neutrophil Elastase FAST 680 probes shows increased activity in Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ lungs as compared to Fibulin-$4^{+/+}$ lungs. (D) Elastin staining of Fibulin-$4^{+/+}$, Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ lungs (n=3, n=3, n=3) shows fragmented elastin layers in Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ lungs, indicated by arrows. Magnification 40x. Scale bar 10 μm.

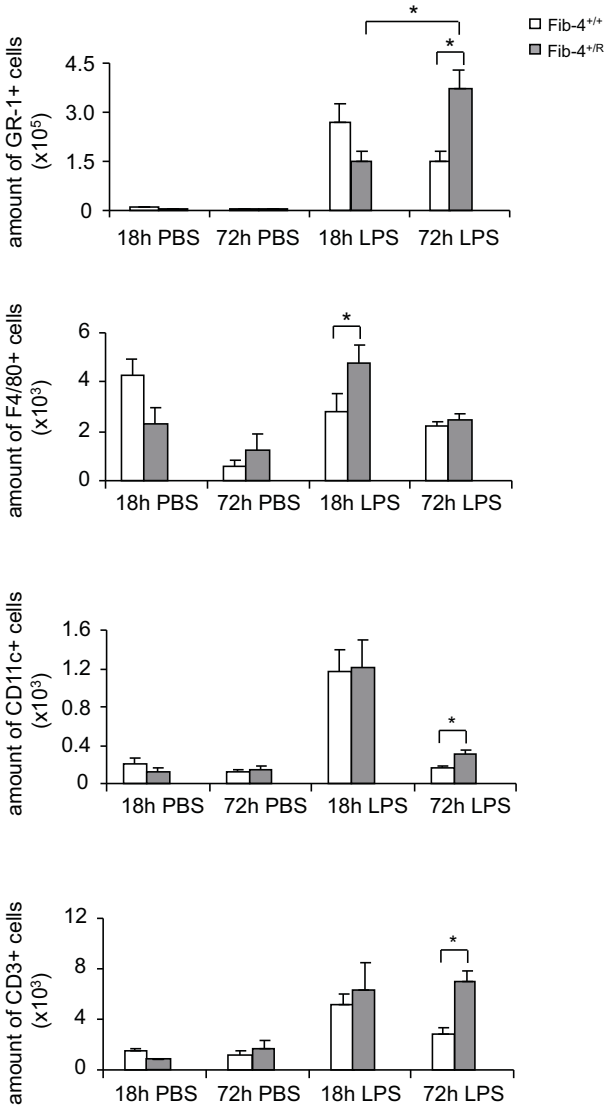


Figure 7 – Increased and prolonged inflammatory response in LPS exposed Fibulin-$4^{+/R}$ mice.

Quantification of immune cells shows significantly increased F4/80+ cells after 18 hours of LPS exposure in Fibulin-$4^{+/R}$ lungs (n=4) and a significantly higher number of Gr1+, CD11c+, and CD3+ cells after 72 hours of LPS exposure as compared to Fibulin-$4^{+/+}$ lungs (n=4, * $p<0.05$).

Increased MMP and neutrophil elastase activity in Fibulin-4 deficient lungs

Fluorescent imaging using protease activatable probes showed increased pulmonary MMP activity in adult Fibulin-4 deficient mice (Figure 6A). The observed decrease in the elastase inhibitor *SERPINA1* and the increased MMP activity, which is also associated with cleavage of α-1 antitrypsin,[30] might lead to increased activity of neutrophil elastase (NE) (also see Figure 5C for this relation). Indeed, we observed a graded increase in NE activity in adult Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ lungs compared to Fibulin-$4^{+/+}$ lungs (Figures 6B and C). In line with this, pulmonary elastin staining demonstrated interruptions in the elastin layers in Fibulin-$4^{+/R}$ lungs and even more in Fibulin-$4^{R/R}$ lungs compared to those of Fibulin-$4^{+/+}$ animals (Figure 6D), as previously found in the aortas of these mice.[14] Newborn Fibulin-$4^{R/R}$ lungs also displayed elastin abnormalities, while newborn Fibulin-$4^{+/R}$ lungs were comparable to those of Fibulin-$4^{+/+}$ mice (data not shown).

Increased and prolonged inflammatory response in lipopolysaccharide (LPS) exposed Fibulin-$4^{+/R}$ mice

Fibulin-$4^{+/R}$ mice developed alveolar airspace enlargement with age together with increased MMP9 and NE activity in the absence of inflammation. However, when adult Fibulin-$4^{+/R}$ lungs were triggered by LPS administration, which mimics bacterial infection in mice by initiating the infiltration of inflammatory cells into the pulmonary alveoli similar to patients with COPD exacerbation,[32, 33] flow cytometric analyses of BAL samples and pulmonary cell suspensions showed an increased and prolonged inflammatory response as compared to Fibulin-$4^{+/+}$ mice. There was a significantly greater influx of macrophages (F4/80+) in the lungs 18 hours after LPS exposure and significantly higher numbers of dendritic cells (CD11c+), T-cells (CD3+), and granulocytes (GR1+) 72 hours after LPS exposure (Figure 7 and Supplemental Figure 3). Moreover, opposite dynamics were observed; in the Fibulin-$4^{+/+}$ lungs the amount of GR1+ cells decreased after 18 hours, while an increase was observed in Fibulin-$4^{+/R}$ lungs 72 hours after LPS exposure. The increase of inflammatory cells after LPS exposure was significantly higher when compared to PBS. The levels of pro-inflammatory cytokines released upon LPS exposure, including IL-1β, TNF-α, and keratinocyte-derived chemokine, were not different between groups (data not shown). These data indicate that Fibulin-$4^{+/R}$ mice exhibit an intensified inflammatory response in the lungs.

DISCUSSION

In this study we show that COPD is more common in patients with AA than in patients with atherosclerotic arterial disease. This relationship was independent of cigarette smoking and other known risk factors. Furthermore, there was no difference in serum hs-CRP levels between patients with and without COPD, indicating that inflammation per se is unlikely to account for the observed relation between COPD and AA. The findings

in this large patient cohort are in line with previous observations of reduced respiratory function in smaller series of AAA patients.[6,7,10] Although some previous studies concluded that the association between COPD and aneurysm formation was related to smoking, medication use or presence of other cardiovascular risk factors, these associations became non-significant after correction in multivariable analyses.[34,35] Our findings suggest that factors other than cardiovascular risk profiles or systemic inflammation contribute to the association between COPD and AA.

Since both diseases, COPD and AA, are characterized by breakdown of the ECM in the airways and –spaces and in the aortic wall, we investigated whether a primary ECM defect provides the pathogenic link between these two diseases. Analogous to the observed degradation of the aortic wall, up-regulation of MMP activity both thoracic as well as abdominal, and previously observed formation of AAs in mice deficient in the ECM component Fibulin-4, we found that gradual downregulation of Fibulin-4 in the lungs correlated with destruction of alveolar walls and airspace enlargement that is characteristic for lung emphysema. Similar to embryonically lethal, complete Fibulin-4 knockout mice,[22] alveolar breakdown was already present in lungs of newborn Fibulin-$4^{R/R}$ mice, and became progressive with age. In contrast, Fibulin-$4^{+/R}$ mice, which have only a 2-fold reduction in the amount of Fibulin-4, had normal elastin structures and alveolar airspaces at birth, but acquired alveolar breakdown with ageing.

Analogous to the activation of the TGF-β pathway in the aortas of Fibulin-4 deficient mice,[14] we here demonstrate enhanced activation of the TGF-β pathway in the lungs of Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ mice. The co-occurrence of lung emphysema and vascular abnormalities in association with deregulated TGF-β signaling has also been shown in another mouse model with a deficiency in an ECM protein, Fibrillin-1, which is a model for Marfan syndrome.[36] Moreover, the combination of pulmonary emphysema and aortic aneurysms coinciding with upregulation of TGF-β signaling has also been observed in autosomal recessive cutis laxa syndrome caused by Fibulin-4 mutations.[37-39] The role for TGF-β in this process is further supported by the development of progressive airspace enlargement in *Smad3* knockout mice, which are deficient for an intracellular regulator of the TGF-β pathway.[40] Overall, these data point to deregulated TGF-β signaling and ECM defects as common underlying factors for aortic and pulmonary abnormalities.

Expression analysis further revealed downregulation of the *SERPINA1* gene, encoding for the serine protease inhibitor α-1 antitrypsin whose targets include elastase. Interestingly, overlapping gene expression profiles of our Fibulin-4 deficient mice with those of COPD patients revealed downregulation of *SERPINA1* as a common denominator. As it is known that patients with α-1 antitrypsin deficiency develop COPD,[28,41] we explored the link between *SERPINA1* and Fibulin-4. Pathway exploration in IPA revealed a direct link to *MMP9*, TGF-β deregulation and 15 other deregulated genes from our dataset. Although *MMP9* itself was not overexpressed, molecular imaging showed that the MMP activity

was gradually higher in Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ lungs. In line with the observed downregulation of *SERPINA1*, fluorescent imaging showed a gradual up-regulation of elastase in Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ lungs, which correlated with elastin fragmentation. The decreased expression of *SERPINA1* may either be a direct effect of Fibulin-4 deficiency, or an indirect effect through its cleavage by *MMP9*.[30] This combination of increased protease activity and decreased antiprotease activity may account for the breakdown of alveolar walls, resulting in emphysema.

Another hallmark of adult Fibulin-4 mice was the inflammatory response in the lungs. Lungs of adult Fibulin-$4^{R/R}$ mice already displayed pulmonary inflammation in a specific pathogen free environment, including influx of a wide range of inflammatory cells with elevated levels of the pro-inflammatory cytokine IL-1β, which coincided with the overexpression of genes involved in inflammatory pathways. In contrast, adult Fibulin-$4^{+/R}$ animals did not exhibit pulmonary inflammation under baseline conditions, but displayed an enhanced respiratory inflammatory response upon LPS inhalation. These findings indicate that although inflammation may contribute to the progressive breakdown of alveolar walls in adult Fibulin-$4^{R/R}$ mice, it is unlikely to be the primary causative factor in Fibulin-$4^{+/R}$ mice. Conversely, ECM degradation by proteases is known to induce the release of bioactive fragments that may act as chemo-attractants for leukocytes and modulate the activity of resident immune cells.[42] Our data suggest that a mild Fibulin-4 deficiency induces disruption of the ECM, which subsequently predisposes to an enhanced inflammatory response with further breakdown of alveolar walls. This vicious circle is further exacerbated by the diminished antiprotease capacity of the lungs and ultimately results in the development of pulmonary emphysema. The Fibulin-$4^{R/R}$ mouse can therefore provide as a model for adverse lung development, while the heterozygous Fibulin-$4^{+/R}$ mouse may serve as a postnatal challenge model.

The traditional inflammatory model of COPD proposes that in susceptible patients cigarette smoking leads to inflammation, which subsequently induces loss of ECM and alveoli, resulting in airspace enlargement. We propose that genetic ECM defects are one of the initiating events contributing to this susceptibility, which are associated with a heightened inflammatory response to environmental triggers, such as microorganisms and smoking. Such a generalized genetic susceptibility to ECM degradation and secondary inflammation in combination with increased protease activity and decreased anti-protease activity might be the common pathophysiologic mechanism underlying the tissue destruction in both COPD and aneurysm formation. Genetic screening for mutations related to ECM defects may be a new strategy to identify people at risk for developing both aneurysms and COPD with age.

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SUPPLEMENTARY MATERIALS

Figure S1. Pseudo coloring of haemotoxylin-eosin (HE) images to identify individual alveolar airspaces in newborn Fibulin-$4^{R/R}$ lungs and adult Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ lungs, and comparison of the architecture of the aortic wall of Fibulin-$4^{+/+}$, Fibulin-$4^{+/R}$, and Fibulin-$4^{R/R}$ mice.

Figure S2. Similar cell structures in wild type and Fibulin-4 knockdown lungs.

Figure S3. LPS infection induces infiltration of inflammatory cells in the alveolar compartments.

Table S1. Clinical characteristics of patients with descending thoracic aortic aneurysm (TAA) or abdominal aortic aneurysm (AAA).

Table S2. COPD in patients with descending thoracic aortic aneurysm (TAA) or abdominal aortic aneurysm (AAA).

Table S3. Association between COPD and aneurysmal disease.

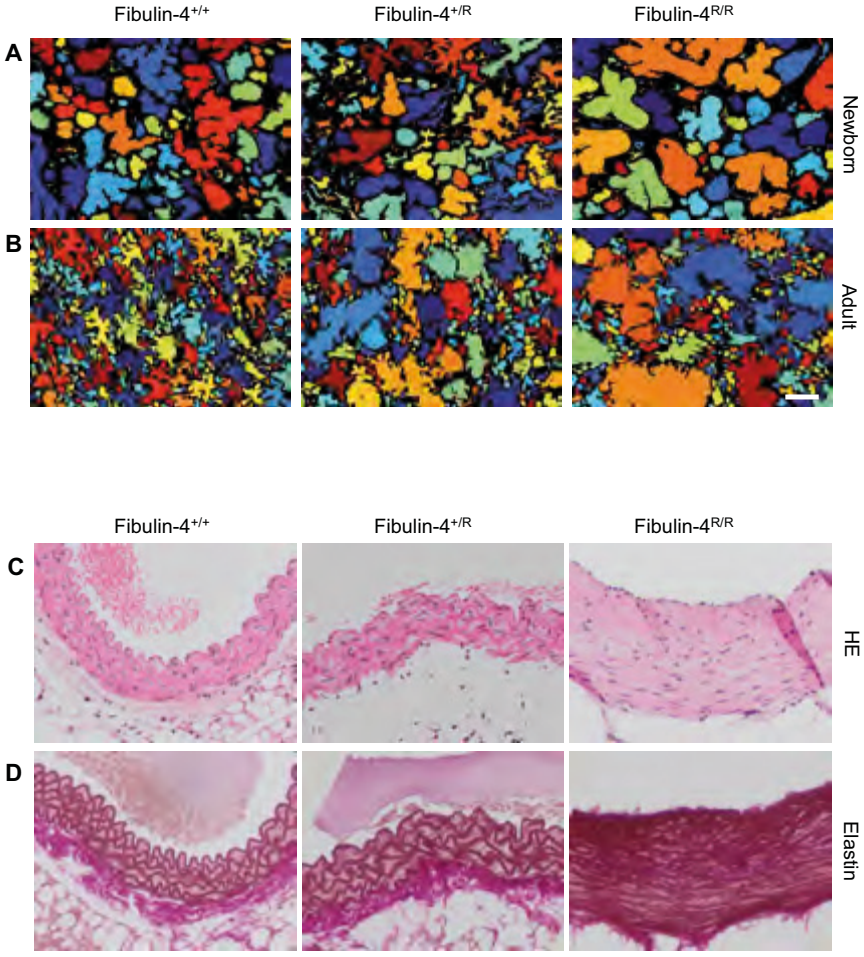
Table S4. The most significantly down-regulated genes in lungs of adult Fibulin-$4^{R/R}$ mice.

Table S5. Over-expressed canonical pathways, based on IPA, in lungs of adult Fibulin-$4^{R/R}$ mice ($p<0.05$).

Table S6. Deregulated TGF-β pathway genes in adult and newborn Fibulin-4 deficient lungs compared to Fibulin-$4^{+/+}$ lungs ($p<0.05$).

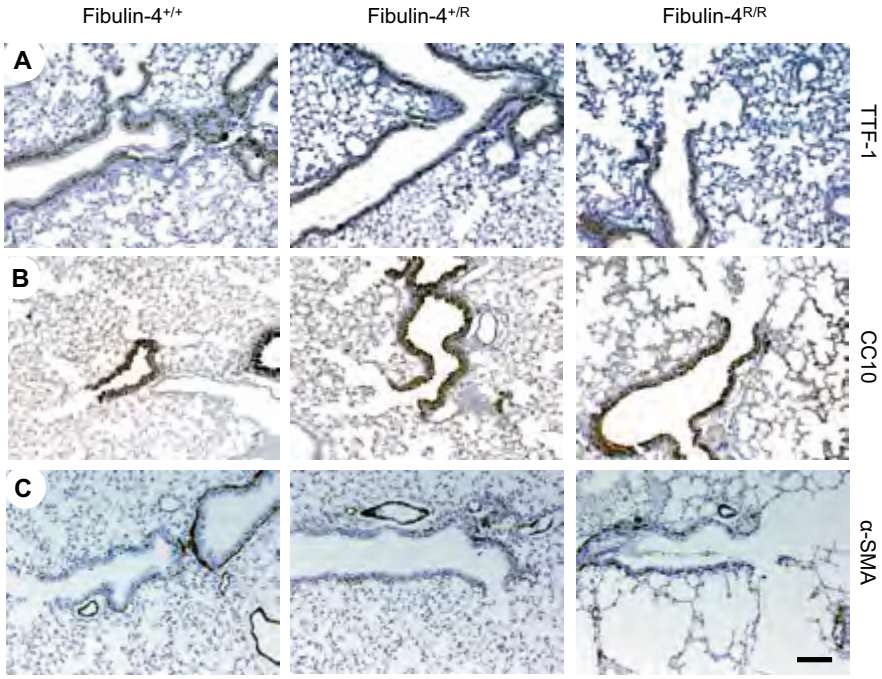
Table S7. Primers used for quantitative real time PCR. Forward and reverse primers are displayed for each gene from 5' to 3'.

SUPPLEMENTAL FIGURES



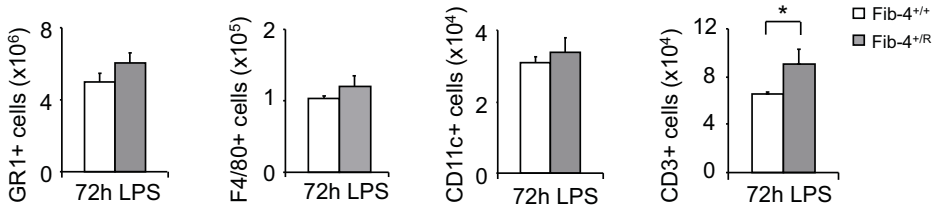
Supplemental Figure S1 – Larger alveolar airspaces in newborn Fibulin-$4^{R/R}$ lungs (A) and adult Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ lungs (B).

To quantify and compare the sizes of the alveolar airspaces, the compartments from the different alveolar airspaces were segmented on the HE images according to the method described in the "Lung morphometry" section of the Material and Methods section. Each segmented compartment was given a different color as shown and subsequently quantified as described. Magnification 10x. Scale bar 100 μm. (C, D) Comparison of the architecture of the aortic wall in Fibulin-$4^{+/+}$, Fibulin-$4^{+/R}$, and Fibulin-$4^{R/R}$ mice used for alveolar airspace analysis in Figure 3C. Haematoxylin- eosin (HE) staining of cross-sections from 120 day-old mice (C). Aberrations in elastic laminae in Fibulin-$4^{R/R}$ mice, consisting of a fragmented and disorganized appearance of elastin in the medial layers of the aorta (D).



Supplemental Figure S2 – Similar cell structures in wild type and Fibulin-4 knockdown lungs.

Stainings for (A) respiratory epithelial cells with TTF-1, (B) Clara cells with CC10 and (C) smooth muscle cells with α-SMA show similar cell structures in Fibulin-$4^{+/+}$ (n=3), Fibulin-$4^{+/R}$ (n=3) and Fibulin-$4^{R/R}$ (n=2) lungs. Magnification 10x. Scale bar 100 µm.



Supplemental Figure S3 – LPS infection induces infiltration of inflammatory cells in the alveolar compartments.

Quantification of immune cells in alveolar compartments shows increased CD3+ cells after 72 hours of LPS exposure in Fibulin-$4^{+/R}$ (n=4) as compared to Fibulin-$4^{+/+}$ lungs (n=4, * $p<0.05$).

SUPPLEMENTAL TABLES

Supplemental Table S1 – Clinical characteristics of patients with descending thoracic aortic aneurysm (TAA) or abdominal aortic aneurysm (AAA).

	TAA	AAA	P-value
	n=62	n=552	
Baseline characteristics			
Male gender (%)	37 (59.7)	488 (88.4)	<.001
Age (years ± SD)	69.0 ± 8.7	71.6 ± 7.6	.013
Body mass index (kg/m2, mean ± SD)	25.2 ± 4.1	26.2 ± 3.8	.054
Cardiovascular comorbidities (%)			
Congestive heart failure	4 (6.5)	62 (11.2)	.264
Ischemic heart disease	16 (25.8)	256 (46.4)	.002
Cerebrovascular disease	5 (8.1)	84 (15.2)	.129
Cardiovascular risk factors (%)			
Kidney disease	4 (6.5)	90 (16.3)	.041
Diabetes mellitus	7 (11.3)	96 (17.4)	.223
Hypertension	44 (71.0)	364 (65.9)	.340
Hypercholesterolemia	53 (85.5)	481 (87.1)	.714
Smoking – current	22 (35.5)	214 (38.8)	.776
Smoking – ever	45 (72.6)	428 (77.5)	.379
Medication (%)			
Statins	44 (71.0)	402 (72.8)	.986
Beta-blockers	48 (77.4)	483 (87.5)	.087
Renin-angiotensin system inhibitors	31 (50.0)	240 (43.5)	.235
Diuretics	14 (22.6)	124 (22.5)	.890
Antiplatelets	27 (43.5)	326 (59.1)	.033

Supplemental Table S2 – COPD in patients with descending thoracic aortic aneurysm (TAA) or abdominal aortic aneurysm (AAA).

	TAA	AAA	P-value
	n=62	n=552	
Total COPD	31 (50.0)	228 (41.3)	.189
GOLD I (%)	12 (19.4)	86 (15.6)	
GOLD II (%)	14 (22.6)	103 (18.7)	.625
GOLD III/IV (%)	5 (8.1)	39 (7.1)	

Supplemental Table S3 – Association between COPD and aneurysmal disease.

	Univariable			Multivariable*		
	odds ratio	**95%CI**	**P-value**	**odds ratio**	**95%CI**	**P-value**
No COPD	1.00			1.0		
COPD	2.08	[1.66 – 2.61]	<0.001	1.56	[1.16– 2.10]	.003
Mild COPD	2.34	[1.67 – 3.28]	<0.001	1.66	[1.08 – 2.57]	.022
Moderate COPD	1.83	[1.36 – 2.46]	<0.001	1.40	[0.97 – 2.04]	.075
Severe COPD	2.38	[1.47 – 3.86]	<0.001	1.63	[0.85 – 3.15]	.142

* Adjusted for: age, gender, BMI, congestive heart failure, ischemic heart disease, cerebrovascular disease, kidney disease, diabetes mellitus, hypertension, hypercholesterolemia, smoking, statins, beta-blockers, renin-angiotensin system inhibitors, diuretics, antiplatelets and hs-CRP.

Supplemental Table S4 – The most significantly down-regulated genes in lungs of adult Fibulin-$4^{R/R}$ mice. The genes are indicated with their ratios compared to Fibulin-$4^{+/+}$ mice and the process involved.

Top down-regulated genes		
Genes	**Ratio**	**Function**
Efemp2	3.56	Extracellular matrix protein
Myrip	2.25	Melanosome transport
Krtap17-1	2.20	Interfilamentous matrix proteins
Fam107a	2.10	Tumor development
Gdpd2	1.87	Hydrolyzes glycerophosphoinositol
Dcdc2	1.82	Microtubule polymerization
Mus81	1.82	Endonuclease
Acot1	1.78	Catalyze the hydrolysis of acyl-CoAs
Apob	1.76	LDL apolipoprotein
Slc15a2	1.74	Proton-coupled peptide transporter in small intestine
Hspa4l	1.70	Chaperone activity
Galntl2	1.69	Oligosaccharide biosynthesis
Hist2h3c	1.67	Nucleosome structure
Hmgcs2	1.67	Mitochondrial enzyme involved in ketogenesis
Slc6a2	1.65	Neurotransmitter transporter
Lonrf3	1.65	Protein-protein and protein-DNA interactions
Ccl20	1.65	Chemotactic factor that attracts lymphocytes and slightly neutrophils
Msc	1.63	Downstream target of the B-cell receptor signal transduction pathway
Fmo3	1.58	Oxidative metabolism

Supplemental Table S5 – Over-expressed canonical pathways, based on IPA, in lungs of adult Fibulin-$4^{R/R}$ mice ($p<0.05$). Genes associated with these pathways are shown with their log ratio changes compared to Fibulin-$4^{+/+}$ lungs.

Canonical pathways	P-value	Involved genes (log ratio)
Cholesterol Biosynthesis	$6.27\ 10^{-5}$	HSD17B7, MSMO1, SC5DL, CYP51A1
Zymosterol Biosynthesis	$1.09\ 10^{-4}$	HSD17B7, MSMO1, CYP51A1
Tumoricidal Function of Hepatic Natural Killer Cells	$8.66\ 10^{-3}$	ENDOG, PRF1, ITGAL
Aldosterone Signaling in Epithelial Cells	$1.03\ 10^{-2}$	HSPA12B, HSPB2, HSP90AA1, PLCL2, DNAJB2, HSPA2, HSPA4L, PRKCB
Sonic Hedgehog Signaling	$1.08\ 10^{-2}$	STK36, PTCH1, HHIP
Granzyme A Signaling	$2.18\ 10^{-2}$	GZMA, PRF1
Acyl-CoA Hydrolysis	$2.18\ 10^{-2}$	ACOT2, ACOT1
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	$3.01\ 10^{-2}$	PRF1, TRA, BCL2
Glucocorticoid Receptor Signaling	$3.15\ 10^{-2}$	FKBP4, SLPI, TGFB2, HSP90AA1,CCL5, CD163, HSPA2, TRA, BCL2
Granzyme B Signaling	$3.24\ 10^{-2}$	ENDOG, PRF1
Hepatic Fibrosis / Hepatic Stellate Cell Activation	$3.32\ 10^{-2}$	MYH10, MYH14, IL10RA, TGFB2, CCL5, BCL2
Atherosclerosis Signaling	$4.19\ 10^{-2}$	APOB, MMP3, SERPINA1, APOC2, ITGA4
p38 MAPK Signaling	$4.29\ 10^{-2}$	HSPB2, MAPT, HIST2H3C, TGFB2, MEF2C
Reelin Signaling in Neurons	$4.17\ 10^{-2}$	MAPT, HCK, ITGAL, ITGA4
LXR/RXR Activation	$4.89\ 10^{-2}$	APOB, VTN, SERPINA1, APOC2, CYP51A1

Supplemental Table S6 – Deregulated TGF-β pathway genes in adult and newborn Fibulin-4 deficient lungs compared to Fibulin-$4^{+/+}$ lungs ($p<0.05$).

Comparison	Deregulated TGF-β pathway genes	Gene symbol	Fold change
Adult Fibulin-$4^{R/R}$ versus Fibulin-$4^{+/+}$	Transforming growth factor β2	Tgfb2	↑ 1.46
	Activin A receptor type 2b	Acvr2b	↓ 1.24
Adult Fibulin-$4^{+/R}$ versus Fibulin-$4^{+/+}$	SMAD specific E3 ubiquitin protein ligase 1	Smurf1	↓ 1.23
Newborn Fibulin-$4^{R/R}$ versus Fibulin-$4^{+/+}$	Protein inhibitor of activated STAT 4	Pias4	↓ 1.25
Newborn Fibulin-$4^{+/R}$ versus Fibulin-$4^{+/+}$	None	-	-

Supplemental Table S7 – Primers used for quantitative real time PCR. Forward and reverse primers are displayed for each gene from 5' to 3'.

Genes	Forward primers	Reverse primers
Fibulin-4	5'-GGGTTATTTGTGTCTGCCTCG-3'	5'-TGGTAGGAGCCAGGAAGGTT-3'
Gapdh	5'-ACCACAGTCCATGCCATCAC-3'	5'-TCCACCACCCTGTTGCTGTA-3'
Hprt	5'-CGAAGTGTTGGATACAGGCC-3'	5'-GGCAACATCAACAGGACTCC-3'

PART II

Features of Familial Abdominal Aortic Aneurysm

5

Lower Atherosclerotic Burden in Familial Abdominal Aortic Aneurysm

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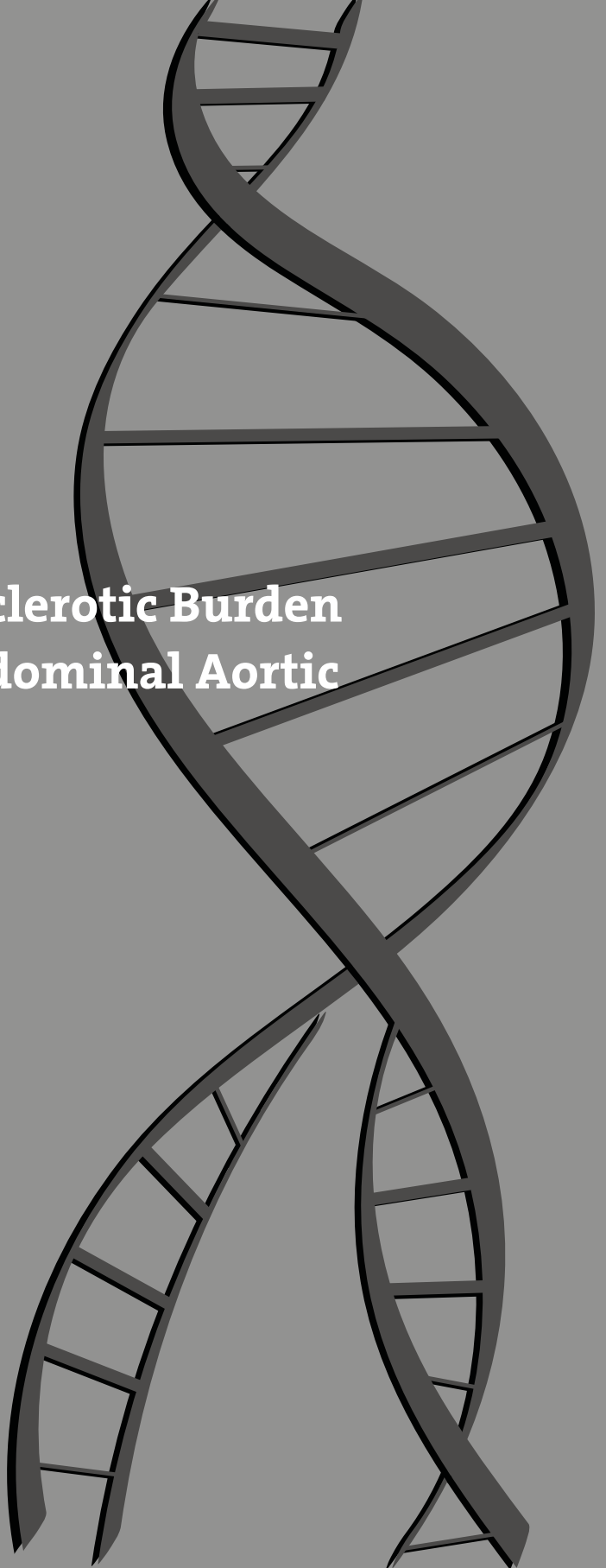
J Vasc Surg. 2013;57(3):642-7.



ABSTRACT

Objective: Despite the apparent familial tendency towards abdominal aortic aneurysm (AAA) formation, the genetic causes and underlying molecular mechanisms are still undefined. In this study we investigated the association between familial AAA and atherosclerosis.

Methods: Data were collected from a prospective database including AAA patients between 2004 and 2012 in the Erasmus University Medical Center, Rotterdam, the Netherlands. Family history was obtained by written questionnaire (93.1% response rate). Patients were classified as familial AAA (fAAA) when at least one affected first-degree relative with an aortic aneurysm was reported. Patients without an affected first-degree relative were classified as sporadic AAA (spAAA). A standardized ultrasound measurement of the common carotid intima-media thickness (CIMT), a marker for generalized atherosclerosis, was routinely performed and patients' clinical characteristics (demographics, aneurysm characteristics, cardiovascular comorbidities and risk factors, and medication use) were recorded. Multivariable linear regression analyses were used to assess the mean adjusted difference in CIMT and multivariable logistic regression analysis was used to calculate associations of increased CIMT and clinical characteristics between fAAA and spAAA.

Results: A total of 461 AAA patients (85% men, mean age, 70 years) were included in the study; 103 patients (22.3%) were classified as fAAA and 358 patients (77.7%) as spAAA. The mean (standard deviation) CIMT in patients with fAAA was 0.89 (0.24) mm and 1.00 (0.29) mm in patients with spAAA (P=.001). Adjustment for clinical characteristics showed a mean difference in CIMT of 0.09 mm (95%CI: 0.02-0.15, P=.011) between both groups. Increased CIMT, smoking, hypertension and diabetes mellitus were all less associated with fAAA as compared to spAAA.

Conclusions: The current study shows a lower atherosclerotic burden, as reflected by a lower CIMT, in patients with familial AAA compared to patients with sporadic AAA, independently of common atherosclerotic risk factors. These results support the hypothesis that although atherosclerosis is a common underlying feature in patients with aneurysms, atherosclerosis is not the primary driving factor in the development of familial AAA.

INTRODUCTION

Abdominal aortic aneurysm (AAA) is characterized by infiltration of inflammatory cells, loss of vascular smooth muscle cells, and extracellular matrix degeneration in the aortic wall.[1] While the causality has been challenged, AAA is associated with atherosclerosis.[2, 3] Approximately 20% of patients with an abdominal aortic aneurysm have a first-degree relative diagnosed with an aortic aneurysm.[4] Despite the apparent familial tendency towards AAA formation, the genetic causes and underlying molecular mechanisms are still undefined.[5]

In this study we investigated the association between familial AAA and atherosclerosis. To this end, we assessed the common carotid intima-media thickness (CIMT) in patients with AAA using B-mode ultrasonography. Thickening of the intimal and medial layers of the common carotid artery is an early expression of generalized atherosclerosis.[6, 7] We evaluated the difference in CIMT between patients with a genetic predisposition to AAA, i.e. familial AAA (fAAA) and patients with sporadic AAA (spAAA), correcting for clinical characteristics. Furthermore, we investigated whether CIMT could serve as a clinical marker to identify patients with fAAA in our population.

METHODS

Study population

The study population consisted of patients with an AAA, defined as an external maximum transverse abdominal aortic diameter ≥30 mm,[8] who underwent either elective open or endovascular repair or remained under surveillance between 2004 and 2012 at the Erasmus University Medical Center in Rotterdam, the Netherlands. The database included a total of 780 patients with AAA. Between 2009 and 2012 all AAA patients were contacted when visiting the outpatient clinic or by mail and asked to complete a semi-structured questionnaire, in order to collect personal data and family histories, and return the questionnaire by mail. Patients who did not respond after one reminder, were contacted and interviewed by telephone (KvdL). In families with multiple AAA patients, only one index patient (i.e. first family member diagnosed with AAA) was included in the study. Patients diagnosed with a known genetic aortic aneurysm syndrome (e.g. Marfan, Loeys-Dietz or vascular Ehlers-Danlos syndrome) were excluded. The study complied with the declaration of Helsinki and was approved by the Institutional Review Board.

Questionnaire and classification of familial AAA

The questionnaire requested information on demographics and the medical history of the index patient. Furthermore, structured questions were included on the occurrence of aortic aneurysms and cardiovascular disease for all known relatives of the index patient.

Patients were classified as fAAA when at least one first-degree relative (parents, siblings or children) was reported to have an aortic aneurysm. Patients who did not report a first-degree relative affected with AAA were classified as spAAA. Patients reporting only second- or third-degree relatives were also classified as spAAA, because the reporting of medical information of second- or third-degree relatives was considered less reliable.

Clinical characteristics

Patients were prospectively enrolled and the following characteristics were recorded for all patients as part of routine clinical practice, including: gender, age, body mass index (BMI), as well as aneurysm characteristics and the cardiovascular comorbidities and risk factors. Aneurysm characteristics included maximal aneurysm diameter and rupture rate. Cardiovascular comorbidities included congestive heart failure, ischemic heart disease (history of myocardial infarction, angina pectoris, coronary revascularisation or pathologic Q-waves on the electrocardiogram), and cerebrovascular disease (history of ischemic/hemorrhagic stroke or transient ischemic attack). Cardiovascular risk factors included kidney disease (serum creatinine ≥2.0 mg/dL), diabetes mellitus (fasting plasma glucose ≥7.0 mmol/L, non-fasting glucose ≥11.1 mmol/L or use of anti-diabetic medication), hypertension (blood pressure ≥140/90 mmHg in non-diabetics, ≥130/80 mmHg in diabetics or use of antihypertensive medication), hypercholesterolemia (low-density lipoprotein [LDL] cholesterol ≥3.5 mmol/L or use of lipid lowering medication). Smoking status was obtained an included current smoking and ever smoking (ie, patients who are currently smoking OR patients with a history of smoking). Prescription medication was recorded, including statins, beta-blockers, renin-angiotensin system (RAAS) inhibitors, diuretics, and antiplatelet drugs. Serum concentrations of the inflammatory biomarker high-sensitivity C-reactive protein (hs-CRP) were measured using immunochemistry (Beckman Coulter, Woerden, the Netherlands).

Atherosclerotic marker

The severity of atherosclerotic disease was assessed by measurements of the CIMT using B-mode ultrasonography according to the guidelines from the 'Mannheim Carotid Intima-Media Thickness Consensus'.[9, 10] Patients were examined in the supine position with the head turned 45° away from the side being scanned and the neck extended slightly. A longitudinal view of the right and left common carotid artery was obtained by a portable Sonosite Titan Ultrasound System (Sonosite Inc., Bothell, WA, USA) with a L38-10-5 MHz linear ultrasound transducer or a portable Vivid-I Ultrasound System (Vivid-I, GE Healthcare, Solingen, Germany) with an 8L-RS transducer. Several measurements were made along a minimum of 10 mm at the posterior wall of the right and left common carotid artery. The intima-media thickness was calculated online by built-in software of the ultrasound system from the interface between lumen and intima to the interface

between media and adventitia. The maximum CIMT value of both common carotid arteries was used for the analysis. Atherosclerotic plaques, defined as focal structures of at least 0.5 mm encroaching into the arterial lumen, were excluded from analysis.[10] The sonographers who performed the measurements were blinded for the clinical characteristics of the patients and had an interobserver correlation of 96.2%.[9]

Statistical analysis

Dichotomous data are presented as numbers and percentages. Continuous variables are presented as mean (standard deviation) or median [interquartile range] when not normally distributed. Categorical data were analysed with chi-square test, and continuous variables with ANOVA or Kruskal-Wallis test, as appropriate. Multivariable linear regression analysis was performed to assess the mean adjusted difference in CIMT between fAAA and spAAA. The CIMT was used as dependent variable and we adjusted for age ≤65 years at diagnosis, gender, BMI, congestive heart failure, ischemic heart disease, cerebrovascular disease, kidney disease, diabetes mellitus, hypercholesterolemia, hypertension, ever smoking, and hs-CRP. Additional analysis was performed using previous adjustments plus medication. Covariates were chosen on the basis of biological plausibility. Multivariable binary logistic regression analysis was used to calculate the associations of increased CIMT (per mm) and clinical characteristics between fAAA and spAAA. Covariates in the model were the same clinical characteristics as used in the multivariable linear regression analysis. To assess any selection bias, baseline characteristics for patients with and without (i.e. those excluded from the study) CIMT measurement were analyzed with chi-square tests.

For all tests a P-value <.05 (two-sided) was considered significant. All analyses were performed using IBM SPSS Statistics version 20.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

The questionnaire was presented to 610 AAA patients, and 482 patients (79.0%) responded after one reminder. Of the remaining 128 patients who did not return the questionnaire, 10 patients were deceased, 108 were interviewed by telephone, and 10 could not be reached. Twenty-two AAA patients were related to other AAA patients participating in the study and were excluded. In this way family histories were obtained from 568 patients, representing a 93.1% response rate. The final study population consisted of 461 of these patients (81.2%) in whom the CIMT was determined.

In total, 103 AAA patients (22.3%) reported to have at least one first-degree relative diagnosed with aortic aneurysm and were classified as fAAA. The remaining 358 patients (77.7%) without first-degree relatives with an aortic aneurysm were classified as spAAA. Clinical characteristics are presented in Table 1. Maximal aneurysm diameters were smaller in patients with fAAA, and there was no difference in ruptured AAA rates

between the two groups. Furthermore, patients with fAAA were younger and had a lower prevalence of diabetes mellitus, hypertension and were less likely to have ever smoked. No significant differences were observed in the use of prescription medication and serum high-sensitivity C-reactive protein levels.

Table 1 – Clinical characteristics of patients with familial AAA and sporadic AAA

	Total population	Familial AAA	Sporadic AAA	P-value
Variable [a]	n=461	n=103	n=358	
Male gender	390 (84.6)	82 (79.6)	308 (86.0)	.112
Age at diagnosis, years	69.7 (8.0)	67.8 (8.0)	70.2 (7.9)	.007
Age ≤65 years at diagnosis	141 (30.6)	40 (38.8)	101 (28.2)	.039
Body mass index, kg/m^2	26.3 (4.2)	26.5 (4.7)	26.2 (3.9)	.707
Aneurysm characteristics				
Aneurysm diameter, mm[b]	59.6 (14.2)	56.9 (15.1)	60.3 (13.9)	.036
Ruptured aneurysm	59 (12.8)	17 (16.5)	42 (11.7)	.201
Cardiovascular comorbidities				
Congestive heart failure	119 (25.8)	30 (29.1)	89 (24.9)	.383
Ischemic heart disease	209 (45.3)	46 (44.7)	163 (45.5)	.879
Cerebrovascular disease	101 (21.9)	23 (22.3)	78 (21.8)	.907
Cardiovascular risk factors				
Kidney disease	96 (20.8)	20 (19.4)	76 (21.2)	.690
Diabetes mellitus	90 (19.5)	13 (12.6)	77 (21.5)	.045
Hypertension	363 (78.7)	71 (68.9)	292 (81.5)	.005
Hypercholesterolemia	437 (94.8)	94 (91.3)	343 (95.8)	.067
Smoking – current	207 (44.9)	44 (42.7)	163 (45.5)	.613
Smoking – ever	422 (91.5)	87 (84.5)	335 (93.6)	.003
Medication				
Statin	361 (78.3)	76 (73.8)	285 (79.6)	.206
Beta-blocker	410 (88.9)	89 (86.4)	321 (89.7)	.353
Renin-angiotensin system inhibitor	267 (57.9)	60 (58.3)	207 (57.8)	.938
Diuretic	145 (31.5)	34 (33.0)	111 (31.0)	.699
Antiplatelet drug	382 (82.9)	81 (78.6)	301 (84.1)	.197
Inflammation marker				
hs-CRP	5.3 [2.6-10.3]	5.2 [2.9-9.9]	5.3 [2.6-10.6]	.793
Atherosclerotic marker				
CIMT, mm	0.97 (0.28)	0.89 (0.24)	1.00 (0.29)	.001

AAA, abdominal aortic aneurysm; CIMT, common carotid intima-media thicknests; hs-CRP, high sensitivity C-reactive protein

[a] Continuous data are presented as the mean (standard deviation) or median [interquartile range] and categorical data as number (%).

[b] Aneurysm diameters were available for 436 patients.

Atherosclerotic marker

The mean CIMT in patients with fAAA was 0.89 (0.24) mm and 1.00 (0.29) mm in patients with spAAA (P=.001). Univariable regression analysis showed a mean difference in CIMT of 0.10 mm (95%CI: 0.04-0.16, P=.001; Table 2). After adjustment for potentially confounding factors, the difference in CIMT decreased to 0.09 mm (95%CI: 0.02-0.15, P=.011). Additional adjustment for medication showed comparable results.

Associations with familial and sporadic AAA

Multivariable binary logistic regression analysis showed that increased CIMT (odds ratio [OR] 0.27, 95%CI: 0.10–0.73, P=.010), ever smoking (OR 0.32, 95%CI: 0.38-0.72, P=.006), hypertension (OR 0.38, 95%CI: 0.21-0.67, P=.001), as well as diabetes mellitus (OR 0.48, 95%CI: 0.23-1.00, P=.049), were all less associated with fAAA (Figure 1).

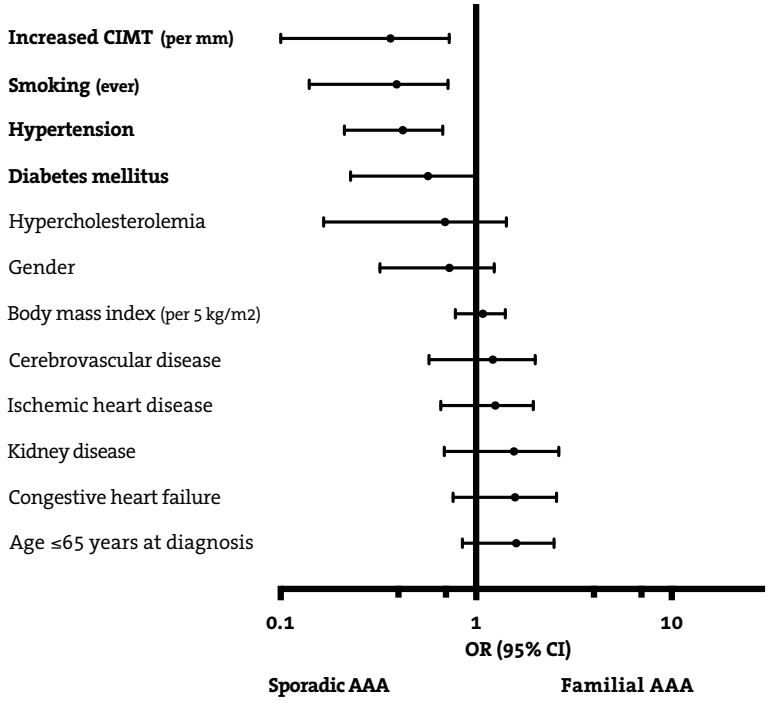


Figure 1 – Multivariable logistic analysis for familial AAA and sporadic AAA

Included in model: age ≤65 years at diagnosis, gender, BMI, congestive heart failure, ischemic heart disease, cerebrovascular disease, kidney disease, diabetes mellitus, hypercholesterolemia, hypertension, ever smoking and high-sensitivity C-reactive protein.

Table 2 – Differences in CIMT between familial AAA and sporadic AAA

Atherosclerotic marker		β	95% CI for β	P-value
CIMT	Unadjusted	0.10	0.04 : 0.16	.001
	Adjusted[a]	0.09	0.02 : 0.15	.011
	Adjusted[b]	0.09	0.03 : 0.16	.007

CI, confidence interval; CIMT, common carotid intima-media thickness
[a] Adjusted for: age ≤65 years at diagnosis, gender, BMI, congestive heart failure, ischemic heart disease, cerebrovascular disease, kidney disease, diabetes mellitus, hypercholesterolemia, hypertension, ever smoking and high-sensitivity C-reactive protein.
[b] Adjusted for [a] and medication.

Assessment of selection bias

We observed a difference in characteristics between patients included and excluded (i.e. those without CIMT measurements) for analysis for age (included: 70 years versus excluded: 67 years, P=.002), congestive heart failure (included: 25.8% versus excluded: 9.3%, P<.001) and hypertension (included: 78.9% versus excluded: 66.4%, P=.006). There was no difference in the percentage of patients classified as fAAA (included: 22.3% versus excluded: 23.4%, P=.820).

Discussion

This study shows that the CIMT was lower in patients with familial AAA than in patients with sporadic AAA. Although patients with familial AAA had a more favorable cardiovascular risk profile (i.e. younger age, less hypertension and less diabetes mellitus), which may have influenced the results, the CIMT remained significantly lower after adjustment for these factors in multivariable analysis. These findings indicate that the atherosclerotic burden is lower in patients with a familial form of AAA and suggest a difference in pathobiology between patients with familial and sporadic AAA.

Thickening of the intimal and medial layers of the common carotid artery is an early expression of generalized atherosclerosis.[6, 11] General cardiovascular risk factors, such as hypertension, smoking, diabetes mellitus, and dyslipidemia, are associated with an increased CIMT.[12] In addition, an elevated CIMT is associated with atherosclerotic diseases,[7] as well as with ischemic events.[13, 14] A systematic review by Aminbakhsh et al. has revealed that the risk of a first myocardial infarction increases with a CIMT of 0.82 mm or higher and the risk of a first stroke with a CIMT of 0.75 mm or higher.[14] Hence, the CIMT is considered a valuable tool to assess early atherosclerosis. In the current study, the mean CIMT was 0.89 mm in patients with fAAA and 1.00 mm in patients with spAAA, with a mean difference of 0.09 mm between the groups after adjusting for cardiovascular comorbidities and risk factors, medication use and the inflammatory marker high-sensitivity C-reactive protein. It has been shown that a rise in CIMT of 0.03 mm per year

is associated with a significant increase in ischemic events and that an absolute CIMT difference of 0.1 mm increases the future risk for myocardial infarction by 10-15% and the risk for stroke by 13-18%.[14, 15] This suggests that the observed 0.09 mm difference in CIMT reflects a lower atherosclerotic load in fAAA patients.

We recently reported that patients with aneurysmal disease have a lower CIMT compared to patients with occlusive arterial disease (0.97 mm versus 1.07 mm, respectively),[16] which is in line with several previous studies.[17-19] We now show that patients with a familial form of AAA have an even lower CIMT than AAA patients without affected relatives. Compared to the healthy aged population, the Rotterdam study showed that the mean CIMT in patients with a mean age of 71 years was 0.79 mm and another study with participants from the United Kingdom over the age of 60 years showed an CIMT of 0.81 mm.[20, 21] Consequently, patients with familial AAA still have higher CIMT values when compared with previously reported CIMT values in the general population, indicating that our patients with AAA were not free of atherosclerosis. Our results further strengthen the idea that even if atherosclerosis may influence the evolution and development of aneurysms, other factors account for divergent pathophysiologic mechanisms underlying familial and sporadic aneurysmal disease.

Although not statistically significant, we observed a trend towards more females affected with fAAA compared to spAAA. A difference in gender was also observed by Darling et al. and supports the hypothesis that female AAA patients may have a higher genetic susceptibility for AAA than males.[22]

Although we did observe a significant difference in CIMT, the high standard deviations and considerable overlap in CIMT between the two groups precludes the clinical use of CIMT measurements for differentiating reliably fAAA from spAAA patients.

Our study has some limitations. First, an important difficulty with the CIMT measurement is the lack of uniformity in its definition and the methodology used, which limits quantitative comparisons of absolute CIMT values between studies.[10] Second, the definition of familial AAA is based on family history and underestimating fAAA is likely because of underreporting of affected relatives and the likelihood of having affected relatives is lower in smaller families. Third, since no systematic molecular screening was performed of the genes associated with Marfan, Loeys-Dietz, or vascular Ehlers-Danlos syndrome, patients with specific genetic aneurysm syndromes might have been included in the study. However, since these syndromes are rare causes for abdominal aortic aneurysms, the contribution of these syndromes to the study population is probably very small. Lastly, since CIMT values were obtained in 81.2% of the AAA patients we cannot exclude the possibility of selection bias. However, since there was no difference in the percentage of patients classified as fAAA, we believe that any potential selection bias was equally divided between the two patient populations and does not hamper the findings of the study.

CONCLUSIONS

The current study shows a lower atherosclerotic burden, as reflected by a lower CIMT, in patients with familial AAA compared to patients with sporadic AAA, independently of common atherosclerotic risk factors. These results support the hypothesis that although atherosclerosis is a common underlying feature in patients with aneurysms, atherosclerosis is not the primary driving factor in the development of familial AAA. Further research on the molecular pathways and genetics of aortic aneurysm formation is warranted to identify the cause(s) of familial and sporadic AAA.

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6

First Genetic Analysis of Aneurysm Genes in Familial and Sporadic Abdominal Aortic Aneurysm

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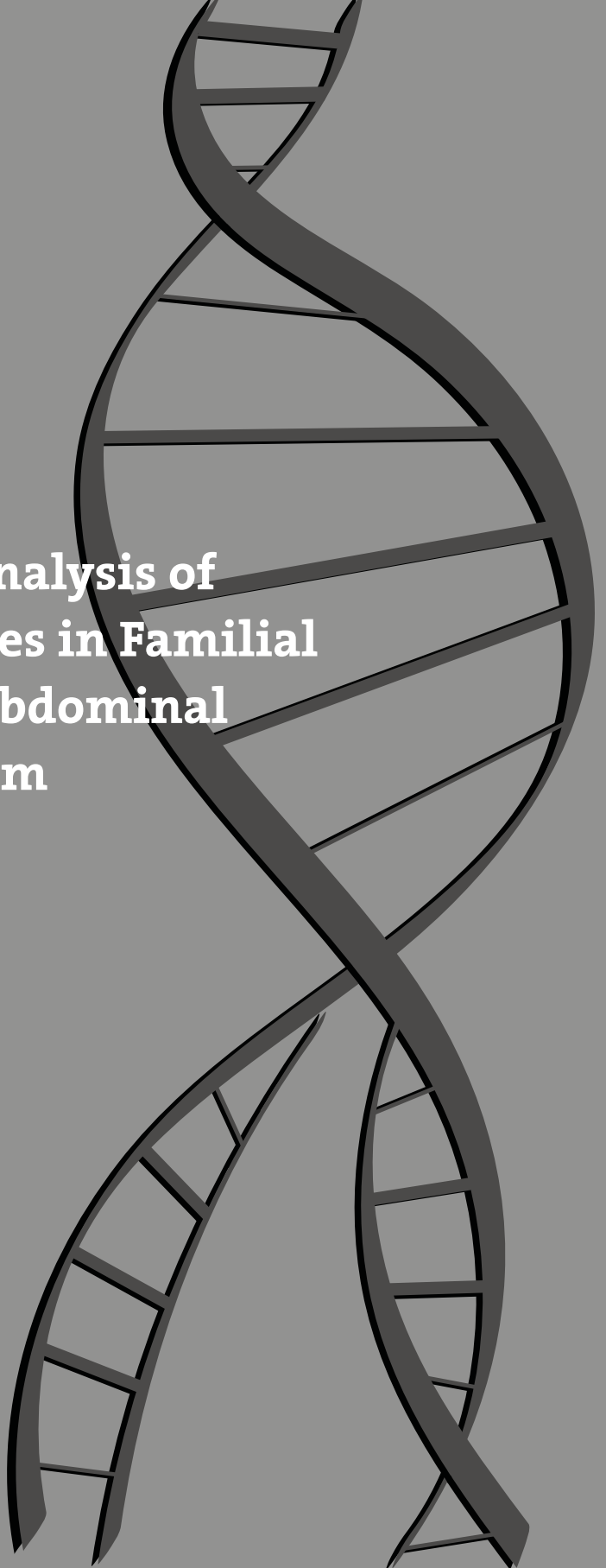

ABSTRACT

Genetic causes for abdominal aortic aneurysm (AAA) have not been identified and the role of genes associated with familial thoracic aneurysms in AAA has not been explored. We analyzed nine genes associated with familial thoracic aortic aneurysms, the vascular Ehlers-Danlos gene *COL3A1* and the *MTHFR* p.Ala222Val variant in 155 AAA patients. The thoracic aneurysm genes selected for this study were the transforming growth factor-beta pathway genes *EFEMP2, FBN1, SMAD3, TGBF2, TGFBR1, TGFBR2,* and the smooth muscle cells genes *ACTA2, MYH11* and *MYLK*. Sanger sequencing was performed of all coding exons and exon-intron boundaries of these genes. Patients with at least one first-degree relative with an aortic aneurysm were classified as familial AAA (n=99), the others as sporadic AAA. We found 47 different rare heterozygous variants in eight genes: two pathogenic, one likely pathogenic, twenty-one variants of unknown significance (VUS) and twenty-three unlikely pathogenic variants. In familial AAA we found one pathogenic and segregating variant (*COL3A1* p.Arg491X), one likely pathogenic and segregating (*MYH11* p.Arg254Cys), and fifteen VUS. In sporadic patients we found one pathogenic (*TGFBR2* p.Ile525Phefs*18) and seven VUS. Thirteen patients had two or more variants. These results show a previously unknown association and overlapping genetic defects between AAA and familial thoracic aneurysms. Indicating that genetic testing may help to identify the cause of familial and sporadic AAA. In this view, genetic testing of these genes specifically or in a genome wide approach may help to identify the cause of familial and sporadic AAA.

INTRODUCTION:

Approximately 20% of the patients with an abdominal aortic aneurysm (AAA) have a positive family history for aneurysms, suggesting a genetic predisposition for AAA in these families.[1-3] The genetic aortic aneurysm syndromes Marfan, Loeys-Dietz, and aneurysms-osteoarthritis (AOS), involving the *FBN1, TGFBR1*, *TGFBR2*, *TGFB2*, and *SMAD3* genes were first identified in patients with pathologic dilatation or aneurysm of the thoracic aorta with multisystem overlapping cardiovascular, skeletal and ocular manifestations.[4-8] The genetic defects in these syndromes affect the integrity of the elastic medial by inference with the transforming growth factor beta (TGF-β) pathway.[4, 9-12] The wide range of variably expressed features in these rare autosomal dominantly inherited syndromic forms of familial thoracic aneurysm, include pectus- and/or spinal deformities, joint laxity, and skin translucency and specifically for AOS, osteoarthritis and for the Loeys-Dietz syndrome, hypertelorism, bifid uvula or cleft palate and arterial tortuosity. Vascular tortuosity, ascending aortic aneurysm, joint laxity and pectus excavatum are also main features of the *EFEMP2* related autosomal recessive juvenile cutis laxa syndrome.[13, 14]

In another group of families with thoracic aneurysm without distinct clinical features, genetic defect were identified, in the so-called non-syndromic familial thoracic aneurysm genes including the *MYH11*, *MYLK* and *ACTA2* that affect smooth muscle cell (SMC) functioning.[15-18] These may also affect TGF-β signaling, like *ACTA2* mutations, occurring in 16% of patients with familial thoracic aortic aneurysm and in sporadic thoracic aortic aneurysms and dissections associated with medial degeneration, focal medial smooth muscle cell hyperplasia and proliferation, and stenotic arteries in the vaso-vasorum.[15, 19, 20] A recent review estimates that approximately 20% of familial thoracic aneurysm cases could be explained by a mutation in one of the thoracic aneurysm genes.[21] Establishing the exact contribution of each of these genes in (familial) thoracic aneurysms has been hampered by the overlap in clinical features. Occasionally isolated abdominal aortic aneurysms have been observed in families with familial syndromic and non-syndromic thoracic aneurysm. Therefore, genes associated with the familial thoracic aortic aneurysm may play a role in the degenerative changes of the extracellular matrix of the abdominal aortic wall underlying the formation of AAA. For this reason, we decided to screen AAA patients for variants in the transforming growth factor-beta pathway genes *EFEMP2, FBN1, SMAD3, TGBF2, TGFBR1*, *TGFBR2,* smooth muscle cells genes *ACTA2, MYH11* and *MYLK,* as well as the vascular Ehlers-Danlos gene *COL3A1*, which is associated with vascular fragility.[22] In addition, we investigated the previously reported association between abdominal aneurysm and the c.665C>T variant in *MTHFR*.[23] We report all the variants found in these analyses, except those classified as clearly not pathogenic (benign). The presented description of variants will convey relevance for classification of variants in future diagnostic setting.

MATERIALS AND METHODS

The study complied with the declaration of Helsinki and was approved by the Institutional Review Board (MEC-2013-265).

Study population

The study population consisted of 155 AAA patients referred for genetic counseling between January 2009 and December 2013 to the Department of Clinical Genetics at the Erasmus University Medical Center in Rotterdam, the Netherlands. Abdominal aortic aneurysm was defined as an external infrarenal abdominal aortic diameter ≥30 mm.[24] Patients were classified as familial AAA when at least one first-degree relative (ie, parent, sibling or child) was confirmed by medical records to be diagnosed with an aortic aneurysm (n=99, 81 male). Patients reporting only affected second- or third-degree relatives were also classified as sporadic AAA, because the reporting of medical information of second- or third-degree relatives was considered less reliable.[25] Patients without an affected first-degree relative were classified as sporadic AAA (n=56, 46 male). In case of familial AAA, the first family member diagnosed with AAA was included as index in the study. Cases of concordant twins were considered as familial AAA. Genetic evaluation of the AAA patients was performed by a clinical geneticist and included ascertainment of a detailed family history and physical examination. All patients consented to DNA testing.

DNA analysis and classification of variants

Sanger sequencing of all coding exons and exon-intron boundaries in *ACTA2* (NM_001613.1), *COL3A1* (NM_000090.3), *EFEMP2* (NM_016938.3), *FBN1* (NM_000138.3), *MYH11* (NM_001040113.1), *MYLK* (NM_053025.3), *SMAD3* (NM_005902.3), *TGFB2* (NM_001135599.2), *TGFBR1* (NM_004612.2) and *TGFBR2* (NM_001024847.2), were performed at the certified laboratories of the Departments of Clinical Genetics of the Erasmus University Medical Center in Rotterdam and the VU Medical Center in Amsterdam. Patients were tested for the p.Ala222Val variant in *MTHFR* (NM_005957.4) at the Department of Clinical Genetics at the University Hospital in Nijmegen, the Netherlands.

Assessment of the pathogenic effect of genetic variants was performed according to the guidelines currently used in the Rotterdam laboratory for DNA diagnostics with the use of Alamut Interactive Biosoftware (Rouen, France). This software incorporates SpliceSiteFinder-like, MaxEntScan, NNSPLICE, GeneSplicer, and Human Splicing Finder for the prediction of splicing variants and the programs Align GVGD, SIFT, Mutation Taster, PolyPhen-2 and KDv4 for *in silico* prediction of the effect of amino acid changes. Additionally, it gives population frequencies for dbSNP and ESP, and shows whether or not a variant has been reported before in Human Gene Mutation Database (HGMD). The

Rotterdam classification system of variants was adapted from the sequence variation classification proposed by Plon et al.[26] The criteria for classification of variants included the allele frequency in the dbSNP/ESP (cut-off 0.01), predicted effects on splicing, the *in silico* prediction of effect on the protein and previously described links to disease. Exonic variants remote from wildtype splice donor and acceptor sites were assessed to have no effect on splicing. For each variant present in HGMD the supporting evidence was reviewed and we evaluated whether previous reports linking specific variants to aneurysm were supported by functional studies or expression assays. Additionally, a variant only predicted by *in silico* prediction to be pathogenic, would not automatically be classified as such, because of lack of functional evidence. This resulted in categorizing variants in five classes: pathogenic, likely pathogenic, unknown significance (VUS), likely benign and benign (Table 1).[27] All variant expect those classified as benign were reported in this paper. A single previous description of a variant in a patient was not considered sufficient evidence for causation and these variants were classified as variants of unknown significance instead of likely pathogenic. In addition, table 2 presents the allele frequencies of the variants in the Dutch population derived from the GoNL cohort which contains data from parent-child combinations.[28] From this source only the information from the parents (n=499) was used to compare the minor allele frequency (MAF) in the Dutch population to the allele frequencies derived from Alamut. We choose to add this information because the study population was predominantly (≥ 95%) of Dutch ancestry and population specific allele frequencies may help categorization of variants. Familial segregation of the variants with aneurysms in families was examined when affected relatives were available and consented for DNA testing.

Table 1 – Classification of variants

Class	Variant
Benign	Frequency in population ≥ 0.01.
Likely benign	Intronic or silent variants with no effect on splicing, missense variants for which 4/5 or 3/4 in silico protein predictions are neutral.
Unknown significance	Intronic, silent or missense variants that affect splicing, in-frame deletions/ insertions, missense variants for which more than 2 in silico protein predictions are damaging.
Likely pathogenic	Frameshift, nonsense or intronic variants that affect splicing in a way that a new in-frame protein is created, missense variants that have previously been linked to disease in HGMD.
Pathogenic	Frameshift, nonsense or intronic variants that affect splicing in a way that no in-frame protein can be created.

Table 2 – Variants in familial and sporadic abdominal aortic aneurysm

Gene/Variant	Protein change	dbSNP ID	MAF/ MAC	Splice site prediction	Predicted pathogenic effect on protein (in silico, n programs)	Amino acid c onservation (n species)	Classification ♦♦	Number of variants (familial/ sporadic AAA) (99/56)	Segregation*	MAF GoNL **
COL3A1								3 (3/0)		
c.812G>A[#34]	p.Arg271Gln	rs112185887	0.001/1	no effect	2/5	moderately conserved (9)	VUS	1 (1/0)	nd	.004
c.898-14A>G	Intronic	-	-	effect	-	-	VUS	1 (1/0)	nd	na
c.1471C>T	**p.Arg491X**	**-**	**-**	**no effect**	**-**	**-**	**P**	**1 (1/0)**	**+**	**na**
EFEMP2								6 (6/0)		
c.160+17G>T	Intronic	-	-	no effect	-	-	LB	1 (1/0)	nd	na
c.277G>A[#]	p.Gly93Ser	rs2234462	0.001/3	no effect	0/4	moderately conserved (10)	LB	3 (3/0)	- / nd	.005
c.368-4G>A	Intronic	rs111550973	0.002/4	no effect	-	-	LB	1 (1/0)	-	.007
c.1047C>T[#]	no-change	-	-	no effect	-	-	LB	1 (1/0)	nd	na
FBN1								***8 (5/3)***		
c.59A>G[#°30]	p.Tyr20Cys	rs201309310	-	no effect	0/4	moderately conserved (11)	VUS	1 (0/1)	na	na
c.248-17C>G[#]	Intronic	-	-	no effect	-	-	LB	1 (1/0)	-	na
c.1108G>A[#]	p.Val370Ile	-	-	no effect	0/5	moderately conserved (11)	LB	1 (1/0)	nd	na
c.2260T>C[#]	p.Tyr754His	-	-	no effect	4/5	highly conserved (11)	VUS	1 (1/0)	nd	na
c.2895G>A[#]	no-change	rs140591	-	no effect	-	-	LB	1 (1/0)	nd	na
c.3455C>T[°31]	p.Ala1152Val	-	-	effect	1/5	highly conserved (11)	VUS	1 (0/1)	na	na
c.6055G>A[°32]	p.Glu2019Lys	-	-	no effect	3/5	highly conserved (11)	VUS	1 (1/0)	nd	.001
c.7412C>G	p.Pro2471Arg	rs193922233	-	no effect	3/5	highly conserved (11)	VUS	1 (0/1)	na	na

Table 2 – Continued

Gene/Variant	Protein change	dbSNP ID	MAF/ MAC	Splice site prediction	Predicted pathogenic effect on protein (in silico, n programs)	Amino acid c onservation (n species)	Classification ♦♦	Number of variants (familial/ sporadic AAA) (99/56)	Segregation*	MAF GoNL **
MYH11								***11 (9/2)***		
c.760C>T*[16]	**p.Arg254Cys**	**rs150759461**	**0.001/2**	**no effect**	**3/4**	**highly conserved (13)**	**LP**	**1 (1/0)**	**+**	**.008**
c.956A>G	p.Asn319Ser	rs149964928	-	effect	1/4	highly conserved (13)	VUS	1 (1/0)	-	na
c.1523G>A	p.Arg508His	rs144244239	0.001/2	no effect	3/4	highly conserved (13)	VUS	1 (1/0)	nd	na
c.1868C>G	p.Ala623Gly	rs140688587	-	no effect	1/4	highly conserved (13)	LB	1 (1/0)	-	na
c.2881-14C>G	Intronic	-	-	no effect	-	-	LB	1 (1/0)	+	na
c.4694C>T#	p.Thr1565Met	rs111854563	0.001/1	no effect	3/4	moderately conserved (13)	VUS	1 (1/0)	gem	.004
c.5587C>T#	no-change	rs142639688	-	no effect	-	-	LB	1 (1/0)	nd	.004
c.5635-7G>A#	Intronic	rs202120792	0.001/1	no effect	-	-	LB	2 (1/1)	nd	na
c.5697G>C#	p.Glu1899Asp	rs113964173	0.005/10	no effect	4/4	highly conserved (13)	VUS	1 (1/0)	-	.008
c.5808-11_-8del	Intronic	-	-	effect	-	-	VUS	1 (0/1)	na	na
MYLK								***19 (13/6)***		
c.312T>C	no-change	rs147597398	-	no effect	-	-	LB	1 (1/0)	gem	na
c.745T>G	p.Ser249Ala	-	-	no effect	2/5	highly conserved (7)	VUS	1 (0/1)	na	na
c.1314C>T	no-change	rs200423954	0.001/2	no effect	-	-	LB	1 (0/1)	na	.001
c.1327C>T#	p.Pro443Ser	rs35156360	0.006/13	no effect	4/5	highly conserved (7)	VUS	4 (4/0)	- / nd	.014
c.2101G>A	p.Ala701Thr	rs142835596	0.003/7	no effect	2/5	highly conserved (7)	VUS	1 (0/1)	na	na
c.3184G>T#	p.Ala1062Ser	rs11558550	-	no effect	0/5	highly conserved (7)	LB	1 (0/1)	na	na
c.3302A>G#	p.Lys1101Arg	-	-	no effect	0/5	highly conserved (7)	LB	1 (1/0)	-	na
c.3403G>A	p.Gly1135Arg	-	-	no effect	4/5	highly conserved (7)	VUS	1 (1/0)	+	na
c.3583A>G#	p.Asn1195Asp	-	-	no effect	3/5	highly conserved (7)	VUS	1 (1/0)	nd	na
c.4179C>T	no-change	-	-	no effect	-	-	LB	2 (1/1)	-	na

Table 2 – Continued

Gene/Variant	Protein change	dbSNP ID	MAF/ MAC	Splice site prediction	Predicted pathogenic effect on protein (in silico, n programs)	Amino acid c onservation (n species)	Classification ♦♦	Number of variants (familial/ sporadic AAA) (99/56)	Segregation*	MAF GoNL **
c.4764G>A#	no-change	rs56056823	0.003/6	no effect	-	-	LB	3 (2/1)	nd	.008
c.4785C>T	no-change	-	-	no effect	-	-	LB	1 (1/0)	nd	na
c.5079G>A#	no-change	rs141467675	0.002/5	no effect	-	-	LB	1 (1/0)	nd	na
TGFB2								***2 (2/0)***		
c.272G>A#	p.Arg91His	rs10482721	0.001/3	no effect	3/4	highly conserved (12)	VUS	1 (1/0)	nd	.005
c.703G>C	p.Val235Leu	rs10482810	0.001/2	no effect	2/4	highly conserved (12)	VUS	1 (1/0)	nd	.006
TGFBR1								***5 (4/1)***		
c.15C>T#	no-change	-	-	no effect	-	-	LB	1 (1/0)	nd	na
c.214A>T	p.Ile72Leu	rs111513627	-	no effect	2/5	highly conserved (12)	VUS	2 (1/1)	-	na
c.927G>C#	no-change	-	-	no effect	-	-	LB	1 (1/0)	nd	na
c.1125A>C#	no-change	rs7861780	0.003/6	no effect	-	-	LB	1 (1/0)	nd	.004
TGFBR2								3 (2/1)		
c.1137C>T	no-change	rs113194608	-	no effect	-	-	LB	1 (1/0)	+	.004
c.1234G>A#°[33]	p.Val412Met	rs35766612	0.001/1	no effect	3/4	highly conserved (14)	VUS	1 (1/0)	gem	.002
c.1573delA#	**p.Ile525Phefs*18**	**-**	**-**	**no effect**	**-**	**-**	**P**	**1 (0/1)**	**na**	**na**

♦♦ P, pathogenic. LP, likely pathogenic. VUS, variant of unknown clinical significance. LB, likely benign.

Abbreviations: MAF, minor allele frequency; MAC, minor allele count; na, not applicable.

In **bold** the variants classified pathogenic or likely pathogenic

* For familial AAA, segregation of the variant in affected relatives was investigated, (+) variant present in all affected relatives in one family, (-) variant did not segregate in a family with AAA, (nd) segregation of the variant in familial AAA was not determined, (gem) affected gemelli with variant.

** Frequency in GoNL based on 499 studied individuals, 998 alleles, (na) variant was not reported in GoNL data.

Variant is involved in complex genotypes.

° Variant reported in aneurysm patient in HGMD.

RESULTS

Forty-seven variants were detected in 31 familial AAA (31%) patients and 12 sporadic AAA (21%) patients in *COL3A1, EFEMP2, FBN1, MYH11, MYLK*, *TGBF2, TGFBR1*, and *TGFBR2*, no variants were found in *ACTA2* and *SMAD3* (Table 2).

Pathogenic variants

Two variants were classified as pathogenic. A *COL3A1* null mutation p.Arg491X was observed, segregating in patients with aneurysms in one family. This null mutation was observed in a in a 49 year old man diagnosed with a small dissection of the arteria lienalis at screening for familial abdominal aneurysms (Supplementary online table). His paternal aunt had a successful repair of an infrarenal aneurysm at age 69 also had the mutation. Her brother was reported with a sudden death at age 32 years. No autopsy was performed. Screening for the *COL3A1* mutation detected one asymptomatic 50-year-old female carrier without signs of vascular pathology on computed tomography angiography. None of the carriers of the null mutations showed distinct loss of subcutaneous fat, skin fragility, abnormal scarring or suffered from complications or bleeding after surgery or childbirth.

A novel heterozygous single basepair deletion in *TGFBR2*, p.Ile525Phefs*18 was found *de novo* in a 47-year-old male presenting with complex vascular pathology. This patient presented at the emergency room with severe acute abdominal pain and was diagnosed with a Stanford type-B aortic dissection associated with a pre-existing large aorto-iliac aneurysm and marked tortuosity with a diameter of 98.5 mm. The ascending aortic diameter was normal measuring 32.0 mm. The patient was treated with b-blockade and blood pressure control, resulting in complete remission of symptoms. Two weeks after onset of the symptoms, the aorto-iliac aneurysm was successfully repaired with an aorto-bifemoral Dacron bypass.[29] Marked arterial elongation and tortuosity of the abdominal aorta and iliac arteries was present without characteristic facial or musculo-skeletal signs of the Loeys-Dietz or Marfan syndrome. DNA analysis of his parents showed that the mutation occurred *de novo* and no other relatives were affected. This patient also had a likely benign *MYH11* intronic variant c.5635-7G>A just before exon 40. The likely benign *MYH11* variant occurred also in his two unaffected sisters and their 74 year old unaffected father.

Likely pathogenic variants

The missense variant in *MYH11* (p.Arg254Cys) was classified as likely pathogenic because a report showing pathogenic effects was available.[16] This variant was initially detected in a 73 year old woman with a symptomatic abdominal aneurysm. The mutation was also present in her 44 year old son diagnosed by family screening with bilateral aneurysm of the iliac arteries and a small abdominal aneurysm.

Variants of unknown significance (VUS)

Twenty-one VUS included 19 missense and 2 intronic variants. In familial cases 15 VUS were observed; 14 missense and one intronic. In sporadic cases, 7 VUS were observed; 6 missense and one intronic. Two VUS were present in multiple patients. The *MYLK* (p.Pro443Ser) variant was found in four patients with familial AAA, but this variant did not segregate in one family and segregation could not be tested in the other families. The *TGFBR1* (p.Ile72Leu) variant was present in one sporadic and one familial case, and did not segregate. Three VUS variants in *FBN1* (p.Tyr20Cys, p.Ala1152Val, and p.Glu2019Lys) were previously reported in patients with Marfan syndrome, without sufficient evidence to be classified as likely pathogenic.[30-32] The *TGFBR2* (p.Val412Met) variant in two affected twin brothers, was previously reported in thoracic aneurysm.[33] The VUS missense variant in *COL3A1* (p.Arg271Gln) was previously linked to Ehlers-Danlos syndrome in literature, however, after critical evaluation of the report, the evidence for this link was considered not sufficient to classify the variant as pathogenic.[34]

Likely benign variants (LB)

Of the twenty-three likely benign variants five were intronic, 13 were synonymous and 5 were missense variants. Four LB variants were observed in more than one patient: the synonymous *MYLK* c.4764G>A was present in three patients (two familial, one sporadic), the synonymous *MYLK* c.4179C>T and the intronic variant *MYH11* c.5635-7G>A occurred in one familial and one sporadic patient, and the missense *EFEMP2* p.Gly93ser was present in three patients with familial AAA without evidence of segregation.

MTHFR c.665C>T

The *MTHFR* c.665C>T (p.Ala222Val) variant, previously reported as C677T, was tested in 130 patients (89 familial and 41 sporadic AAA patients). Twelve patients (9%) were homozygous for the variant allele: ten (11%) familial and two (5%) sporadic. Forty-five (38%) patients were heterozygous for the variant allele: thirty one (35%) familial and fourteen (34%) sporadic patients. The MAF in our study population was 0.265 compared to 0.320 in the Dutch GoNL cohort (Table 3).

Complex genotypes

Thirteen AAA patients (11 familial, including one pair of concordant monozygotic twins, and 2 sporadic) had two or more variants (Table 4). One pathogenic *TGFBR2* was involved in a complex genotype with a likely benign variant in *MYH11*.

Table 3 – Frequencies of the *MTHFR* c.665C>T variant in AAA and control populations

First author	Patients (N)	Diagnosis	MAF	Normal (CC)	Heterozygote (CT)	Homozygote (TT)	CT and TT
Brunelli et al.	58	AAA	0.483	14 (24%)	32 (55%)	12 (21%)	44 (76%)
	60	Control	0.392	19 (32%)	35 (58%)	6 (10%)	41 (68%)
Strauss et al.	63	AAA	0.365	21 (33%)	38 (60%)	4 (6%)	42 (67%)
	75	Control	0.231	49 (65%)	20 (27%)	6(8%)	26 (35%)
Jones et al.	428	AAA*	0.310	211 (49%)	169 (40%)	48 (11%)	217 (51%)
	282	Control (healthy)	0.309	134 (48%)	122 (43%)	26 (9%)	148 (52%)
	271	Control (CVD)	0.303	137 (51%)	104 (38%)	30 (11%)	134 (49%)
	226	Control (PAD)	0.332	106 (47%)	90 (40%)	30 (13%)	120 (53%)
Sofi et al.	438	AAA	0.430	141 (32%)	217 (50%)	80 (18%)	297 (68%)
	438	Controls	0.380	166 (38%)	211 (48%)	61 (14%)	272 (62%)
Ferrara et al.	42	AAA > 60 years	0.298	18 (43%)	23 (55%)	1 (2%)	24 (57%)
	46	AAA < 60 years	0.424	10 (22%)	33 (72%)	3 (6%)	36 (78%)
	45	Control	0.133	34 (75%)	10 (23%)	1 (2%)	11(24%)
Current study	130	AAA total	0.265*	73 (56%)	45 (35%)	12 (9%)	57 (44%)
	89	*AAA familial*	*0.287*	*48 (54%)*	*31 (35%)*	*10 (11%)*	*41 (46%)*
	41	*AAA sporadic*	*0.220*	*25 (61%)*	*14 (34%)*	*2 (5%)*	16 (39%)
Overall	1205	AAA	0.364	488 (40%)	557 (46%)	160 (13%)	717 (60%)
	1397	Control	0.326	645 (46%)	592 (42%)	160 (11%)	752 (54%)

*MAF in Dutch population: 0.320.

Frequency of variants in the Dutch population

For 16 of the variants found in this study, the allele frequency in the Dutch population was available from GoNL. The MAF of the VUS variant in *MYLK* (p.Pro443Ser) was 0.014 in the Dutch population, and was reported in dbSNP with a MAF of 0.006. This indicates that this variant would be reclassified as a class 1 variant, using the GoNL frequency information instead of the frequency reported by dbSNP/ESP used in Alamut.

DISCUSSION

The genetic defects causing familial abdominal aortic aneurysm are poorly understood. This study showed that genes known to be associated with inherited thoracic aortic aneurysm also have a role in abdominal aortic aneurysm. Our study is based on a group of AAA patients referred for counselling. Therefore, the observed results do not represent prevalence of variants in the Dutch AAA population. Although familial cases were overrepresented in the current study, a referral bias for genotype can be excluded, since there was no prior information on genetic defects in familial or sporadic AAA. The validation of our family history data showed that no relatives were reported incorrectly as affected, indicating that risk in relatives was not overestimated. On the other hand,

Table 4 – AAA patients with multiple variants in aneurysm genes

Patient	familial / sporadic AAA	COL3A1	EFEMP2	FBN1	MTHFR	MYH11	MYLK	TGFB2	TGFBR1	TGFBR2
1.	familial	-	-	LB c.1108G>A	-	-	-	-	LB c.15C>T	-
2.	sporadic	-	-	VUS c.59A>G	-	-	LB c.3184G>T	-	-	-
3.	familial	-	-	LB _c.2895G>A	-	-	VUS c.4764G>A	-	-	-
4.[b]	gemelli	-	-	-	-	VUS c.4694C>T		-	-	VUS c.1234G>A
5.	sporadic	-	-	-	-	LB c.5635-7G>A	-	-	-	**P c.1573delA[a]**
6.	familial	-	-	-	-	VUS c.5697G>C		-	LB c.1125A>C	-
7.	familial	-	LB c.277G>A	-	-	-	LB c.3302A>G	-	-	-
8.[b]	familial	VUS c.812G>A	-	-	-	-	VUS c.1327C>T	-	-	-
9.	familial	-	LB c.277G>A	-	-	LB c.5587C>T	VUS c.1327C>T	-	-	-
10.	familial	-	-	LB c.248-17C>G	-	VUS c.956A>G	-	-	-	-
11.	familial	-	LB c.1047C>T and (3)c.277G>A	-	-	-	-	-	LB c.927G>C	-
12.[b]	familial			VUS c.2260T>C				VUS c.272G>A		
13.	familial	-	-	-	-	-	LB c.3583A>G and VUS c.5079G>A	-	-	-

P, pathogenic. LP, likely pathogenic. VUS, variant of unknown clinical significance. LB, likely benign
[a] *de novo* mutation
[b] Patient with multiple variants of VUS or higher.
Variants of class 4 or higher are shown in bold.

underreporting of familial disease may have happened, in particular for a disease like AAA, where aneurysms in relatives may go unnoticed and relatives could have undiagnosed aneurysms or may have died before age of onset. It is therefore important to bear in mind that familial AAA cannot be excluded when family history of aneurysm is uninformative or missing.

This study investigated the association between AAA and the thoracic aneurysm genes *ACTA2*, *COL3A1, EFEMP2, FBN1, MYH11, MYLK*, *SMAD3*, *TGBF2, TGFBR1*, *TGFBR2,* and *MTHFR* (p.Ala222Val). There have been several large GWAS studies that found AAA risk alleles in *LRP1*,[35] *DAP21P*,[36] *ANRIL*,[37] and *SORT1*.[38] These genes have not been tested in this study, but it would be useful to do so in future studies.

In this study three variants were observed classified as pathogenic or likely pathogenic amongst a total of 47 unique rare variants in our AAA study population of 155 patients. Lack of a comprehensive overview of genetic variants in thoracic aneurysms precluded comparison of our findings in the abdominal aneurysm population to thoracic aneurysms population.

Assessment of pathogenicity of genetic variants remains a major challenge.[39] Comprehensive guidelines are needed to distinguish true pathogenic from ambiguous variants with unknown clinical significance which constitutes a large part of the results of molecular analyses.[27] Variants listed in HGMD which reports whether variants and/or genes have been described in literature as requires critical review of evidence presented to justify classification as likely pathogenic. Additional searches may be needed because not all known variants are listed in HGMD. Establishing a causal effect of variants involves finding a method of choice for functional testing of variants in aneurysm genes, which is complicated giving the likelihood of tissue specific gene expression. Especially since nowadays abdominal aortic aneurysms are mostly restored by an endovascular procedure, no aortic aneurysm tissue from patients can be collected for functional testing. Alamut incorporates allele frequency reported by dbSNP/ESP. The use of population-specific control cohorts, as GoNL in the current study, may improve correct classification of variants and prevent associating population specific polymorphisms with disease.

Significant co-segregation of a variant with disease provides evidence to support pathogenicity. In our study population it was often not possible to detect co-segregation because AAA is a late onset disorder, where the majority of patients, familial AAA and sporadic AAA alike, are older than 65 years and most affected relatives are no longer alive. Although our results suggest that more variants occur in familial cases (31%) than in sporadic cases (21%), the available sample size of the study population did not provide sufficient statistical power to test the difference between familial and sporadic AAA (table 5).

Table 5 – Genetic variants in in 99 familial and 56 sporadic abdominal aortic aneurysm patients

	Total AAA patients			Familial AAA patients			Sporadic AAA patients			p-value*	
Gene/ variants	**n**	**ALL**	**LP/P**	**n**	**ALL**	**LP/P**	**n**	**All**	**LP/P**	**ALL**	**LP/P**
ACTA2	139	0	0	92	0	0	47	0	0	1	1
COL3A1	122	3(2%)	1(1%)	82	3(2%)	1(1%)	40	0	0	1	1
EFEMP2	89	5(7%)	0	65	5(8%)	0	24	0	0	0.19	1
FBN1	127	8(6%)	0	85	5(6%)	0	42	3(7%)	0	1	1
MYH11	133	11(8%)	1(1%)	90	9(10%)	1(1%)	43	2(5%)	0	0.50	1
MYLK	136	18(13%)	0	90	12(13%)	0	46	6(13%)	0	1	1
SMAD3	142	0	0	94	0	0	48	0	0	1	1
TGFB2	65	2(3%)	0	40	2(5%)	0	25	0	0	0.52	1
TGFBR1	141	5(4%)	0	93	4(4%)	0	48	1(2%)	0	0.66	1
TGFBR2	140	3(2%)	1(1%)	94	2(2%)	0	46	1(2%)	1(2%)	1	0.33

*P-value was calculated using the two-tailed Fisher's exact test.
LP, likely pathogenic. P, pathogenic. All variants: pathogenic, likely pathogenic, unknown clinical significance and likely benign

***COL3A1**:* The vascular type of the Ehlers-Danlos syndrome is caused mutations in type III procollagen encoded by the *COL3A1* gene.[22] Abnormal type III procollagen results in altered connective tissue in particular of the vascular wall, skin and inner organs. The Ehlers-Danlos type IV syndrome is associated with vascular fragility, thin and translucent skin, typical facial features, rupture uterus or intestines, and variably hypermobility or contractures. In the *COL3A1* gene we found one familial pathogenic null mutation and two VUS variants in familial AAA. Null mutations in *COL3A1* cause haploinsufficiency and were previously associated with attenuated clinical features of the vascular type of Ehlers-Danlos syndrome, like in the family described in the current study.[40]

***EFEMP2**:* No pathogenic *EFEMP2* variants were detected in AAA patients. Four LB variants were observed in five familial AAA patients (8%). One patient had two different variants in *EFEMP2*. One LB variant with an allele frequency of 0.005 in the Dutch population occurred in three patients.

***FBN1**:* The Marfan syndrome (MFS) was the first well recognized genetic aortic aneurysm syndrome described in 1896,[41] and has an estimated prevalence of 2 to 3 per 10.000 individuals equally affecting men and women.[42] We found five *FBN1* VUS, two in familial and three in sporadic patients including three VUS previously reported in aneurysm patients in HGMD (table 2).

***MYH11**:* One likely pathogenic segregating variant and five VUS were observed among the ten rare variant in this gene.

***MYLK**:* The *MYLK* gene harboured the most variants of all examined genes in this study in

familial and sporadic patients. We found 13 unique variants in 18 patients, in 13% of the familial and 13% of the sporadic AAA patients. Of these variants, five were VUS and the rest was classified as likely benign.

TGFB2: Boileau and Lindsay et al. described simultaneously a mutation in the *TGFB2* gene causing familial thoracic aortic aneurysm and dissections and overlapping clinical features with Loeys-Dietz syndrome.[4,11] We observed two VUS in the *TGFB2* in two patients with familial AAA (6%).

TGFBR1 and ***TGFBR2***: In *TGFBR1* we found variants in 4% of familial and 2% of sporadic patients, one VUS and three likely benign. To our knowledge no pathogenic variants in *TGFBR1* or *TGFBR2* have been linked to familial AAA. Specific alleles were previously associated with risk for AAA.[43,44]

In *TGFBR2*, we found one de novo pathogenic novel single basepair deletion leading to a truncated protein. We also found one missense variant that was reported in HGMD and was classified as VUS, and one variant that we classified as likely benign.

MTHFR: The role in the susceptibility for abdominal aortic aneurysms of the *MTHFR* c.665C>T (p.Ala222Val) variant, previously reported as C677T, was investigated by a number of case control studies showing more robust associations in some than in others.[45] More recently genome wide association studies endorsed that this variant was associated with an increased risk.[46-50] In the current study, the MAF of the risk allele was lower (0.265) than in the Dutch control population (0.320), indicating that our data did not support a link with AAA.

CONCLUSIONS

This study identified three causal variants in a set of genes previously associated with familial thoracic aortic aneurysms in 155 familial and sporadic AAA patients. Our results showed that diagnostic testing of these aneurysm genes might help find the cause for AAA and help to accurately identify relatives at risk. It is important to note the occurrence of *de novo* mutations, indicating that a negative family history should not preclude genetic testing. Pathogenic variants were found in two younger male patients with complex vascular features and an elderly female AAA patient. Although we cannot exclude an effect of referral bias, these observations merit further studies addressing the question whether age, gender and clinical features define a risk profile for molecular defects in AAA patients.

The identification of 44 other variants in genes associated with hereditary thoracic aneurysms suggests a more important contribution of these genes in AAA than known before. We expect that additional aneurysm associated genes will be detected in the future because the majority of familial AAA patients had no variants in the examined genes.

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SUPPLEMENTARY ONLINE TABLE

Clinical features of abdominal aortic aneurysm patients with variants in aneurysm genes and the results of molecular an clinical family studies.

Gene/Variant	Class*	familial/ sporadic AAA (99/56)	Segregation	Family #	Age (yrs) index (relatives)[r]	M/F	Aneurysm in index and relatives	Body length/ armspan [+] (ratio)[++]	Beighton score	Skin Extensible=1 Scarring=2** Translucent=3 N=normal	Skeletal Pectus excavatum=1 kyphoscoliosis=2 arthrosis=3 N= none	Other
COL3A1												
c.812G>A[a]	VUS	1 (1/0)	nd	1/0	71	M	infrarenal ,dissection	-	-	-	3	
					70[r]	F	type B infrarenal	-	-	-	-	
c.898-14A>G	VUS	1 (1/0)	nd	5[b]/2[c]	67	M	infrarenal, iliac	178/188	1	3	3	retrognathia
					60[r]	M	descendens,	((1.05)	-	1	2	
					71[r]	F	ascendens dilatation descendens, iliac rupture		-	-	-	
c.1471C>T	**P**	1 (1/0)	**+**	4/2	49	M	art lienalis	172/182 (1.05)	0	N	N	
					69[r]	F	infrarenal	-	-	-	-	
					59[r]	F	infrarenal	-	-	-	-	
EFEMP2		***6 (6/0)***										
c.160+17G>T	LB	1 (1/0)	nd	5/2	68	M	infrarenal	183/189 (1.03)	0	3	3	
					72[r]	M	juxtarenal	173/191 (1.10)	0	1	3	
					67[r]	M	infrarenal, iliac	172/178 (1.03)	0	1,3	3	inguinal hernia
c.277G>A	LB	3 (3/0)[d]	-	6/4	51	M	infrarenal, iliac	177/182	6	2	N	
					66[r]	F	infrarenal	-	-	-	-	
					48[r]	F	rupture infrarenal	-	-	-	-	
					70[r]	M	rupture infrarenal	-	-	-	-	
					55[r]	M	infrarenal	-	-	-	-	
			nd[f]	1/1	56	M	infrarenal	176/185	5	1	N	
					71[r]	M	infrarenal	-	-	-	-	
			nd[e]	1/0	72	M	infrarenal	-	3	N	N	

Supplementary Online Table – Continued

Gene/Variant	Class*	familial/ sporadic AAA (99/56)	Segregation	Family #	Age (yrs) index (relatives)[r]	M/F	Aneurysm in index and relatives	Body length/ armspan [+] (ratio)[++]	Beighton score	Skin Extensible=1 Scarring=2** Translucent=3 N=normal	Skeletal Pectus excavatum=1 kyphoscoliosis=2 arthrosis=3 N= none	Other
c.368-4G>A	LB	1 (1/0)	-	6/5	69	M	infrarenal, dissection	170/185 (1.08)	2	N	N	wrist sign
					69[r]	F	type B	-	-	-	-	
					61[r]	M	ascendens	-	-	-	-	
					43[r]	F	dissection type A	-	-	-	-	
					36[r]	F	descendens	-	-	-	-	
					71[r]	F	dilatation ascendens dilatation descendens	-	-	-	-	
c.1047C>T	LB	1 (1/0)	nd[f]	1/1	56	M	infrarenal	176/185	5	1	3	hypertelorism
					71[r]	M	infrarenal	(1.05) -	-	-	-	inguinal hernia
FBN1		***8 (5/3)***										
c.59A>G[g30]	VUS	1 (0/1)			72	M	infrarenal	-	0	N	N	inguinal hernia
c.248-17C>G[h]	LB	1 (1/0)	-	1/1	65	M	infrarenal	180/180	0	2,3	3	
					69[r]	F	infrarenal	-	-	-	-	
c.1108G>A[i]	LB	1 (1/0)	nd	3/1	44	M	rupture infrarenal, iliac infrarenal	172/181 (1.05)	0	N	3	
					74[r]	M			-	-	-	
c.2260T>C[j]	VUS	1 (1/0)	nd	1/0	58	M	infrarenal, dilatation iliac	171/175	0	N	N	
c.2895G>A[k]	LB	1 (1/0)	nd	3/0	50	M	infrarenal	173/173	0	2,3	N	
c.3455C>T	VUS	1 (0/1)			61	F	thoraco-abdominal	-	-	-	-	
c.6055G>A	VUS	1 (1/0)	nd	1/0	75	F	infrarenal	180/179	6	1	N	wrist sign
c.7412C>G	VUS	1 (0/1)			64	M	infrarenal, iliac	185/177	7	N	N	inguinal hernia

Supplementary Online Table – Continued

Gene/Variant	Class*	familial/ sporadic AAA (99/56)	Segregation	Family #	Age (yrs) index (relatives)[r]	M/F	Aneurysm in index and relatives	Body length/ armspan [+] (ratio)[++]	Beighton score	Skin Extensible=1 Scarring=2** Translucent=3 N=normal	Skeletal Pectus excavatum=1 kyphoscoliosis=2 arthrosis=3 N= none	Other
MYH11		11 (9/2)										
c.760C>T	LP	1 (1/0)	-	1/1	73	F	infrarenal	173/179 (1.03)	3	N	N	
					44[r]	M	infrarenal, iliac	197/199	2	2	N	
c.956A>G[h]	VUS	1 (1/0)	-	1/1	65	M	infrarenal	180/180	0	2,3	3	
					69[r]	F	infrarenal	-	-	-	-	
c.1523G>A	VUS	1 (1/0)	nd	1/0	77	M	infrarenal	-	-	-	-	
c.1868C>G	LB	1 (1/0)	-	2/1	62	F	infrarenal	171/169	9	N	N	high palate,
					64[r]	M	infrarenal	-	-	-	-	wrist sign
c.2881-14C>G	LB	1 (1/0)	-	4[b]/1	63	M	infrarenal	185/186	-	2	-	
					62	F	descendens	-	-	-	-	
c.4694C>T[l]	VUS	1 (1/0)	gem	1/1	60	M	rupture infrarenal,	189/193	1	1,2,3	2	wrist sign
					61	M	iliac, popliteal, juxtarenal, infrarenal, popliteal	183/192(1.04)	2	1,2		
c.5587C>T[e]	LB	1 (1/0)	nd	1/0	72	M	infrarenal	-	3	N	N	
c.5635-7G>A	LB	2 (1/1)	nd	1/1	48	F	rupture renal art	176/178	3	1	2	high palatum
					54[r]	M	rupture renal art	-	-	-	-	
			[o]		47	M	infrarenal, iliac, dissection type B	189/195 (1.03)	7	N	3	vascular tortuosity
c.5697G>C[m]	VUS	1 (1/0)	-	4/0	75	F	infrarenal	174/178	1	2	3	
c.5808-11-8del	VUS	1 (0/1)			77	M	rupture iliac, infrarenal	175/178	0	N	N	inguinal hernia
MYLK		19 (13/6)										
c.312T>C	LB	1 (1/0)	gem	1/1	49	F	infrarenal, ascendens	175/188 (1,07)	4	2,3	N	wrist sign,
					50	F	dilatation infrarenal, iliac, popliteal	178/191 (1,09)	9	2	N	thumb sign

Supplementary Online Table – Continued

Gene/Variant	Class*	familial/ sporadic AAA (99/56)	Segregation	Family #	Age (yrs) index (relatives)[r]	M/F	Aneurysm in index and relatives	Body length/ armspan [+] (ratio)[++]	Beighton score	Skin Extensible=1 Scarring=2** Translucent=3 N=normal	Skeletal Pectus excavatum=1 kyphoscoliosis=2 arthrosis=3 N= none	Other
c.745T>G	VUS	1 (0/1)			92	M	infrarenal	192/195	0	3	1	inguinal hernia
c.1314C>T	LB	1 (0/1)			57	M	rupture thoraco-abdominal, iliac	176/177	0	2,3	1	
c.1327C>T	VUS	4 (4/0)	-	3/3	73	M	infrarenal	170/182(1.07)	4	2,3	N	inguinal hernia
					72[r]	F	infrarenal, juxtarenal	-	-	-		
					77[r]	M	infrarenal	-	-	-		
					71[r]	M	infrarenal	-	-	2,3	N	
			nd[e]	1/0	72	M	infrarenal	-	3	N	N	
			nd[a]	1/0	71	M	infrarenal, dissection	-	-	-	3	
					70[r]	F	type B	-	-	-	-	
			nd	1/1	82	M	infrarenal	181/189 (1.04)	0	2,3	3	
					84[r]	M	infrarenal, iliac infrarenal	-	-	-	-	inguinal hernia
c.2101G>A	VUS	1 (0/1)			74	M	suprarenal, arteria renalis	172/172	8	1,2	N	gout
c.3184G>T[g]	LB	1 (0/1)			72	M	infrarenal	-	0	N	N	inguinal hernia
c.3302A>G[d]	LB	1 (1/0)		6/4	51	M	infrarenal, iliac	177/182	6	2	N	
					66[r]	F	infrarenal	-	-	-	-	
			-		48[r]	F	rupture infrarenal	-	-	-	-	
					70[r]	M	rupture infrarenal	-	-	-	-	
					55[r]	M	infrarenal	-	-	-	-	
c.3403G>A	VUS	1 (1/0)	+	1/1	49	F	infrarenal	185/181	9	N	3	wrist sign
					85[r]	M	infrarenal,iliac	-	-	-	3	
c.3583A>G[n]	VUS	1 (1/0)	nd	1/0	64	F	infrarenal, iliac	177/188 (1.06)	2	N	3	
c.4179C>T	LB	2 (1/1)	nd	1/0	74	M	infrarenal	181/189 (1.03)	9	3	2	
					67	M	infrarenal	-	-	-	-	

Supplementary Online Table – Continued

Gene/Variant	Class*	familial/ sporadic AAA (99/56)	Segregation	Family #	Age (yrs) index (relatives)[r]	M/F	Aneurysm in index and relatives	Body length/ armspan [+] (ratio)[++]	Beighton score	Skin Extensible=1 Scarring=2** Translucent=3 N=normal	Skeletal Pectus excavatum=1 kyphoscoliosis=2 arthrosis=3 N= none	Other
c.4764G>A[k]	LB	3 (2/1)	nd	3/0	50	M	infrarenal	173/173	0	2,3	N	
			nd	2/0	82	M	infrarenal	-	-	-	-	
					62	M	thoraco- abdominal	180/188 (1.04)	2	2	1,2	
c.4785C>T	LB	1 (1/0)	nd	1/1	59	F	juxtarenal	-	-	-	-	
					63	M	infrarenal	-	-	-	-	
c.5079G>A[n]	LB	1 (1/0)	nd	1/0	64	F	infrarenal, iliac	177/188 (1.06)	2	N	3	
TGFB2		***2 (2/0)***										
c.272G>A[i]	VUS	1 (1/0)	nd	1/0	58	M	infrarenal, iliac	171/175	2	3	3	
c.703G>C	VUS	1 (1/0)	nd	5/2	79	M	infrarenal	172/184 (1.06)	0	N	2,3	inguinal hernia
					66[r]	M	infrarenal	172/184 (1.06)	0	3	1	
					62[r]	M	infrarenal	-	-	-	-	
TGFBR1		5 (4/1)										
c.15C>T[i]	LB	1 (1/0)	nd	3/2	44	M	rupture infrarenal, iliac infrarenal	172/181 (1.05)	0	N	3	
					74[r]	M		-	-	-	-	
c.214A>T	VUS	2 (1/1)			78	M	infrarenal	178/180	0	N	3	
			nd	1/1	69	M	infrarenal	177/185 (1.04)	0	N	3	
					68[r]	F	infrarenal	-	-	-	-	
c.927G>C	LB	1 (1/0)	nd	1/0	66	M	infrarenal	176/184 (1.05)	9	1	3	inguinal hernia
c.1125A>G[m]	LB	1 (1/0)	nd	4/0	75	F	infrarenal	174/178	1	2	3	
TGFBR2		***3 (2/1)***										
c.1137C>T	LB	1 (1/0)	+	2/1	71	M	infrarenal	178/174	1	1,2	1	inguinal hernia
					72[r]	M	ascendens	173/168	0	N	3	
c.1234G>A[l]	VUS	1 (1/0)	gem		60	M	rupture infrarenal, iliac, popliteal juxtarenal, infrarenal, popliteal	189/193	1	1,2,3	2	wrist sign
					61	M		183/192 (1.06)	2	1,2		
c.1573delA°	P	1 (0/1)	de novo	-	47	M	infrarenal, iliac, dissection type B	189/195 (1.03)	7	N	3	vascular tortuosity wrist sign

* Classification of variants: P, pathogenic. LP, likely pathogenic. VUS (variant of unknown clinical significance). LB likely benign.
nd, not determined.
In bold the variants classified as pathogenic or probably pathogenic
** skin signs: scarring, abnormal wide or paper thin
# family: total number of relatives reported to be affected with aortic aneurysm (excludes index patient) / the number of relatives confirmed to be affected
[+] body measurement in cm, [++] ratio body length/ arm span < 1.03 (normal) not mentioned
[r] age relative diagnosed with an aortic aneurysm
[a] patient with *COL3A1* c.812G>A and *MYLK* c1327C>T
[b] aortic aneurysm in families of both parents
[c] mother, father
[d] patient EFEMP2 c.277G>A and MYLK c.3302A>G variant
[e] patient with EFEMP2 c.277G>A, *MYH11* c.5587C>T and *MYLK* c.1327C>T variant
[f] patient with *EFEMP2* c.277G>A and *EFEMP2* c.1047C>T variant
[g] patient with *FBN1* c.59A>G and *MYLK* c.3184G>T variant
[h] patient *FBN1* c.248-17C>G and *MYH11* c.956A>G variant
[i] patients with *FBN1* c.1108G>A and *TGFBR1* c.15C>T variant
[j] patient with *FBN1* c.2260T>C and *TGFB2* c.272G>A variant
[k] patient *FBN1* c.2895G>A and *MYLK* c.4764G>A variant
[l] patient with *MYH11* c.4694C>T variant and pathogenic *TGFBR2* c.1234G>A variant
[m] patient with *MYH11* 5697G>C and *TGFBR1* 1125A>G variant
[n] patient with *MYLK* c.3583A>G and *MYLK* c.5079G>A variant
[o] patient with *MYH11* c.5635-7A and the pathogenic *TGFBR2* c.1572delA variant

PART III

Clinical Outcome of Familial Abdominal Aortic Aneurysm

7

Familial Abdominal Aortic Aneurysm is Associated with More Complications after Endovascular Aneurysm Repair

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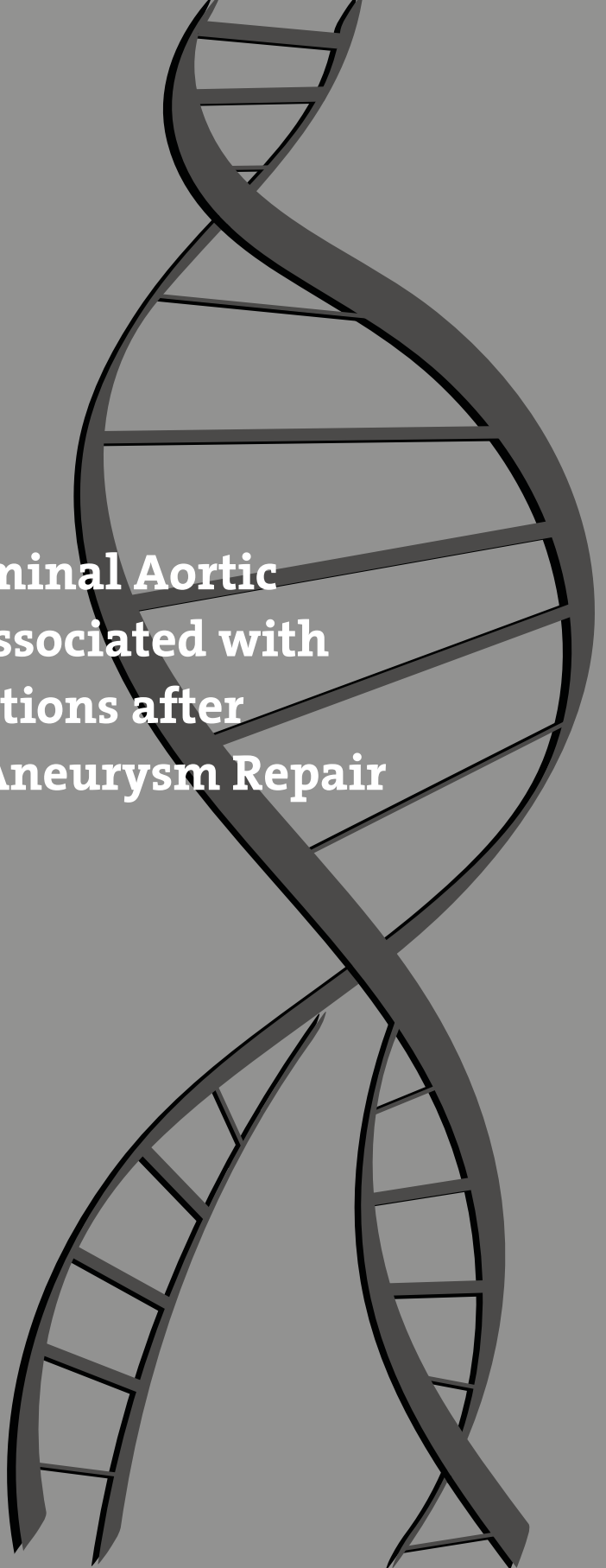

ABSTRACT

Objective: A familial predisposition to abdominal aortic aneurysms (AAA) is present in approximately one-fifth of patients. Nevertheless, the clinical implications of a positive family history are not known. We investigated the risk of aneurysm-related complications after endovascular aneurysm repair (EVAR) for patients with and without a positive family history of AAA.

Methods: Patients treated with EVAR for intact AAAs in the Erasmus University Medical Center between 2000 and 2012 were included in the study. Family history was obtained by written questionnaire. Familial AAA (fAAA) was defined as patients having at least one first-degree relative affected with aortic aneurysm. The remaining patients were considered sporadic AAA (spAAA). Cardiovascular risk factors, aneurysm morphology (aneurysm neck, aneurysm sac and iliac measurements), and follow-up were obtained prospectively. The primary endpoint was complications after EVAR, a composite of endoleaks, need for secondary interventions, aneurysm sac growth, acute limb ischemia and post-implantation rupture. Secondary endpoints were; specific components of the primary endpoint (presence of endoleak, need for secondary intervention and aneurysm sac growth), aneurysm neck growth and overall survival. Kaplan-Meier estimates for the primary endpoint were calculated and compared using log-rank (Mantel-Cox) test of equality. A Cox-regression model was used to calculate the independent risk of complications associated with fAAA.

Results: 255 patients were included in the study (88.6% men; age 72±7 years, median follow-up 3.3 years [IQR: 2.2-6.1]). A total of 51 patients (20.0%) were classified as fAAA. Patients with fAAA were younger (69 versus 72 years, P=.015) and were less likely to have ever smoked (58.8% versus 73.5%, P=.039). Preoperative aneurysm morphology was similar in both groups. Patients with fAAA had significantly more complications after EVAR (35.3% versus 19.1%, P=.013), with a 2-fold increased risk (adjusted HR 2.1, 95%CI 1.2-3.7). Secondary interventions (39.2% versus 20.1%, P=.004) and aneurysm sac growth (20.8% versus 9.5%, P=.030) were the most important elements accounting for the difference. Furthermore, a trend towards more type I endoleaks during follow-up was observed (15.6% versus 7.4%, P=.063) and no difference in overall survival.

Conclusions: The current study shows that patients with a familial form of AAA develop more aneurysm-related complications after EVAR, despite similar AAA morphology at baseline. These findings suggest that patients with fAAA form a specific subpopulation and create awareness for a possible increase in the risk of complications after EVAR.

INTRODUCTION

Approximately 20% of the abdominal aortic aneurysm (AAA) patients have a positive family history for aneurysms, with a prevalence ranging largely from 6% to 35%, depending on ethnicity and method of data collection.[1-4] This suggests that in these families there is a genetic predisposition to AAA and that patients can be classified as familial AAA (fAAA), whereas patients without a clear inherited risk can be classified as sporadic AAA (spAAA). Despite the apparent familial tendency towards AAA formation and results from some genetic studies, the exact underlying genetic defects and their contribution to the development, growth, and severity of complications are unknown.[5] The molecular and clinical well-delineated genetic aortic aneurysm syndromes, including Marfan, Loeys-Dietz, the vascular Ehlers-Danlos syndrome and defects in the smooth muscle cell genes *MYH11* and *ACTA2*, are mostly associated with thoracic aortic aneurysms, but occasionally AAA may be observed in the affected families.[6-10] Like in most known syndromes, in AAA there are recognized defects both in the connective tissue components and in cellular elements affecting all layers of the aortic wall.[11]

In the last decade, endovascular aneurysm repair (EVAR) has proven to be a valid treatment modality for AAA and the majority of elective patients are now treated endovascularly.[12] Generally, endovascular repair in patients with known genetic aortic aneurysm syndromes is not advised, since patients have a higher chance of complications.[13, 14] At present, little is known on clinical outcome after EVAR for patients with an inherited risk for AAA and no data on aneurysm morphology of this particular group is available to date. One may hypothesize that AAA patients with a positive family history may develop more seal and fixation problems, and also post-implantation sac growth due to inherited aortic wall defects. Furthermore, differences in aneurysm morphology for fAAA patients, if present, could also influence outcome.

In the present study, we evaluated aneurysm-related complications after EVAR for patients with fAAA and spAAA, and explored possible differences in aneurysm morphology in these groups.

METHODS

The study population was derived from a prospective database including all EVAR procedures performed at the Erasmus University Medical Center in Rotterdam, the Netherlands. From January 2000 until March 2012, 473 patients were treated with EVAR at our institution. Exclusion criteria for this study were isolated iliac artery aneurysm, traumatic aneurysm, anastomotic aneurysm, infectious aneurysm and ruptured aneurysm. Between 2009 and 2012, all AAA patients at our institution were contacted when visiting the outpatient clinic or by mail and asked to fill out a semi-structured questionnaire, in order to collect

personal data and family histories. Patients who did not respond after one reminder were contacted and interviewed by telephone (KvdL). In families with multiple AAA patients, only one index patient (i.e. first family member diagnosed with AAA) was included in the study. Patients previously diagnosed with a genetic aortic aneurysm syndrome (e.g. Marfan, Loeys-Dietz, or vascular Ehlers-Danlos syndrome) were excluded, but no specific genetic testing was routinely performed. A flow diagram of patient inclusion is presented in Figure 1. The study complied with the declaration of Helsinki and was approved by the Institutional Review Board.

Questionnaire and classification of familial AAA

The questionnaire requested information on demographics and the medical history of the index patient. Furthermore, structured questions were included on the occurrence of aortic aneurysms and cardiovascular disease for all known relatives of the index patient. Patients were classified as fAAA when at least one first-degree relative (parents, siblings or children) was reported to have an aortic aneurysm.[1] Patients who did not report a first-degree relative affected with AAA were classified as spAAA. Patients reporting only second- or third-degree relatives were also classified as spAAA, because the reporting of medical information of second- or third-degree relatives was considered less reliable.

Image processing

All patients were preoperatively assessed using computed tomography angiography (CTA) and entered the institutional surveillance protocol that included an early postoperative CTA (typically before hospital discharge), a CTA at 6 months and 1 year, and then CTA scans yearly after. Since 2007, the 6 month examination has been waived and CTA surveillance replaced by duplex ultrasound (DUS) examinations in selected patients considered a lower risk according to the treating physician's experience in concurrence with Clinical Practice Guidelines of the European society for Vascular Surgery. Also, DUS examinations or non-contrast CT scans were performed as an alternative to CTA in patients with impaired renal function.

Computed tomography angiography was performed according to standardized institutional protocols. Morphologic analyses and measurements were performed using dedicated software with center lumen line (CLL) reconstruction (3Mensio, Vascular 4.2 software, 3Mensio Medical Imaging BV, Bilthoven, the Netherlands). Center lumen lines were semi-automatically constructed and followed the center of the aortic and iliac permeable lumen.

The preoperative, early (<30 days) postoperative, and last follow-up CTA scans were analyzed in all patients. In patients with complications after EVAR, all CTA scans were analyzed.

Interobserver variability was previously assessed and agreement was high for AAA diameter (R^2 linear = 0.996), neck length (R^2 linear = 0.991), and neck diameter (R^2 linear = 0.935).[15]

Definitions

Aneurysm related definitions used in the study were derived from the reported standards for EVAR and/or were previously described.[12, 15-18] Briefly, aneurysm and neck diameters were determined after CLL reconstructions. Aneurysm neck length was defined as the length of the lowermost renal artery to the level where the aortic diameter increases with at least 10 percent. Aneurysm angulation (suprarenal and infrarenal) were defined after CLL reconstruction. Aneurysm neck thrombus and calcification were defined as having more than 25 percent of the cross sectional area of the neck being affected. Iliac stenosis was defined as having at least one focal stenosis in the one of the iliac arteries. Iliac tortuosity was defined as absent, minor or major by one experienced observer (FBG) using 3-D reconstruction. Iliac aneurysm was defined as having an iliac diameter over 3 cm measured after CLL reconstructions. Aneurysm sac behaviour and proximal neck dilatation during follow-up was calculated for patients with at least two suitable imaging surveillance exams. Aneurysm neck growth was defined as an increase of ≥2 mm between the maximum neck diameter at first postoperative and last available CTA scan during follow-up. Aneurysm sac growth was defined as an increase of in diameter ≥5 mm and aneurysm sac shrinkage as a decrease in diameter ≥5 mm between the maximal aneurysm diameter at first postoperative and last available imaging (i.e. 2 available CTA scans or 2 available DUS examinations) during follow-up.

End points

The primary study endpoint was freedom from complications after EVAR. Complications after EVAR was defined as a composite of one of the following: endoleak during follow-up (i.e. type Ia, type Ib, type III or undetermined type endoleaks on postoperative examinations), secondary intervention (i.e. proximal stent/cuff, limb extension, coil/glue embolization, open ligation of collaterals, conversion to aorto-uni-iliac device, conversion to open repair and relining), aneurysm sac growth, acute limb ischemia or post-implantation aneurysm rupture. Type II endoleak was not included as a complication after EVAR, because we consider intervention for type II endoleak only when in combination with aneurysm sac growth which is included as complication after EVAR.[12] In case the primary endpoint was met by multiple criteria, the date of the first event was considered for the purpose of survival analysis.

The secondary endpoints were; individual components of the primary endpoint (endoleak during follow-up, secondary interventions and aneurysm sac growth), aneurysm neck growth and overall survival after EVAR.

Clinical characteristics

The medical histories of the patients were obtained from medical files. The demographic characteristics included gender and age. The cardiovascular comorbidities included ischemic heart disease (history of myocardial infarction, coronary revascularisation or pathologic Q-waves on the electrocardiogram), cerebrovascular disease (history of ischemic/hemorrhagic stroke or transient ischemic attack), and cardiac arrhythmia. The cardiovascular risk factors included kidney disease (estimated glomerular filtration rate <60 ml/min per 1.73 m^2), diabetes mellitus (fasting plasma glucose ≥7.0 mmol/L, non-fasting glucose ≥11.1 mmol/L or use of anti-diabetic medication), hypertension (blood pressure ≥140/90 mmHg in non-diabetics, ≥130/80 mmHg in diabetics or use of antihypertensive medication), chronic obstructive pulmonary disease (COPD; history of COPD or stage ≥1 according to the GOLD classification). Smoking was obtained and included current smoking and ever smoking (ie, patients who are currently smoking OR patients with a history of smoking). Prescription medications were recorded and included the use of statins, beta-blockers, antiplatelets and anticoagulant therapy.

Statistical analysis

Dichotomous data are described as counts and percentages. Continuous variables are described as mean (standard deviation) or median with interquartile range [IQR] when not normally distributed. Categorical data were analysed with chi-square tests and continuous variables with ANOVA or Kruskal-Wallis tests, as appropriate. A multivariable Cox regression was used to assess the hazard ratio (HR), along with the 95% confidence interval (CI), for complications after EVAR between fAAA and spAAA. Variables entered into the multivariate Cox regression model were selected on basis of univariable significant differences at baseline between fAAA and spAAA (i.e. age and ever smoking). Kaplan-Meier estimates were calculated for freedom from complications after EVAR. Estimates for fAAA and spAAA were compared using log-rank (Mantel-Cox) test of equality. To assess a possible selection bias, we tested for differences in complications after EVAR, for included and excluded patients of the complete EVAR database, using chi-square tests.

For all tests, a P-value <.05 (two-sided) was considered significant. All analyses were performed using IBM SPSS Statistics version 20.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

A total of 373 patients were treated with EVAR for intact degenerative aorto-iliac aneurysms (Figure 1). Since 84 patients died before receiving the questionnaire and 34 patients did not respond to the questionnaire and could not be reached, the total study population consisted of 255 patients. No patients were identified with a genetic aortic

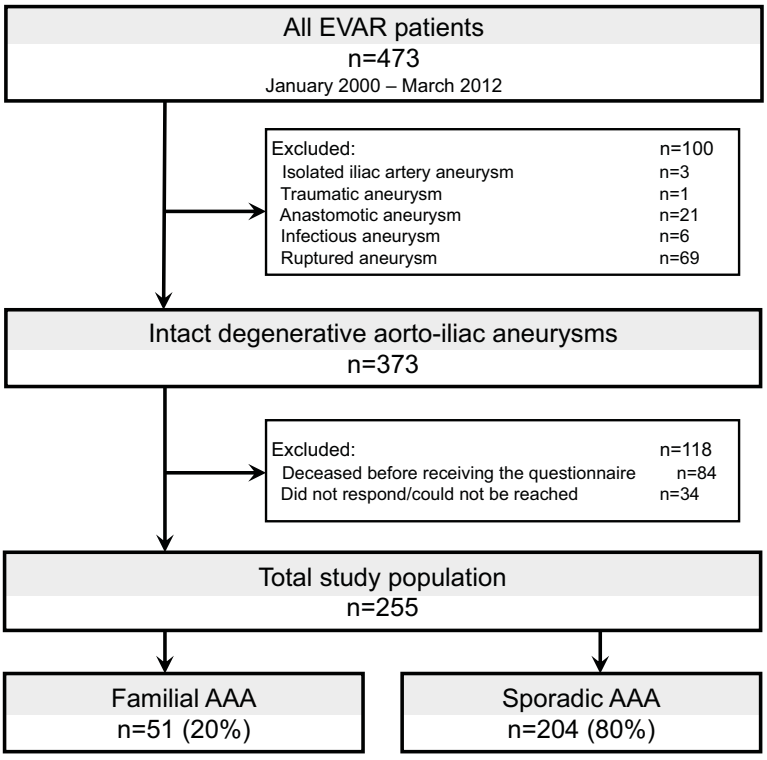

Figure 1 – Flow diagram of patient inclusion

aneurysm syndrome. The mean age of the population was 71.5 (±7.4) years and 226 patients (88.6%) were of male gender.

Clinical characteristics and aneurysm morphology at baseline

Of the 255 included patients, 51 (20.0%) had at least one affected first-degree relative and were classified as fAAA. The remaining 204 patients (80.0%) had no affected first-degree relative and were classified as spAAA. All clinical characteristics at baseline are presented in Table 1. Patients with fAAA were younger compared to spAAA patients (69 versus 72 years, P=.015) and were less likely to ever smoked (58.8% versus 73.5%, P=.039). There were no differences in aneurysm morphology between the two groups (Table 2). Preoperative neck and aneurysm diameter were similar, as well as the presence of iliac stenosis, iliac tortuosity and iliac aneurysms.

Table 1 – Clinical characteristics at baseline

Variable[a]	Familial AAA n=51	Sporadic AAA n=204	P-value
Male gender	44 (86.3)	182 (89.2)	.554
Age at diagnosis, years	69.3 ± 8.1	72.1 ± 7.1	.015
Age ≤65 years at diagnosis	14 (27.5)	31 (15.2)	.040
Cardiovascular comorbidities			
Ischemic heart disease	20 (39.2)	72 (35.3)	.619
Cerebrovascular disease	6 (11.8)	25 (12.3)	.905
Cardiac arrhythmia	7 (13.7)	17 (8.3)	.243
Cardiovascular risk factors			
Kidney disease	8 (15.7)	51 (25.0)	.186
Diabetes mellitus	10 (19.6)	39 (19.1)	.961
Hypertension	31 (60.8)	138 (67.6)	.285
COPD	18 (35.3)	83 (40.7)	.435
Smoking – current	17 (33.3)	81 (39.7)	.403
Smoking – ever	30 (58.8)	150 (73.5)	.039
Medication			
Statins	40 (78.4)	148 (72.5)	.393
Beta-blockers	42 (82.4)	152 (74.5)	.240
Antiplatelets	43 (84.3)	150 (73.5)	.108
Anticoagulants	5 (9.8)	27 (13.2)	.508

COPD: chronic obstructive pulmonary disease
[a] Continuous data are presented as the mean ± standard deviation and categorical data as number (%).

Table 2 – Aneurysm morphology at baseline

Variable[a]	Familial AAA n=51	Sporadic AAA n=204	P-value
Neck diameter, mean ± SD, mm	26.2 ± 4.2	25.4 ± 3.5	.194
Neck length, mean ± SD, mm	31.2 ± 17.5	31.5 ± 13.9	.982
AAA diameter, mean ± SD, mm	61.6 ± 12.8	60.3 ± 13.3	.533
Aneurysm angulation			
Suprarenal, mean degrees of angulation ± SD	22.3 ± 17.7	24.0 ± 18.1	.723
Infrarenal, mean degrees of angulation ± SD	37.5 ± 20.3	40.6 ± 24.8	.415
Neck thrombus, n (%)	14 (27.5)	70 (34.3)	.263
Neck calcification, n (%)	11 (21.6)	50 (24.5)	.558
Iliac stenosis, n (%)	8 (15.7)	38 (18.6)	.553
Iliac tortuosity, n (%)	28 (54.9)	110 (53.9)	.985
Iliac aneurysms, n (%)	15 (29.4)	65 (31.9)	.736

AAA: abdominal aortic aneurysm
[a] Preoperative CTA scans were available for 242 patients.

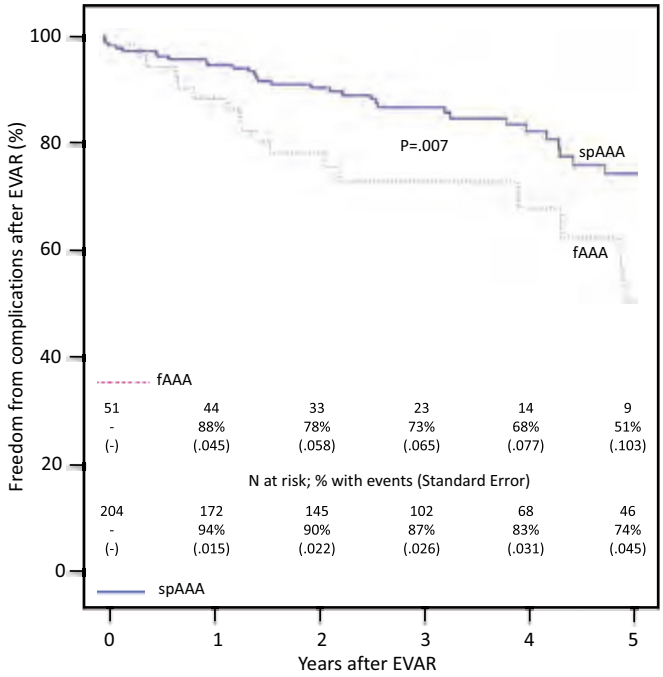

Figure 2 – Kaplan-Meier estimates are shown for complications after EVAR between familial AAA (*dashed red line)* and sporadic AAA (*solid blue line*).

7

Complications after EVAR

The median duration of follow-up was similar for fAAA and spAAA patients (3.9 years [IQR, 2.4-6.9] and 3.3 years [IQR, 2.1-5.5], P=.163). During this period, a total of 57 patients (22.4%) had complications after EVAR; 18 fAAA patients and 39 spAAA patients (35.3% versus 19.1%, P=.013, Table 3. Kaplan-Meier estimates for freedom of complications after EVAR were significantly different between both groups, with a 5-year estimate of 51% in fAAA and 74% in spAAA (P=.007, Figure 2).

A total of 19 patients (37.3% of fAAA) had two or more affected relatives. Patients with two or more affected relatives had more complications after EVAR compared to those with only one affected relative (42.1% versus 31.2%, respectively), although it did not reach statistical significance (P = .443).

Patients with fAAA had a 2.1-fold increased risk of complications after EVAR as compared to spAAA patients after adjustment for age and ever smoking (HR 2.1, 95% CI 1.2-3.7, Table 4). Age HR 0.99 (0.95 – 1.02, P=.405) and ever smoking HR 0.84 (0.49 – 1.45, P=.538) did not predict for complications after EVAR in the multivariable model

Table 3 – Complications after EVAR

Variable[a]	Familial AAA n=51	Sporadic AAA n=204	P-value
Complications after EVAR, patients	**18 (35.3)**	**39 (19.1)**	**.013**
Endoleak during follow-up, events	**8 (15.7)**	**18 (8.8)**	**.147**
Type Ia	5	9	
Type Ib	3	6	
Type III	0	1	
Type undetermined	0	2	
Secondary intervention	**20 (39.2)**	**41 (20.1)**	**.004**
Proximal stent/cuff	4	10	
Limb extension	5	18	
Coil/glue embolization	2	2	
Open ligation of collaterals	3	4	
Conversion to AUI	1	0	
Conversion to open repair	2	5	
Relining	3	2	
Aneurysm sac growth[b]	**10 (20.8)**	**18 (9.5)**	**.030**
Acute limb ischemia	**0**	**4**	**.313**
Post-implantation aneurysm rupture	**0**	**0**	**...**

EVAR: endovascular aneurysm repair, AAA: abdominal aortic aneurysm, AUI: aorto-uni-iliac device
[a] Categorical data are presented as number (%).
[b] Aneurysm sac measurements were available for 237 patients with ≥2 postoperative imaging exams (i.e. 2 CTA scans or 2 DUS examinations).

Table 4 – Uni- and multivariable analysis for complications after EVAR associated with familial AAA

	Univariable			Multivariable[a]		
	HR	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
Sporadic AAA	Ref			Ref		
Familial AAA	2.15	1.22 - 3.81	.008	2.05	1.15 - 3.66	.015

HR: hazard ratio
[a] Adjusted for: age and ever smoking

Endoleaks during follow-up

Patients with fAAA had more endoleaks during follow-up (15.7% versus 8.8%), although it did not reach statistical significance (P=.147). The difference appeared to be caused mainly by more type Ia and Ib endoleaks (15.6% versus 7.4%, P=.063).

Table 5 – Aneurysm sac behaviour and proximal neck dilatation during follow-up

Variable[a]	Familial AAA	Sporadic AAA	P-value
Aneurysm neck diameter[b]			
Growth	25 (59.5)	103 (63.2)	.662
Aneurysm sac diameter[c]			
Growth	10 (20.8)	18 (9.5)	.030
Stability	15 (31.2)	52 (27.5)	.608
Shrinkage	23 (47.9)	119 (63.0)	.057

[a] Categorical data are presented as number (%).
[b] Aneurysm neck measurements were available for 205 patients with ≥2 postoperative CTA scans.
[c] Aneurysm sac measurements were available for 237 patients with ≥2 postoperative imaging exams (i.e. 2 available CTA scans or 2 available DUS examinations).

Secondary interventions during follow-up

Patients with fAAA had a significantly higher secondary intervention rate after EVAR than spAAA patients (39.2% versus 20.1%, P=.004). Proximal stent/cuff, coil/glue embolization, open ligation of collaterals and relining were more common in patients with fAAA. Detailed data regarding elements of secondary interventions are presented in Table 3.

Aneurysm sac behaviour and proximal neck dilatation during follow-up

Aneurysm sac growth was more common in patients with fAAA than those with spAAA (20.8% versus 9.5%, P=.030, Table 5). Notably, this was independent of type II endoleaks, which occurred in 13.7% of the fAAA patients and 11.8% of the spAAA patients (P=.713). Patients with fAAA also tended to have less aneurysm sac shrinkage (47.9% versus 63.0%, P=.057). There was no difference in aneurysm neck growth, which occurred in 59.5% of the fAAA patients and 63.2% in patients with spAAA (P=.662).

Overall long-term survival

During follow-up, 41 patients died; 7 (13.7%) in the fAAA group and 34 (16.7%) in the spAAA group (P=.609).

Assessment of selection bias

As mentioned above, no difference in survival was observed between the two groups. However, we observed a difference in complications after EVAR for patients included and excluded from analysis (22.4% versus 15.1%, respectively, P=.046).

DISCUSSION

The main finding of the study was that patients with fAAA have a 2-fold higher risk of developing aneurysm-related complications after EVAR than patients with spAAA, despite similar AAA morphology. Although Brewster et al. showed several years ago a trend towards more aneurysm-related mortality in patients with a history of aneurysmal disease,[19] this is the first report focussing on the association between family history and complications after EVAR.

In his study we chose not to include patients with isolated iliac, traumatic, anastomotic or infectious aneurysms, because they either have different EVAR related complication risk or have other pathofysiological mechanisms leading to aneurysm formation compared to "typical" AAA. Additionally, we excluded the ruptured aneurysms because they have a high rate of non-responders due to high mortality, which could be an important source of bias. Also, the purpose of this study was primarily to determine the contribution of family history to preoperative risk assessment and modification, which is essentially directed at elective (preventive) situations. For ruptured aneurysms, family history of AAA is most likely not going to change the immediate attitude, which is to offer a life-saving procedure.

We found that 20% of our AAA population had a positive family history, which is similar to other studies reporting on the prevalence of fAAA.[1, 20-22] Furthermore, patients with fAAA were younger and were less likely to have a history of smoking compared to patients with spAAA in our population. Previous studies similarly suggested that fAAA patients are slightly younger but studies on the effect of smoking are scarce.[1, 21, 23]

Since it is well known that adverse AAA morphology may result in increased number of adjunctive procedures,[24] and it is also known that some genetic aortic aneurysm syndromes are associated with specific anatomical features like arterial elongation and tortuosity,[25-27] we determined aneurysm morphology before stent implantation. Maximum AAA diameter and presence of iliac tortuosity or stenosis were comparable between the two groups. Similarly, aneurysm neck characteristics such as diameter, length, angulation as well as the presence of thrombus and calcification were not different for fAAA and spAAA patients. Consequently, the observed disparities in complications cannot be attributed to morphological differences between groups.

Secondary interventions and aneurysm sac growth were the most important elements accounting for the difference in the composite primary endpoint of complications after EVAR. Although patients with fAAA also tended to have more endoleaks, in particular proximal and distal type I endoleaks, this difference failed to reach statistical significance due to limited patient numbers in the two groups. Patients with fAAA displayed more aneurysm sac growth, independent of the presence of type II endoleaks, and less aneurysm sac shrinkage than patients with spAAA. It may be hypothesized that an

intrinsic weakness of the aortic wall results in more rapid progression of aneurysm disease and contributes to a higher need for secondary interventions in fAAA patients. These observations suggest that -as yet unknown- inherited connective tissue disorders may underlie aneurysm formation in patients with familial AAA.

Over the median follow-up period of 3 years, aneurysm neck growth was quite common (about 60%) in both groups. This high rate results from a low threshold definition and is comparable to other reports on contemporary stent grafts.[28, 29]

In patients with known connective tissue disorders, endovascular therapies have been shown to result in much higher failure rates due to rapid dilatation or dissection of the aorta, and are generally unadvised.[13, 14] Nevertheless, we still believe that EVAR is a valid treatment alternative over open repair in patients with a family history, since most complications observed in our study in fAAA patients could be treated with minimally invasive techniques. Also, low morbidity and the early survival advantage of EVAR appear to be unchanged in the fAAA group. Although standard postoperative surveillance is still recommended, our study should create awareness for the fact that patients with fAAA may develop more complications after EVAR. New prospective studies are needed for clarification of our findings and should determine which postoperative surveillance program suits fAAA patients best. Apart from the surveillance program, all fAAA patients in our institute receive genetic counseling to provide information on the hereditary of aortic aneurysms and are offered screening for all first-degree relatives.

There are several limitations that need to be considered. First, the single-center nature of this study limits the generalization of the results. A second limitation is the classification of familial AAA based on self reported family history alone. The chance of having affected relatives is lower in small families compared to large families. Also, since objective screening of relatives was not performed, underreporting of fAAA is likely. Thirdly, no systematic molecular screening was performed for the known genetic aortic aneurysm syndromes. However, since these syndromes are rare causes for abdominal aortic aneurysms and generally present at a younger age, their contribution to the study population is probably negligible. Additionally, the relative short follow-up of 3.3 years should be taken into account, since it is known that endoleaks may develop in a later stage. Long-term follow-up is therefore warranted. Lastly, our study is also limited by its retrospective design, therefore, we evaluated possible selection bias. The mortality of fAAA and spAAA patients was similar for both groups, which suggests homogeneity between the two included groups, but we observed small difference in complications after EVAR for included and excluded patients. This was probably explained by the fact that patients treated for ruptured aneurysms died more frequently in the perioperative period and consequently could not develop a complication. Also, patients with a small anastomotic aneurysm treated with a covered stent are less likely to develop an EVAR

related complication as defined in the study. Therefore, we believe that bias might be present due to study design but was minimized by the chosen inclusion criteria and does not invalidate the main findings of the study.

CONCLUSIONS

The current study shows that patients with a familial form of AAA develop more aneurysm-related complications after EVAR, despite similar AAA morphology at baseline. Although the limitations of this study suggest caution in interpretation of the results, the 2-fold higher aneurysm-related complication rate after EVAR should create awareness for a possible incremental risk in this subgroup. Our findings emphasize the need for further research on genetic causes and underlying molecular mechanisms of AAA.

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8

Clinical Outcome of Endovascular Aneurysm Repair in Familial Abdominal Aortic Aneurysm

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In progress

ABSTRACT

Objective: To determine the influence of a positive family history for aneurysms on the clinical success after endovascular aneurysm repair (EVAR) from a large worldwide EVAR registry.

Methods: From March 2009 to April 2011, 1262 AAA patients with abdominal aortic aneurysms (AAA) treated with EVAR were enrolled in the Endurant Stent Graft Natural Selection Global Postmarket Registry (ENGAGE). Patients were classified into familial and sporadic AAA patients according to baseline clinical report forms. Clinical characteristics, aneurysm morphology and follow-up were obtained prospectively. The primary endpoint was clinical success after EVAR, a composite of technical success and freedom from the following complications: AAA increase > 5 mm, endoleak type I and III, aneurysm rupture, conversion, secondary procedures, migration and occlusion. Secondary endpoints were the individual components of clinical success, aneurysm-related mortality and all-cause mortality. Duration of follow-up was 2 years for all patients and 3 years for the first 500 patients enrolled.

Results: Of the 1262 AAA patients (89.5% male and mean age 73.1 years), 6.7% (85) patients reported a positive family history and were classified as familial AAA. Patients with familial AAA were more often female (18.8% versus 9.9%; *P* = .010), and no difference in aneurysm morphology was observed. There was no significant difference in clinical success between patients with familial and sporadic AAA (at two-years: 81.2% versus 86.7%; *P* = .156; at three-years 79.1% versus 85.1%; *P* = .296). Patients with familial AAA suffered from more stent graft occlusion (at two-years: 7.1% versus 3.5%; *P* = .095; at three-years: 9.5% versus 2.2%; *P* = .007) and there was a trend towards more secondary procedures in patients with familial AAA (Kaplan-Meier estimate 17.8% versus 10.9% after three years of follow-up, *P* = .06). Furthermore, patients with familial AAA had a higher aneurysm-related mortality (5.9% versus 1.2% after three years of follow-up; *P* = <.001).

Conclusions: The current study shows no significant difference in clinical success after endovascular repair in familial or sporadic AAA patients. However, the higher aneurysm-related mortality and the trend towards more aneurysm-related secondary procedures in patients with a positive family history suggest that these patients react differently to endovascular repair. Longer follow-up is needed to determine if family history should be accounted for when determining patient suitability for EVAR. For the time being, patients with familiar forms of AAA should be offered a close follow-up regime.

INTRODUCTION

Approximately 20% of the patients with abdominal aortic aneurysm (AAA) have a positive family history for aortic aneurysms and suggests a genetic susceptibility for aneurysm formation in these families.[1-3] It is unknown if this familial predisposition has clinical consequences, nor if it has any influence on the results after endovascular aneurysm repair (EVAR) in these patients. Patients with familial AAA might behave differently after stent graft placement because of altered aneurysm morphology or accelerated degeneration of the aortic wall. As a consequence, inherited aortic wall deficits may influence seal and fixation of implanted stent grafts.

Recently, we described that patients with a positive family history (ie, familial AAA patients) developed significantly more aneurysm-related complications after EVAR compared to patients with a sporadic AAA (35% versus 19% respectively).[4] This was the first study addressing this issue and due to the single-center nature of the study, the results need verification using larger EVAR patient cohorts.

The aim of the current study was to evaluate clinical success of endovascular repair for patients with and without a positive family history of AAA from a large worldwide EVAR registry. Additional analyses were performed for specific components of clinical success, aneurysm morphology and mortality.

METHODS

The study population was derived from consecutive patients enrolled in the Endurant Stent Graft Natural Selection Global Post-market Registry (ENGAGE). The ENGAGE study was initiated to evaluate the real life performance of the Endurant Stent Graft System (Medtronic Vascular, Santa Rosa, CA, USA) and is registered on http://clinicaltrials.gov (NCT00870051). From March 2009 to April 2011, eligible patients from 79 high-volume sites in 30 countries throughout the world were enrolled. Information on study design, data collection, monitoring, and statistical methods has been published previously.[5]

Classification of familial AAA

Patients were classified into familial and sporadic AAA based on responses to the baseline questionnaires. The specific question asked in the questionnaire was: "Family history of aneurysms? Yes or No. If yes, specify..." All patients with a positive family history for aneurysms were subsequently classified as familiar AAA, whereas patients who reported a negative family history for aneurysms were classified as sporadic AAA.

Clinical characteristics

Study data were recorded by each participating site prior to surgery and included demographic characteristics, baseline symptoms, physical measurements, risk factors and

comorbidities, and preoperative risk assessment. Demographic characteristics included; gender and age. Baseline symptoms included; abdominal/back or other complaints or asymptomatic. Physical examination included; height, weight and blood pressure. Risk factors and comorbidities included; tobacco use, hypertension, hyperlipidemia, history of diabetes mellitus, cancer, cardiac diseases, myocardial infarction, arrhythmia, angina pectoris, congestive heart failure, coronary artery disease, cardiac revascularization and valvular heart disease, chronic pulmonary diseases, renal insufficiency, carotid artery disease, cerebrovascular disease transient ischemic attack and cerebral vascular accident, peripheral arterial diseases and gastrointestinal complications. Furthermore, preoperative risk assessment was classified according to the American Society of Anesthesiologists Classification of health (ASA class-I-IV).[6]

Aneurysm morphology

Prior to surgery, all patients received computed tomography angiography (CTA) imaging in order to determine the eligibility for endovascular repair. The aneurysm morphologic characteristics included maximal aneurysm diameter, proximal and distal non-aneurysm aortic neck diameter, length of non-aneurysmal aortic neck, distal diameter of non-aneurysm neck, angle between proximal AAA neck & main axis, length from lowest renal artery to aortic bifurcation, length from aortic bifurcation to the end of the seal zone, diameter of iliac aneurysm, length of iliac aneurysm, iliac artery tortuosity (mild/moderate/severe), iliac artery stenosis and aortic mural thrombus/calcification at the site of the proximal neck.

Follow-up

The study follow-up was designed according to standard practice at each clinical site, with the exception of the requirement for 30-day and 1-year imaging studies. No other tests outside a site's standard regimen for AAA follow-up were required. Diagnostic images were analyzed at both time points for technical outcomes and AAA changes.

Endpoints

The primary endpoint of the study was clinical success and was classified as recommended in the reporting standards for EVAR.[7] It was defined as a composite of technical success (defined as successful delivery and deployment of the Endurant stent graft in the planned position without unintentional coverage of one or both internal iliac arteries or visceral aortic branches and with successful removal of the delivery system) and freedom from the following complications during follow-up: AAA increase > 5 mm, endoleak type I and III, aneurysm rupture, conversion, secondary procedures, migration and occlusion (defined as 100% obstruction). Secondary endpoints were the individual components of clinical success, aneurysm-related mortality and all-cause mortality. All deaths within 30 days

postoperative were considered aneurysm-related. Duration of follow-up was two years for all patients and three years for the first 500 patients enrolled.

Data collection

Data on each patient were recorded on a web-based electronic case report form (Viracity™ clinical Asset management, MERGE Healthcare, Chicago, IL, USA). Data were imported by, or under supervision of, institutions` principal investigators. Research analysts from Medtronic Bakken Research Centre BV (Maastricht, The Netherlands) verified the quality of the entered data during monitoring visits. Each Institutional Review Board from all participating centers approved data collection and analysis and informed consent was obtained from all patients.

Statistical analysis

All statistical analyses were performed by qualified statisticians from Medtronic Inc. Dichotomous data are described as counts and percentages. Continuous variables are described as mean (standard deviation). Categorical data were compared with chi-square tests and continuous variables with ANOVA. Freedom from secondary procedures, aneurysm-related and all-cause mortality were separately assessed using Kaplan-Meier estimates. Missing values were excluded from analysis.

For all tests, a *P* value <.05 (two-sided) was considered statistically significant. All analyses were performed using SAS® version 9.0 software for windows (SAS institute Inc., Cary, NC, USA).

RESULTS

A total of 1266 patients were included in the study. Four patients were excluded (refusal of immediate treatment (n=1), received open repair (n=1), operation at a non-participating site (n=1), and missing informed consent (n=1)), as previously described.[8]

The final study population consisted of 1262 patients. The mean age was 73.1 (8.1) years and 89.5% was male. A total of 85 patients (6.7%) reported a positive family history and were classified as familial AAA, whereas 1177 patients (93.3%) were classified as sporadic AAA. The clinical characteristics of familial and sporadic AAA patients are presented in Table I. Patients with familial AAA were more often female (18.8% versus 9.9%; *P* = .010), had a higher systolic blood pressure (142 mmHg versus 136 mmHg; *P* = .008), had less prior history of cancer (10.7% versus 21.2%; *P* = .022), and had more prior GI complications (28.2% versus 19.1%; *P* = .041). Patients with familial AAA had also a trend towards a lower ASA classification.

Table 1 – Clinical characteristics of patients with familial and sporadic AAA

	Familial AAA	Sporadic AAA	P *value*
Variable[a]	n=85	n=1177	
Gender			.010
Female	18.8% (16/85)	9.9% (117/1177)	
Male	81.2% (69/85)	90.1% (1060/1177)	
Age (years)	71.6 ± 8.4	73.2 ± 8.1	.059
Baseline symptoms			
None	82.4% (70/85)	83.9% (988/1177)	.701
Abdominal pain	11.8% (10/85)	10.6% (125/1177)	.742
Back pain	5.9% (5/85)	5.2% (61/1177)	.780
Other symptoms	1.2% (1/85)	2.5% (30/1177)	.430
Physical measurements			
Height (cm)	174.3 ± 9.8	172.7 ± 8.1	.071
Weight (kg)	82.5 ± 15.8	81.1 ± 15.3	.689
Systolic blood pressure (mmHg, mean)	142.2 ± 21.1	135.9 ± 18.7	.008
Diastolic blood pressure (mmHg, mean)	79.4 ± 12.7	78.2 ± 11.2	.385
Risk factors and comorbidities			
Tobacco use	47.0% (39/83)	49.4% (568/1150)	.672
Hypertension	77.4% (65/84)	75.3% (875/1162)	.669
Hyperlipidemia	64.2% (52/81)	60.3% (668/1108)	.487
Diabetes	16.5% (14/85)	19.1% (222/1160)	.545
Cancer	10.7% (9/84)	21.2% (245/1158)	.022
Alcoholism	4.8% (4/84)	3.1% (36/1145)	.420
Cardiac disease	56.5% (48/85)	53.4% (628/1177)	.578
MI	33.3% (28/84)	26.0% (293/1126)	.143
Arrhythmia	17.9% (15/84)	15.9% (183/1150)	.639
Angina	11.9% (10/84)	16.0% (184/1151)	.321
Congestive heart failure	1.2% (1/85)	6.2% (71/1145)	.057
Coronary artery disease	30.9% (25/81)	35.1% (400/1138)	.434
Cardiac revascularization (CABG or PTCA)	31.3% (26/83)	26.9% (312/1162)	.376
Valvular Heart disease	5.9% (5/85)	6.2% (71/1151)	.916
COPD	22.4% (19/85)	25.5% (295/1157)	.520
Renal insufficiency	8.2% (7/85)	16.0% (187/1167)	.055
Carotid artery disease	14.1% (10/71)	10.7% (105/984)	.373
Cerebrovascular disease	15.3% (13/85)	12.5% (147/1177)	.453
Transient ischemic attack	4.8% (4/84)	4.9% (57/1166)	.959
Cerebral vascular accident	8.2% (7/85)	5.1% (60/1171)	.218
Paraplegia	0.0% (0/85)	0.3% (3/1171)	.640
Paraparesis	0.0% (0/85)	0.9% (10/1172)	.393
Vascular disease	37.6% (32/85)	30.5% (359/1177)	.169

Table 1 – Continued

Variable[a]	Familial AAA n=85	Sporadic AAA n=1177	P *value*
Previous abdominal aortic aneurysm	1.2% (1/85)	1.6% (19/1171)	.751
Any thoracic aneurysm	3.7% (3/82)	1.8% (20/1126)	.229
Peripheral vascular disease	21.2% (18/85)	18.3% (212/1160)	.506
Thromboembolic event	6.0% (5/83)	3.1% (36/1154)	.153
Bleeding disorder	3.5% (3/85)	1.7% (20/1177)	.223
Liver disease	3.5% (3/85)	2.2% (26/1177)	.433
GI complications	28.2% (24/85)	19.1% (225/1177)	.041
ASA classification			.063
Class I	2.4% (2/85)	6.4% (75/1177)	
Class II	51.8% (44/85)	41.0% (483/1177)	
Class III	41.2% (35/85)	41.5% (489/1177)	
Class IV	4.7% (4/85)	10.0% (130/1177)	

Abbreviations: ASA, American Society of Anesthesiologists; MI, myocardial infarction; COPD, chronic obstructive pulmonary disease; CABG, coronary artery bypass surgery; PTCA, percutaneous transluminal coronary angioplasty; GI, gastrointestinal.
[a] Continuous data are presented as the mean ± standard deviation and categorical data as percentages (denominator differs in case of missing values).

Aneurysm morphology

The majority of the aneurysm morphologic characteristics were similar between both groups, as presented in Table 2. Minor significant differences were observed for aneurysm diameter (58 mm versus 61 mm; $P = .040$) and right iliac stenosis (5.5 mm versus 8.6 mm, $P = .044$). No differences were observed for proximal neck angulation and iliac tortuosity.

Clinical outcome

There was no statistically significant difference in clinical success after two-years of follow-up between familial and sporadic AAA patients (81.2% versus 86.7%; $P = .156$, Table 3). Follow-up at three-years showed similar results (79.1% versus 85.1%; $P = .296$). Technical success was reached for 98.8% in familial AAA and 99.1% in sporadic AAA ($P = .824$). Patients with familial AAA suffered from more stent graft occlusion (at two-years: 7.1% versus 3.5%; $P = .095$; at three-years: 9.5% versus 2.2%; $P = .007$). There was a trend towards more secondary procedures in patients with familial AAA during follow-up as compared to patients with sporadic AAA, but this difference did not reach statistical significance (Kaplan-Meier estimate 17.8% versus 10.9% at three years of follow-up, $P = .06$; Figure 1). The increased amount of secondary procedures in familial AAA was mainly explained by procedures for stenosis or occlusion of the stent graft or limb, as presented in supplemental Table S1.

Table 2 – Aneurysm morphology of patients with familial and sporadic AAA

	Familial AAA	Sporadic AAA	P value
Variable[a]	**n=85**	**n=1177**	
Maximal aneurysm diameter, mm	58.0 ± 10.2 (85)	60.5 ± 11.7 (1161)	.040
Proximal non-aneurysm aortic neck diameter, mm	23.6 ± 3.3 (85)	23.7 ± 3.5 (1172)	.928
Distal non-aneurysm aortic neck diameter, mm	24.9 ± 4.5 (84)	24.9 ± 4.0 (1161)	.894
Length of non-aneurysmal aortic neck, mm	28.6 ± 13.1 (84)	26.9 ± 12.3 (1166)	.268
Distal diameter of non-aneurysm neck of right iliac, mm	14.5 ± 4.1 (74)	14.1 ± 3.5 (1032)	.492
Distal diameter of non-aneurysm neck of left iliac, mm	13.7 ± 3.6 (76)	13.8 ± 3.5 (1031)	.840
Angle between proximal AAA neck & main axis, degrees	27.1 ± 24.7 (83)	30.6 ± 23.7 (1143)	.130
Length from lowest renal artery to aortic bifurcation, mm	121.9 ± 19.0 (84)	120.3 ± 19.3 (1169)	.407
Length of right iliac from aortic bifurcation to end of seal zone, mm	59.0 ± 27.0 (78)	57.5 ± 24.3 (1052)	.562
Length of left iliac from aortic bifurcation to end of seal zone, mm	59.0 ± 27.0 (76)	58.8 ± 24.7 (1040)	.430
Diameter of right iliac aneurysm, mm	35.2 ± 18.3 (11)	29.3 ± 11.5 (167)	.190
Diameter of left iliac aneurysm, mm	26.7± 8.8 (8)	29.8 ± 13.1 (153)	.864
Length of right iliac aneurysm, mm	46.7 ± 23.9 (12)	42.6 ± 20.8 (147)	.426
Length of left iliac aneurysm, mm	32.5 ± 14.3 (9)	43.4 ± 22.8 (131)	.180
Right iliac tortuosity			.057
Mild	62.4% (53/85)	49.2% (579/1168)	
Moderate	32.9% (28/85)	41.5% (485/1170)	
Severe	7.1% (6/85)	9.1% (106/1170)	
Left iliac tortuosity			.155
Mild	56.5% (48/85)	46.7% (545/1168)	
Moderate	36.5% (31/8)	41.5% (485/1170)	
Severe	7.1% (6/85)	9.1% (106/1170)	
Right iliac stenosis, mm	5.5 ± 15.1 (85)	8.6 ± 17.3 (1158)	.044
Left iliac stenosis, mm	7.8 ± 18.5 (85)	9.5 ± 18.0 (1156)	.088
Aortic mural thrombus/calcification at the proximal neck, mm	6.9 ± 11.3 (85)	10.6 ± 17.7 (1157)	.086

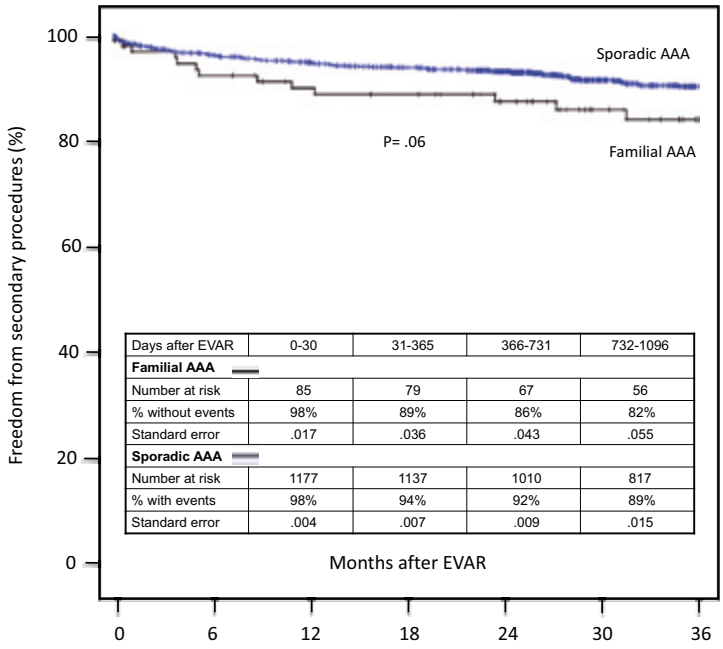
Abbreviations: AAA, abdominal aortic aneurysm

[a] Continuous data are presented as the mean ± standard deviation (number of patients available for analysis) and categorical data as percentage (number of patients available for analysis).

Table 3 – Clinical success through follow-up

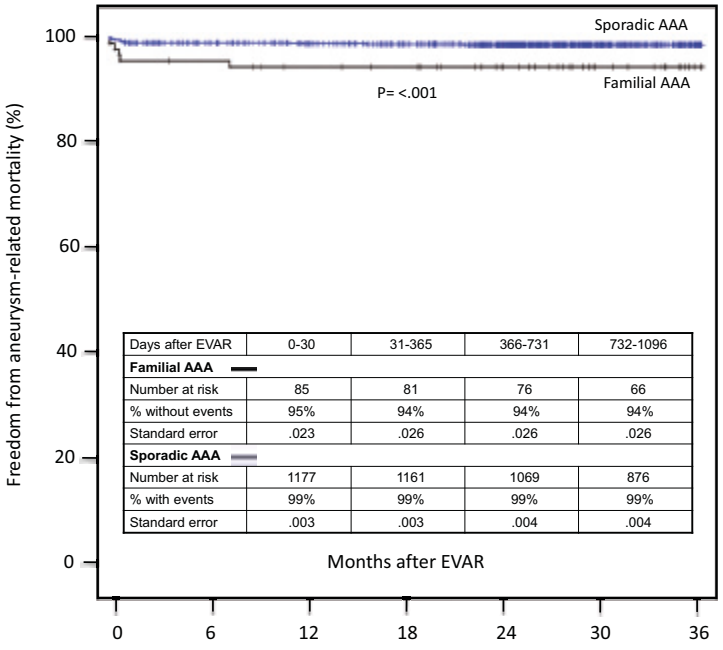
	Two-years			Three-years		
	Familial AAA	Sporadic AAA	P value	Familial AAA	Sporadic AAA	P value
Variable[a,b]	**n=85**	**n=1177**		**n=43**	**n=456**	
Clinical success	**81.2% (69/85)**	**86.7% (1020/1177)**	**.156**	**79.1% (34/43)**	**85.1% (388/456)**	**.296**
Technical success	98.8% (84/85)	99.1% (1166/1177)	.824	97.7% (42/43)	99.1% (452/456)	.362
AAA increase >5 mm	6.4% (4/62)	5.1% (37/727)	.643	7.4% (2/27)	8.9% (23/259)	.797
Endoleak type I and III	0.0% (0/84)	1.6% (19/1158)	.237	0.0% (0/42)	1.8% (8/447)	.382
Aneurysm rupture	0.0% (0/85)	0.3% (4/1177)	.590	0.0% (0/43)	0.2% (1/456)	.759
Conversion	1.2% (1/85)	0.8% (9/1177)	.679	2.3% (1/43)	0.7% (3/456)	.241
Secondary procedures	12.9% (11/85)	7.3% (86/1177)	.060	16.3% (7/43)	7.7% (35/456)	.052
Migration	0.0% (0/84)	0.4% (5/1158)	.546	0.0% (0/42)	0.7% (3/447)	.594
Occlusion	7.1% (6/84)	3.5% (41/1158)	.095	9.5% (4/42)	2.2% (10/447)	.007

Abbreviations: AAA, abdominal aortic aneurysm
[a] All variables represents follow-up through two years.
[b] The number of patients available for analysis is presented between brackets.



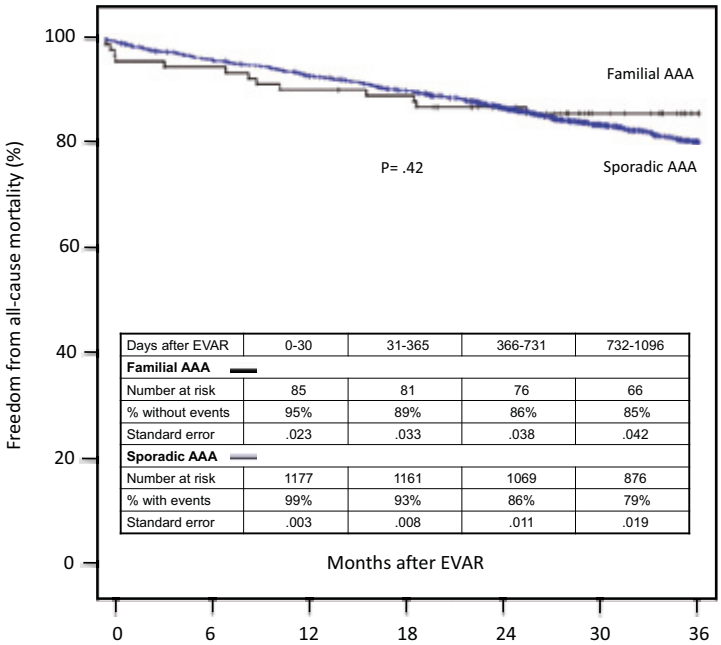
Days after EVAR	0-30	31-365	366-731	732-1096
Familial AAA				
Number at risk	85	79	67	56
% without events	98%	89%	86%	82%
Standard error	.017	.036	.043	.055
Sporadic AAA				
Number at risk	1177	1137	1010	817
% with events	98%	94%	92%	89%
Standard error	.004	.007	.009	.015

Figure 1 – Kaplan-Meier estimates for freedom from secondary procedures



Days after EVAR	0-30	31-365	366-731	732-1096
Familial AAA				
Number at risk	85	81	76	66
% without events	95%	94%	94%	94%
Standard error	.023	.026	.026	.026
Sporadic AAA				
Number at risk	1177	1161	1069	876
% with events	99%	99%	99%	99%
Standard error	.003	.003	.004	.004

Figure 2 – Kaplan-Meier estimates for freedom from aneurysm-related mortality



Days after EVAR	0-30	31-365	366-731	732-1096
Familial AAA				
Number at risk	85	81	76	66
% without events	95%	89%	86%	85%
Standard error	.023	.033	.038	.042
Sporadic AAA				
Number at risk	1177	1161	1069	876
% with events	99%	93%	86%	79%
Standard error	.003	.008	.011	.019

Figure 3 – Kaplan-Meier estimates for freedom from all-cause mortality ilial and sporadic AAA

No differences for AAA diameter increase > 5 mm, type I or III endoleak, aneurysm rupture, conversion to open repair, or stent graft migration were observed between the groups, as presented in Table 3.

Mortality

There was a significant difference in aneurysm-related mortality between familial and sporadic AAA (5.9% versus 1.2% after three years of follow-up; P = <.001; Figure 2). The causes of death are presented in supplemental Table S2. No significant difference for all-cause mortality was observed (15.5% versus 21.5% after three years of follow-up; P = 0.42; Figure 3).

DISCUSSION

The current study shows no statistically significant difference in clinical success after three-years of follow-up between patients with a positive and negative family history. There was a 5% difference however, suggesting a clinical relevant difference between the groups. This is supported by the significantly higher aneurysm-related mortality in patients with familial AAA (6% vs 1%). The patients with familial AAA were also more

likely to suffer from stent graft occlusion and required almost twice as many secondary procedures, mostly to treat the occluded stent graft or limb.

The results of the current study support our previous finding that patients with familial AAA have more aneurysm-related complications after EVAR.[4] In that study, aneurysm sac growth and secondary interventions were the most important elements accounting for the difference. Increased aneurysm sac growth was not observed in the current study however. The outcome of these studies do not preclude endovascular repair in patients with familial AAA but should create awareness that these patients may react differently on endovascular repair and may require more additional interventions to maintain adequate aneurysm exclusion.

The question remains why familial AAA patients develop more complications like stent graft occlusion and need more secondary procedures. We previously hypothesized that an intrinsic weakness of the aortic wall may result in more rapid progression of aneurysm disease contributing to a higher need for secondary interventions observed in familial AAA patients.[4] This does not seem to explain the observed difference in stent graft occlusion in this study. Furthermore, we did not observe a higher rate of type I (or III) endoleak in familial AAA patients. Another hypothesis for higher rates of secondary procedures (or occlusions) in familial AAA is a higher complexity in aneurysm morphology, since it is known that hostile anatomy is associated with more adjunctive procedures,[9] and that genetic aortic aneurysm syndromes are associated with specific morphologic features.[10-12] Similar to our previous study however, no major morphologic differences between both groups were observed in any registered parameters. Disparities in secondary procedures can therefore not be attributed to any morphologic differences in the commonly evaluated parameters between the groups. The results also preclude conventional aneurysm morphology parameters as markers to identify patients with familial AAA. A possible explanation for our results may lie in the high prevalence of females in familial AAA patients (19% versus 10%), since recent studies showed that women have more perioperative complications after endovascular repair and require more adjunctive procedures.[13, 14] A higher prevalence of females is also observed in more studies reporting on familial AAA,[15] but did not reach statistical significance is most studies.[1, 3, 16, 17] Although this may be an explanation for our results, the reason for this phenomenon remains to be elucidated.

We also observed a higher aneurysm-related mortality in familial AAA patients. This is in agreement with previous findings described by Brewster et al in 2006.[18] They concluded that family history of aneurysmal disease was associated with a trend towards a higher aneurysm-related mortality (odds ratio 9.5) over a course of twelve-years. In the current study, the major cause of death responsible for the difference was increased 30-day mortality and seemed unrelated to EVAR implantation since technical success was similar in both groups. The reason for this needs to be clarified and may not be directly

associated with the type of repair. It may also be associated with the higher proportion of females, known to have a higher perioperative mortality.[19-21]

In this study we used self-reported family history to define familial AAA and observed a low degree of familial AAA of only 7%. Previous studies using semi-structured questionnaires on the occurrence of aneurysms among relatives showed a much higher degree of familial disease of approximately 20%.[3, 4] Under-classification of familial AAA patients in the current study is therefore likely and may have influenced our results.
Patients with familial AAA were found to have a (although marginally) higher systolic blood pressure and less cancer. While most studies report variable results on hypertension in patients with familial AAA,[3, 15-17] data on cancer prevalence in familial AAA is scarce. Furthermore, patients with familial AAA were also younger of age, but this did not reach statistical significance as it did in some previous studies.[3, 4, 16] Overall, the current study supports the view of previous observations that familial AAA patients are more often females, tend to be younger and seem to have a lower atherosclerotic risk profile.

Another important matter is that the higher need for secondary interventions negatively impacts the cost-effectiveness of endovascular repair. Secondary procedures are costly and were one of the driving forces in the DREAM trial for EVAR not to be cost-effective.[22, 23] As a consequence, endovascular repair may be less cost-effective for patients with familial AAA. Although standard postoperative surveillance is recommended for all patients treated with endovascular repair for AAA, the current studies implies that patients with familial AAA may need more secondary interventions to maintain adequate aneurysm exclusion. Familial AAA patients may therefore need a closer follow-up regime to identify early complications.

There are several limitations that need to be considered. Firstly and as reported previously, under-classification of familial AAA patients is the largest limitation and may have influenced our results. Ideally, familial AAA is being established based on a semi-structured questionnaire. Nevertheless, the data presented in this study represent the definition of familial AAA used in most outpatient clinic settings. Secondly, the study has relative short follow-up of three years in only 500 patients. Long-term follow-up is needed before definite conclusions can be drawn. Thirdly, no systemic molecular screening was performed for aneurysm related diseases such as Marfan, Loeys-Dietz, or vascular Ehlers-Danlos syndrome. However, these syndromes are a rare cause for AAA and they usually reveal at a far younger age. Therefore we think that the potential contribution of these syndromes was exceedingly small.

CONCLUSIONS

The current study shows that endovascular repair is a valid treatment in familial AAA patients. However, the higher aneurysm-related mortality and the trend towards more aneurysm-related secondary procedures in patients with a positive family history suggest that these patients have an marginal but significant increase in risk after EVAR and underline the importance of a family history in our aneurysm patients. Longer term data is required to determine the role of family history in suitability for EVAR, and to establish the need for adaptation of follow-up in these patients. For the time being, patients with familiar forms of AAA should be offered a close follow-up regime.

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Supplemental Table S1 – Reason and type of secondary procedures through follow-up

	Two-years		Three-years	
	Familial AAA	Sporadic AAA	Familial AAA	Sporadic AAA
	n=11	n=86	n=7	n=35
Reason for secondary procedure				
Type I endoleak	1 (9.1%)	21 (24.4%)	0 (0%)	7 (20.0%)
Type II endoleak	3 (27.3%)	11 (12.8%)	2 (28.6%)	5 (14.3%)
Type III endoleak	0 (0%)	5 (5.8%)	0 (0%)	6 (17.1%)
Undefined endoleak	0 (0%)	2 (2.3%)	0 (0%)	0 (0%)
Stenosis or occlusion of stent graft or limb	6 (54.5%)	33 (38.4%)	5 (71.4%)	10 (28.6%)
Stent graft kinking	0 (0%)	4 (4.7%)	0 (0%)	3 (8.6%)
Aneurysm expansion	0 (0%)	1 (1.2%)	0 (0%)	0 (0%)
Ruptured AAA	0 (0%)	1 (1.2%)	0 (0%)	0 (0%)
Other	1 (9.1%)	8 (9.3%)	0 (0%)	5 (11.4%)
Type of secondary procedure				
Coiling	2 (18.2%)	14 (16.3%)	1 (14.3%)	6 (17.6%)
Embolectomy of thrombectomy	1 (9.1%)	7 (8.1%)	1 (14.3%)	3 (8.8%)
PTA without stent	2 (18.2%)	14 (16.3%)	2 (28.6%)	5 (14.7%)
PTA with stent	5 (45.5%)	34 (39.5%)	2 (28.6%)	14 (41.2%)
Bypass	1 (9.1%)	16 (18.6%)	1 (14.3%)	6 (17.6%)
Other	0 (0%)	1 (1.2%)	0 (0%)	0 (0%)

Abbreviations: AAA, abdominal aortic aneurysm; PTA, percutaneous transluminal angioplasty.

Supplemental Table S2 – Causes of death

	Familial AAA	Sporadic AAA
	n=13	n=215
Cause of death		
Cardiac disease	1 (7.7%)	37 (17.2%)
Pulmonary disease	0 (0%)	17 (7.9%)
Renal disease	0 (0%)	2(0.9%)
Neurologic disease	0 (0%)	13 (6.4%)
Infection/sepsis	0 (0%)	36 (16.7%)
Cancer	3 (23.1%)	46 (21.4%)
Other vascular disease	0 (0%)	4 (1.9%)
Other	1 (7.7%)	25 (11.6%)
Unknown	1 (7.7%)	31 (14.4%)
Aneurysm related mortality	5 (38.5%)	14 (6.5%)
Cardiac disease	1	7
Renal disease	0	1
Infection/sepsis	2	1
Cancer	0	1
Other	1	2
Unknown	0	1
Aneurysm related	1	1

PART IV

Clinical Management of Familial Abdominal Aortic Aneurysm

9

What a Vascular Surgeon Should Know about Familial Abdominal Aortic Aneurysm

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Hence J.M. Verhagen

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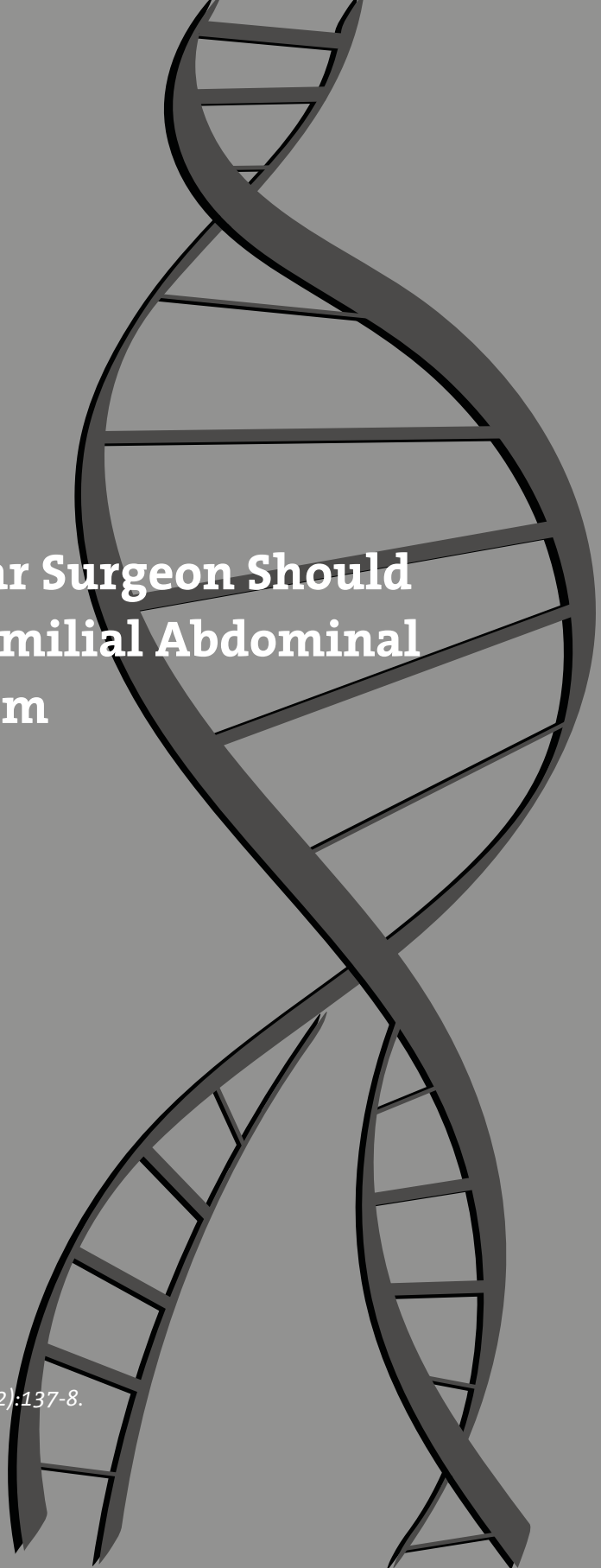

A genetic susceptibility for abdominal aortic aneurysm (AAA) may explain the high prevalence of patients with a positive family history for aortic aneurysms (i.e. familial AAA). The results from recent studies have implications for daily practice with regard to identifying familial AAA patients, screening of relatives and treatment strategies.

Classifying patients as 'familial' is important, as relatives have an increased risk of developing an AAA with time. Several definitions have been used in the literature, the most common being when at least one first-degree relative (parent, sibling, offspring) is diagnosed with an aortic aneurysm.[1] Patients with a negative (or uninformative) family history are usually classified as having a sporadic AAA. Familial AAA can be assessed by information from family history alone, or by screening relatives. However, both methods have shortcomings. Family history alone usually underestimates the true prevalence, as patients may not be aware that relatives have been diagnosed with an AAA or the relatives are too young to have developed an aneurysm yet. Screening relatives with a single ultrasound scan may be of limited benefit, as they may develop aneurysms over time. Furthermore, this can only be undertaken in relatives who are alive, willing and capable of undergoing screening.

Based upon family history review, the proportion of AAA patients with familial AAA is around 13% [range: 6-36%].[2] Ultrasound screening of AAA patient relatives suggest a prevalence of 20% [range: 9-43%] in males and 4% [range: 0-11%] in females.[2] The prevalence of AAA in age-selected general populations is around 6% [range: 4-8%] in males and 1% [range: 1-2%] in females.[3] Accordingly, relatives of AAA patients have a two-three fold excess risk of developing an AAA in their lifetime.

One question is whether familial AAA patients are clinically different. One may hypothesize that in AAA patients with high genetic risk, other risk factors may be less important. Studies suggest that familial AAA patients are significantly more likely to be female, younger, have less cardiovascular risk factors like hypertension and diabetes mellitus, and possibly have a lower carotid intima thickness (CIMT).[1] However, a distinctive and clinically useful phenotype for accurately predicting familial AAA patients has not been established. Studies also show a high rate of thoracic aortic aneurysms and an increased rate of bilateral iliac aneurysms in familial AAA patients,[4] and this could partly explain the high prevalence of thoracic aneurysms in AAA patients.[5] Relatives of familial AAA patients also appear to have increased aortic diameters,[6] supporting the hypothesis of a systemic involvement. Additional analyses on familial AAA patients and their relatives are needed to increase knowledge on this matter.

To answer the question as to why patients with familial AAA are different, one must understand the mode of inheritance of possible genetic defects. In some families, a major genetic defect with a high risk of recurrence (eg an autosomal dominant inheritance pattern with reduced penetrance), is expected.[7] Other families exhibit more complex

genetics with lower recurrence risks. In any case, additional environmental effects, such as smoking, hypertension, and hypercholesterolemia could enhance the risk of aneurysm formation and, therefore, explain the variability in expression of the disease. It is also crucial to realize that these families have many environmental risk factors in common, leading to a possible overestimation of any genetic effect.

Most accepted genetic aortic aneurysm syndromes are caused by defects in genes involved in the TGF-ß pathway,[8] and are associated with syndromes like Marfan's disease and Loeys-Dietz syndrome. These predominantly affect the thoracic aorta, but can affect the abdominal aorta as well. Interestingly, no causative genes have been found (as yet) in familial AAA. The main reason for this is that familial AAA is a late-onset disease, making it difficult to collect DNA samples from a sufficient number of affected relatives to perform genetic linkage studies. Until now, family studies have detected a linkage with the 19q13 and 4q31 regions in the genome,[9] but without identifying specific genes in these regions.[10] In order to further establish the genetics of AAA formation, other strategies including genome-wide association studies (GWAS) have been explored. Currently described associations of a genome wide single nucleotide polymorphism with AAA include *DAB2IP, LRP1, LDLR, ANRIL,* and *SORT1*.[10] Having one of these alleles results in an additional ±20% risk for developing AAA, but the causal relationship of these polymorphisms still needs to be elucidated. Novel improvements in next generation sequencing techniques should enable the identification of causative genes in the near future, which would lead to a significant impact on treatment strategies and screening of high-risk populations.

Because relatives of AAA patients have an increased risk for developing aneurysms and are a relatively easy population to identify, they may form an attractive subgroup to offer screening. Adequate estimates of risk per relative (parents, siblings and children) stratified by gender are needed, but are currently lacking. The present ACC/AHA guidelines recommend ultrasound screening for male relatives aged 60 years or older, while the ESVS guidelines recommend ultrasound screening of both male and female relatives over 50 years who have a family history of AAA. Recent data from large screening programs show a lower incidence of AAA than expected,[11] questioning the efficacy of population screening. Screening relatives of AAA patients may therefore be a more efficient alternative.

Another issue for debate is the optimal timing of any intervention. Patients with familial AAA seem to show accelerated aneurysm growth,[12] which could partly be explained by the higher rate of females, where increased growth rates have been observed. Interestingly, this could partly explain the observed higher rate of aneurysm rupture in familial AAA,[13] raising the question as to whether familial AAA patients should be treated at a younger age.

It is not known whether endovascular aneurysms repair (EVAR) or open repair (OR) is the optimal treatment for patients with familial AAA. Only very limited data is available, but since familial AAA patients tend to be younger, they may benefit from OR, which

carries a lower secondary intervention risk with time. One study has, however, reported that late failure after OR was independently associated with a positive family history for AAA.[14] Regarding outcomes after EVAR, a trend towards a higher aneurysm-related mortality in patients with familial AAA has been reported,[15] while others have shown that familial AAA patients seem to develop more aneurysm-related complications without an increase in all-cause mortality.[16] Accordingly, the available data suggest worse outcomes for familial AAA patients undergoing both EVAR and OR, but longer term follow-up and data from large international registries will be needed to define the optimal treatment and follow-up strategies in these patients.

In conclusion, accumulating knowledge suggests a genetic component in the formation of AAA and new genetic techniques may lead to the identification of causative genes which could have a significant impact on treatment strategies and the screening of high-risk populations. Familial AAA patients may react differently to types of operative repair and may need modified postoperative surveillance programmes, emphasizing the need to collect family histories.

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10

Screening Families for Abdominal Aortic Aneurysm; Don't Forget the Women

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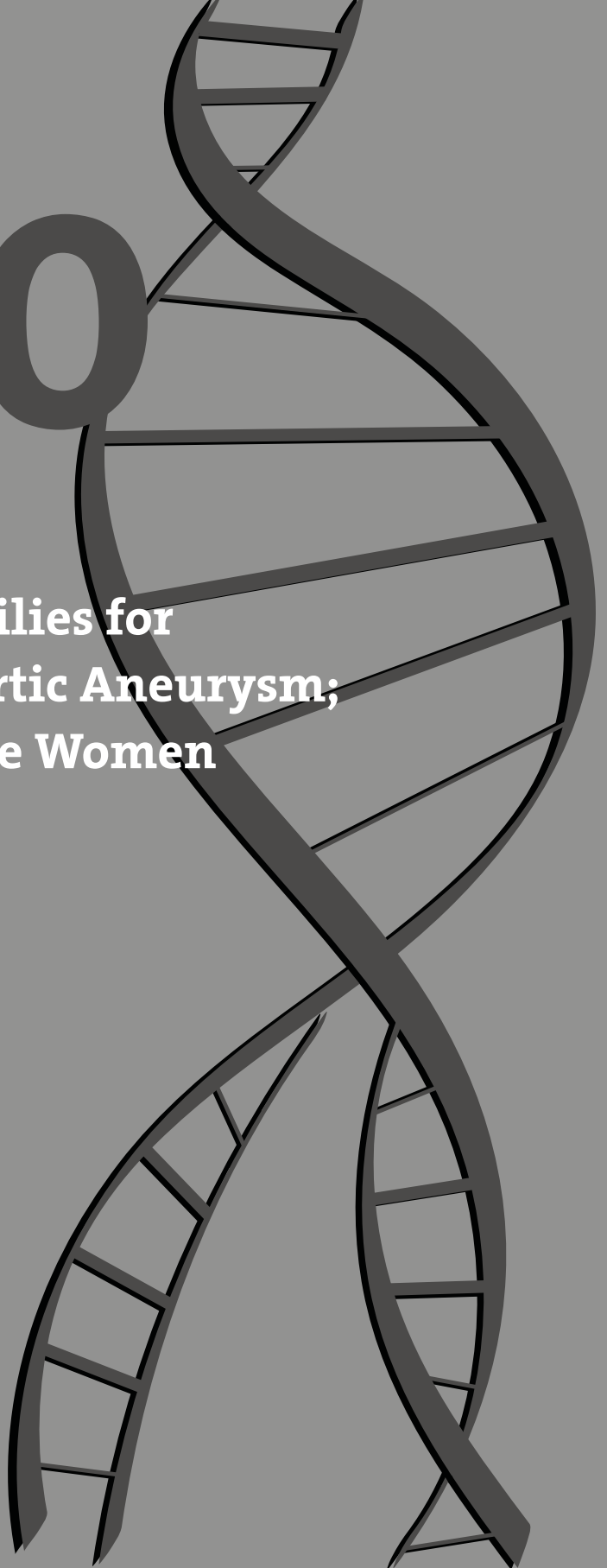

ABSTRACT

Purpose: Early detection of abdominal aortic aneurysm (AAA) occurring in 6% of males and 1% of females above 55 years improves survival significantly. Since genetic predisposition plays an important role in AAA, early diagnosis of relatives of patients is important. And guidelines for family screening based on valid estimates of risk in relatives are needed. We investigated risk for relatives by gender of patients and relatives. We also investigated if specific clinical characteristics of cases could predict high risk for relatives.

Methods: Semi-structured family history questionnaires were used to collect detailed family histories. Patients were classified as familial AAA when at least one first-degree relative had an aortic aneurysm. Risk for relatives was stratified by gender of cases and of relatives. Multivariable regression analysis was performed to find characteristics predicting high risk for relatives.

Results: Familial AAA occurred in 23% cases: 22% male, 27% female (P = .283). Overall, relatives of female cases had higher risk than relatives of male cases (9.0% versus 5.9%, P = .022). In familial AAA risk for relatives of male cases was 21% compared to 29% in relatives of female cases (ns). AAA patients younger than 65 years, without diabetes mellitus or hypertension had a fourfold higher risk of familial AAA.

Conclusions: Male and female relatives have high risk for AAA, in particular the relatives of female AAA cases. Therefore, family based targeted screening for abdominal aortic aneurysms should be extended from familial cases to the families of all AAA cases.

INTRODUCTION

Abdominal aortic aneurysm (AAA) occurs in approximately 6% [range 4%-8%] of the male and 1% [range: 1%-2%] of the female population above the age of 55 years,[1-6] and is a major cause of mortality in the aged population.[7] Clinical risk factors for AAA include age, male gender, smoking and a positive family history.[8] Population based screening programs targeting the high risk category of men over the age of 65 years showed a decrease in mortality from rupture,[1-3, 5] and elective repair is associated with a tenfold reduction in risk.[9] It is important to establish if relatives of AAA patients represent a specific high risk category that may benefit from screening, and if screening of relatives may be a cost-effective way to reduce AAA related mortality in the population.

Familial clustering of aneurysm disease was first described 30 years ago,[10] and has since been described at rates around 14% (Table S1, Supplemental data). Familial AAA may occur in the genetic aortic aneurysm syndromes, caused by genetic defect in genes involved in the TGF-ß pathway and smooth muscular cell homeostasis, predominantly affecting the thoracic part of the aorta.[11-14] We recently reported that two percent of familial and sporadic AAA were caused by a mutation (*TGFBR2, MYH11,* and *COL3A1*,) from a diagnostic testing panel of nine aneurysm genes, that were previously associated with syndromal and non-syndromal thoracic aneurysms. Indicating that apart from rare overlapping genetic defects between AAA and thoracic aortic aneurysms, for the majority of AAA the major genetic causes remain unknown.[15]

In familial AAA, relatives have an increased risk for abdominal aneurysm compared to the general population. Therefore, similar to the other highly prevalent hereditary cardiovascular disorders hypertrophic cardiomyopathy and hypercholesterolemia, screening of relatives of familial cases is indicated allowing early diagnosis and improving prognosis of relatives.[16] There are large differences in the approach of screening families for AAA in the current international guidelines (Table 1).[17-21] In particular in terms of gender, age and surveillance interval. The present ACC/AHA guidelines recommend ultrasound screening for male siblings and offspring older than 60 years of any patient with AAA and the ESVS guidelines recommend ultrasound screening of male and female relatives over 50 years with a family history of AAA.[17, 19]

The goal of this study was to assess gender related risk for aortic aneurysm in relatives of AAA patients using a retrospective cohort analysis of family history data. Furthermore, we investigated whether specific clinical characteristics of AAA cases may help predict high risk for relatives.

Table 1 – Summary of current international guidelines regarding screening for AAA.

Organisation	Year	Recommandations	Level of recommendation[a]	Level of evidence
ACC/AHA[17]	2005	Men 60 years of age or older who are either the siblings or offspring of patients with AAAs should undergo physical examination and ultrasound screening for detection of aortic aneurysms.	I	B
		Men who are 65 to 75 years of age who have ever smoked should undergo a physical examination and 1-time ultrasound screening for detection of AAAs.	IIa	B
SVS[18]	2009	One-time ultrasound screening for AAA is recommended for all women at or older than 65 years with a family history of AAA or who have smoked.	Strong	Moderate
		Re-screening patients for AAA is not recommended if an initial ultrasound scan performed on patients 65 years of age or older demonstrates an aortic diameter of 2.6 cm.	Strong	Moderate
ESVS[19]	2011	Population screening of older men for AAA, in regions where the population prevalence is 4% or more, reduces aneurysm-related mortality by almost half within 4 years of screening, principally by reducing the incidence of aneurysm rupture.	A	1a
		Population screening of older women for AAA does not reduce the incidence of aneurysm rupture.	B	1b
		Population screening of older female smokers for AAA may require further investigation.	B	3c
		Ever-smoking increases the risk of developing AAA 4- to 5-fold. Screening only smokers might improve the cost- effectiveness of aneurysm screening.	D	5
		A family history of AAA increases the risk of AAA about 2-fold. Screening of older men and women having a family history of AAA might be recommended.	C	3a
		Screening Asian men for AAA may not be cost-effective.	B	2b
		Opportunistic screening of patients with peripheral arterial disease should be considered.	B	2a
ACPM[20]	2011	Recommends one-time screening in men aged 65–75 years who have ever smoked.	na	na
		Routine AAA screening in women not recommended.	na	na
USPSTF[21]	2014	Men aged 65 to 75 years who have ever smoked; Screen once for AAA by ultrasound.	B	na
		Men aged 65 to 75 years who have never smoked; Selectively screen for AAA.	C	na
		Women aged 65 to 75 years who have ever smoked; No recommendation.	I statement	na
		Women who have never smoked; Do not screen for AAA.	D	na

ACC/AHA, American College of Cardiology/American heart association; SVS, Society for Vascular Surgery; ESVS, European Society for Vascular Surgery; ACPM, American College of Preventive Medicine; USPSTF, U.S. Preventive Services Task Force; na, not applicable
Highlighted: recommendations regarding family screening.
[a]Classification as provided by each guideline grading system.
ACC/AHA: Level of recommendation: I) Conditions for which there is evidence for and/or general agreement that a given procedure or treatment is beneficial, useful, and effective. II) Conditions for which there is conflicting evidence and/or a divergence of opinion about the usefulness/efficacy of a procedure or treatment, IIa: Weight of evidence/opinion is in favor of usefulness/efficacy, IIb: Usefulness/efficacy is less well established by evidence/opinion. III) Conditions for which there is evidence and/or general agreement that a procedure/treatment is not useful/effective and in some cases may be harmful. Level of evidence: A) Data derived from multiple randomized clinical trials or meta-analyses. B) Data derived from a single randomized trial or nonrandomized studies. C) Only consensus opinion of experts, case studies, or standard-of-care.
SVS: Strength of a recommendation: Strong) Benefits > Risks. Weak) Benefits ~ Risks. Grading quality of evidence: High) Additional research is considered very unlikely to change confidence in the estimate of effect. Moderate: Further research is likely to have an important impact on in the estimate of effect. Low) Further research is very likely to change the estimate of the effect.
ESVS: Grades of recommendation: A) Consisted level 1 studies. B) Consisted level 2 or 3 studies or extrapolations from level 1. C) Level 4 studies or extrapolations from level 2 or 3 studies. D) Level 5 evidence or troublingly inconsistent or inconclusive studies of any level. Level of evidence: 1a) SR (with homogeneity) of RCTs. 1b) Individual RCT (with narrow Confidence Interval). 1c) All or none. 2a) SR (with homogeneity) of cohort studies. 2b) Individual cohort study (including low quality RCT; e.g., <80% follow-up). 2c) "Outcomes" Research; Ecological studies. 3a) SR (with homogeneity) of case-control studies. 3b) Individual Case-Control Study. 4) Case-series (and poor quality cohort and case control studies). 5) Expert opinion without explicit critical appraisal, or based on physiology, bench research or "first principles"
ACPM: not applicable
USPSTF: Definitions of Grades. A) The USPSTF recommends the service. There is high certainty that the net benefit is substantial. B) The USPSTF recommends the service. There is high certainty that the net benefit is moderate or there is moderate certainty that the net benefit is moderate to substantial. C) The USPSTF recommends selectively offering or providing this service to individual patients based on professional judgement and patient preferences. There is at least moderate certainty that the net benefit is small. D) The USPSTF recommends against the service. There is moderate or high certainty that the service has no net benefit or that the harms outweigh the benefit. I statement) The USPSTF concludes that the evidence is insufficient to assess the balance of benefits and harms of the service. Evidence is lacking, of poor quality, or conflicting, and the balance of benefits and harms cannot be determined.

MATERIALS AND METHODS

The study complied with the declaration of Helsinki and was approved by the Institutional Review Board (MEC-2012-078).

Study population and questionnaire

The study population was derived from a prospective database of vascular surgery patients treated between 2004 and 2012 at the Erasmus University Medical Center in Rotterdam, the Netherlands. Patients with an abdominal aortic aneurysm, defined as an external maximum transverse abdominal aortic diameter ≥30 mm,[19] undergoing elective open or endovascular repair or remaining under surveillance, were included in the study. The Dutch civil registry was consulted to establish whether patients eligible for inclusion were alive and exclude deceased cases. Between 2009 and 2012 all remaining AAA patients were contacted when visiting the outpatient clinic or by mail and we asked to answer a semi-structured written questionnaire. The questionnaire requested information on demographics, family history and the medical history of the index patient. The following questions were used to ascertain the occurrence of aneurysm for each relative (father/ mother/brother/sister/son/daughter/uncle/aunt/grandmother/grandfather) separately: "Did your (relative) had an aortic aneurysm?" and also "Do you have any relative that has been diagnosed with an aortic aneurysm or operated for this? Was this your father/ mother/brother/ sister/son/daughter/uncle/aunt/grandmother/grandfather?" In case a relative was reported to be affected we asked for an informed consent to request the medical records to confirm the family history. The questionnaire also included questions on the medical histories of each of the parents, siblings and children of the index patient individually, with a specific focus on the occurrence of cardiovascular disease in each of the first-degree relatives. Patients who did not respond after one reminder, were contacted and interviewed by phone (KvdL). In families with multiple AAA patients, only the index patient (ie, first family member diagnosed with AAA) was included as index in the study. No routine molecular testing was performed of aneurysm genes (ie, for Marfan, Loeys-Dietz or vascular Ehlers-Danlos syndromes). None of the patients in this study was previously diagnosed with these genetic disorders.

Classification of familial AAA

Patients were classified as familial AAA when at least one first-degree relative (ie, parent, sibling or child) was reported to have an aortic aneurysm. Patients who did not report an affected first-degree relative were classified as sporadic AAA. Patients reporting only affected second- or third-degree relatives were also classified as sporadic AAA, because the reporting of medical information of second- or third-degree relatives was considered less reliable and it is difficult to retrieve medical records of distantly related relatives.[22]

All patients classified as familial AAA patients were subsequently invited for genetic counseling. In this way consent was obtained to retrieve medical records of affected relatives.

Clinical characteristics

The clinical characteristics of the index AAA patients were obtained from medical files and included gender, age at diagnosis, age at surgery, body mass index (BMI), as well as AAA specifics, cardiovascular comorbidities and risk factors. AAA specifics included location of the aortic aneurysm (ie, suprarenal or infrarenal AAA) and whether the AAA was ruptured or not. Cardiovascular comorbidities included congestive heart failure, ischemic heart disease (history of myocardial infarction, angina pectoris, coronary revascularisation or pathologic Q-waves on the electrocardiogram), and cerebrovascular disease (history of ischemic/hemorrhagic stroke or transient ischemic attack). Cardiovascular risk factors included kidney disease (serum creatinine ≥2.0 mg/dL), diabetes mellitus (fasting plasma glucose ≥7.0 mmol/L, non-fasting glucose ≥11.1 mmol/L or use of anti-diabetic medication), hypertension (blood pressure ≥140/90 mmHg in non-diabetics, ≥130/80 mmHg in diabetics or use of antihypertensive medication) and hypercholesterolemia (low-density lipoprotein [LDL] cholesterol ≥3.5 mmol/L or use of lipid lowering medication). Smoking status was obtained and included current smoking and ever smoking (ie, patients who are currently smoking OR patients with a history of smoking). Prescription medications were recorded and included the use of statins, beta-blockers, renin-angiotensin system inhibitors, diuretics, and antiplatelets.

Statistical analysis

Dichotomous data are described as counts and percentages. Continuous variables are described as mean (standard deviation). Categorical data were compared using chi-square tests. Continuous variables were compared using ANOVA. Increase in risk was estimated for male and female relatives of AAA patients. The risks were compared to the pooled estimate risk in the general population (ie, 5.6% in males over the age of 55 and 1.4% in females over the age of 55).[1-4,6] A multivariable binary logistic regression analysis was used to calculate odds ratios for familial AAA. Variables included in the analysis were age ≤65 years at diagnosis, gender, BMI, location of aneurysm, congestive heart failure, ischemic heart disease, cerebrovascular disease, kidney disease, diabetes mellitus, hypertension, hypercholesterolemia, and smoking history. Additional analysis was performed using previous adjustments plus medication. Variables were chosen on the basis of biological plausibility.

A P value < .05 (two-sided) was considered significant. All analyses were performed using IBM SPSS Statistics version 20.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

The vascular surgery database included a total of 780 patients diagnosed with AAA (Figure 1). A total of 600 patients were alive and were sent the questionnaire. Four hundred eighty-two patients (79.0%) responded after one reminder. Questionnaires were administered by telephone to 108 patients. Ten patients could not be reached by telephone. Twenty-two patients were relatives of other AAA patients participating in the study and were included as affected relatives of the index patient. In total 568 patients (85.7% male) were included in the study, representing a response rate of 94.6% (568/600).

Risk for relatives

In the study population of 568 AAA patients 183/2873 (6.4%) relatives (parents and siblings) were reported to have an aortic aneurysm (Table 2). Overall, 9.5% (130/1375) of the male relatives and 4.0% (53/1331) of the female relatives were affected with aortic aneurysm ($P < .001$, Table 2), showing an increase in risk of 1.7 for male relatives and 2.8 for female relatives compared to population risk. Taking the gender of the index cases into account we observed that risk for relatives of female cases was significantly higher (9.0%, 36/402) than for relatives of male cases (5.9%, 147/2471, $P = .022$).

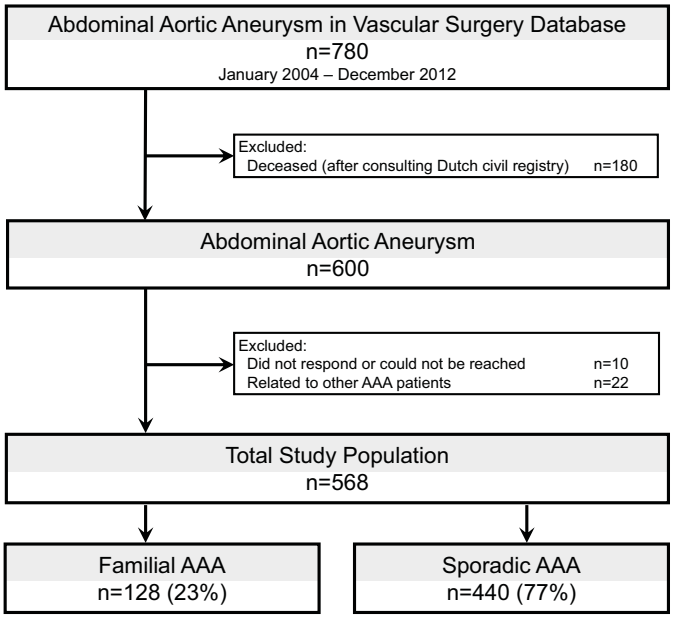

Figure 1 – Flow diagram of patient inclusion

One-hundred and twenty-eight patients had a positive family history and were classified as familial AAA. In these 128 families, 22.4% (183/817) of the relatives were reported to be affected with AAA (Table 3). In familial AAA, 33.1% (130/393) of the male relatives and 15.4% (53/344) of the female relatives were affected with aortic aneurysm ($P < .001$), showing an 5.9 increase in risk for male relatives and a 11 time increase in risk for AAA for female relatives compared to population risk (table 4). In familial AAA, there was no significant difference in risk between relatives of female cases (28.6%, 36/126) and relatives of male cases (21.3%, 147/691) ($P = .071$).

Table 2 –First-degree relatives with aortic aneurysm of 568 AAA patients

First-degree relatives	Relatives with aortic aneurysm n (%)	Relatives with aortic aneurysm 81 female AAA patients n (%)	Relatives with aortic aneurysm 487 male patients n (%)	*P* value
Parents	68/1136 (6.0)	14/162 (8.6)	54/974 (5.5)	.124
Fathers	40/568 (7.0)	6/81 (7.4)	34/487 (7.0)	.890
Mothers	28/568 (4.9)	8/81 (9.9)	20/487 (4.1)	.026
Siblings	115/1737 (6.6)	22/240 (9.2)	93/1497 (6.2)	.087
Brothers	90/807[a] (11.2)	16/113 (14.2)	74/694 (10.7)	.274
Sisters	25/764[a] (3.3)	6/101 (5.9)	19/663 (2.9)	.106
Total	183/2873 (6.4)	36/402 (9.0)	147/2471 (5.9)	.022

[a] Specified data were available for 1571 siblings.

Table 3 – First-degree relatives with aortic aneurysm of 128 familial AAA patients

First-degree relatives	Relatives with aortic aneurysm n (%)	Relatives with aortic aneurysm 22 female familial AAA patients n (%)	Relatives with aortic aneurysm 106 male familial AAA patients n (%)	*P* value
Parents	68/256 (26.6)	14/44 (31.8)	54/212 (25.5)	.386
Fathers	40/128 (31.3)	6/22 (27.3)	34/106 (32.1)	.658
Mothers	28/128 (21.9)	8/22 (36.4)	20/106 (18.9)	.071
Siblings	115/561 (20.5)	22/82 (26.8)	93/479 (19.4)	.124
Brothers	90/265[a] (34.0)	16/45 (35.6)	74/220 (33.6)	.805
Sisters	25/216[a] (11.6)	6/32 (18.8)	19/184 (10.3)	.169
Total	183/817 (22.4)	36/126 (28.6)	147/691 (21.3)	.071

[a]Specified data were available for 481 siblings.

Table 4 – Gender related familial risk for abdominal aorta aneurysm (AAA)

	Relatives				
	All relatives	**Male relatives[a]**		**Female relatives[b]**	
	Risk	**Risk**	**Increase[c]**	**Risk**	**Increase[c]**
All AAA cases					
Male cases	5.9%	9%	*1.6 x*	3.4%	*2.4x*
Female cases	9.0%	11%	*2x*	7.6%	*5.5x*
All AAA	**6.4%**	**9.5%**	***1.7x***	**4%**	***2.8x***
Familial AAA cases					
Male cases	21.3%	33%	*5.9x*	13%	*9.6x*
Female cases	28.6%	33%	*5.9x*	26%	*18.5x*
Familial AAA	**22.4%**	**33%**	***5.9x***	**15.4%**	***11x***

[a] Fathers and brothers.
[b] Mothers and sisters.
[c] Risk in current study compared to estimated population risk in age ≥55 years of 5.6% in men and 1.4% in women.[1-4, 6]
(%) Risk for relatives in current study.

Prevalence and characteristics of familial AAA

Familial AAA was reported by 128 of 568 index AAA patients indicating a prevalence of 22.5% in this study population. Since only three children (two sons and one daughter) were reported with aortic aneurysm, offspring was not reported in Table 2 and 3. Familial AAA was reported more frequently by female cases (27.2%) than by male cases (21.8%) although the difference was not statistically significant (P = .282, Table 5). Risk for relatives was significantly higher for relatives of female cases than relatives of male cases. Eighty-seven patients (67.9%) reported one affected relative, 30 patients (23.4%) reported two affected, and 11 patients (8.6%) reported three or more affected relatives. There was a small difference in family size (number of parents and siblings) between familial AAA and sporadic AAA patients, being on average 4.7 in sporadic AAA and 6.4 in familial AAA (P < .001).

The mean age at diagnosis in the study population was 69.2 (8.1) years, and was not different between males and females. Infrarenal AAA was diagnosed in 452 patients (79.6%) and suprarenal AAA in 116 patients (20.4%). Female patients had a higher rate of suprarenal AAA compared to male patients (38.3% versus 17.5%, P < .001). A total of 507 patients (89.3%) were treated by endovascular or open repair, and 61 patients (10.7%) were managed conservatively. Comparing the clinical characteristics of patients with familial and sporadic AAA showed that patients with familial AAA were diagnosed at a younger age (Table 5). In unadjusted analysis, significant differences were also observed for diabetes mellitus, hypertension and smoking. The AAA location was not different between the two groups. Figure 2 presents the differences in clinical characteristics between familial AAA

Table 5 – Clinical characteristics of patients with familial and sporadic AAA

Variable[a]	Total population n=568	Familial AAA n=128	Sporadic AAA n=440	*P* value
Male gender	487 (85.7)	106 (82.8)	381 (86.6)	.282
Age at diagnosis, years	69.2 (8.1)	67.3 (8.2)	69.7 (8.0)	.003
Age at surgery, years	69.7 (7.9)	67.8 (7.9)	70.2 (7.9)	.005
Age ≤65 years at diagnosis	184 (32.4)	53 (41.4)	131 (29.8)	.013
Body mass index, kg/m2	26.4 (4.2)	26.6 (4.5)	26.4 (4.1)	.608
AAA characteristics				
Suprarenal AAA	116 (20.4)	26 (20.3)	90 (20.5)	.972
Infrarenal AAA	452 (79.6)	102 (79.7)	350 (79.5)	
Ruptured AAA	67 (11.8)	18 (14.1)	49 (11.1)	.364
Cardiovascular comorbidities				
Congestive heart failure	129 (22.7)	30 (23.4)	99 (22.5)	.824
Ischemic heart disease	262 (46.1)	57 (44.5)	205 (46.6)	.681
Cerebrovascular disease	126 (22.2)	30 (23.4)	96 (21.8)	.698
Cardiovascular risk factors				
Kidney disease	113 (19.9)	22 (17.2)	91 (20.7)	.383
Diabetes mellitus	119 (21.0)	18 (14.1)	101 (23.0)	.030
Hypertension	434 (76.4)	85 (66.4)	349 (79.3)	.002
Hypercholesterolemia	538 (94.7)	118 (92.2)	420 (95.5)	.146
Smoking – current	253 (44.5)	56 (43.8)	197 (44.8)	.838
Smoking – ever	520 (91.5)	111 (86.7)	409 (93.0)	.026
Medication				
Statins	495 (87.1)	108 (84.4)	387 (88.0)	.287
Beta-blockers	508 (89.4)	113 (88.3)	395 (89.8)	.629
Renin-angiotensin system inhibitors	318 (56.0)	75 (58.6)	243 (55.2)	.499
Diuretics	182 (32.0)	45 (35.2)	137 (31.1)	.391
Antiplatelets	460 (81.0)	100 (78.1)	360 (81.8)	.349

[a] Categorical data are presented as number (%) and continuous data as mean (standard deviation).

and sporadic AAA as calculated in multivariable logistic regression analysis. Familial AAA was independently associated with an age ≤65 years at diagnosis (Odds ratio [OR] 1.9, 95% confidence interval [CI]: 1.23-2.89, *P* = .004) and inversely associated with diabetes mellitus (OR 0.52, 95% CI: 0.29-0.91, *P* = .023) and hypertension (OR 0.53, 95% CI: 0.33-0.85, *P* = .008). Additional adjustment for medication did not alter the outcome. Patients having these three discriminating variables (ie, age ≤65 years at diagnosis, without diabetes and without hypertension) had a 4.4 fold increased risk for familial AAA (6.9% versus 24.3%, 95% CI: 1.54-12.22, *P* = .006).

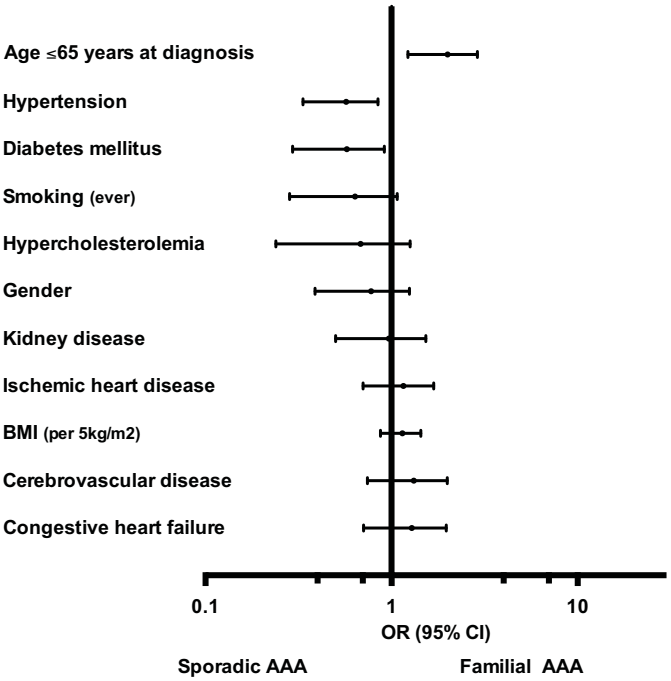

Figure 2 – Multivariable logistic analysis for clinical characteristics according to familial and sporadic AAA

Included variables: age ≤65 years at diagnosis, gender, BMI, location of aneurysm, congestive heart failure, ischemic heart disease, cerebrovascular disease, kidney disease, diabetes mellitus, hypertension, hypercholesterolemia and smoking history.

Validation of familial AAA classification

Eighty-five of the 128 index patients (66%) classified as familial AAA were seen by a clinical geneticist. In this way consent was obtained was obtained from 72 families to review the medical records allowing to confirm that relatives were diagnosed with AAA. We did not find false positive reports of aortic aneurysms in relatives of AAA patients. In 13 families the diagnosis in relatives could not be confirmed because the medical records were no longer available conform the current the Health Council in the Netherlands.[23] Medical records of relatives were not available from 14 familial AAA patients refusing genetic counseling and of 29 patients who died during the course of the study.

DISCUSSION

The main results of this study were that in approximately one in four AAA patients familial disease is expected. An important finding was that all relatives of AAA patients have an increased risk (6.4%) compared to general population. Relatives of female cases are at greater risk (9%) than relatives of male patients (5.9%). In familial AAA risk these risks are increased to 28.6% for relatives of female cases and 21.3% for relatives of male cases. These findings have important clinical implications. The current ACC/AHA guidelines recommends screening of male relatives over the age of 60 years and the ESVS guidelines advice screening of all relatives over the age of 50 years with a known family history of AAA.[17, 19] Our findings suggest that all relatives of all AAA patients should be screened, irrespective of gender and family history, since the risk remained elevated when family history was not taken into account. Adding that particularly families of women with AAA and familial cases have significantly increased risks.

Given the overall higher prevalence of AAA in men than in women, population screening for AAA focuses currently on the elderly male population.[24] Trials showed that screening of men over the age of 65 years was effective in reducing mortality from rupture,[19, 25-27] recent data from large national screening programs across the United Kingdom, Sweden and United States showed however a lower incidence of AAA than expected, raising questions on the efficacy of population screening.[28-30] When population risk would become lower than currently assumed, the difference with familial risk becomes even more prominent. This indicates that relatives of AAA patients are for now the easiest identifiable high-risk population who may benefit from screening in order to reduce aneurysm-related mortality in the population.

The prevalence of familial AAA in this study was higher than described in previous family history studies which was on average 14% [range: 6%-36%, Supplemental data Table S1]. Using a semi-structured questionnaire to obtain family histories may have improved detection of familial AAA compared to other, less reliable methods of taking family histories. Studies using ultrasound screening of relatives reported on average a prevalence of 11% [range: 0%-38%, Supplemental data table SII], and clearly showed the major limitations of these studies, i.e. that few relatives of patients 65 years and older were available for screening.

The reported prevalence of an aortic aneurysm in 15% of the female relatives in familial AAA, irrespective of the patient's gender, in this and previous studies is much higher than the expected prevalence in women. Among the twelve previously conducted AAA family history studies, only four analyzed risk for relatives by gender of case and gender of the relatives. [31-34] All four showing that relatives of female patients had a higher risk (mean 30%) than relatives of male patients (mean 17.3) (Supplemental data Table S1). Similarly of the nineteen studies presenting the results of echographic AAA family

screening, only two studies reported separate risk for male and female relatives of male and female index patients (Supplemental Table S2). The results of these two studies corroborated that families of female patients had higher recurrence rate of AAA.[35, 36]

These finding support a genetic component in aortic aneurysm formation, in particular in women. It is well known that fewer women have AAA than men, suggesting a gender specific susceptibility for AAA in men. As hypothesized for complex genetic disorders occurring more frequently in men (eg, pyloric stenosis), the genetic susceptibility that could be described as a genetic 'burden', necessary to develop the disease, is lower for men than for women. According to this theory, affected women are expected to have a higher genetic 'burden'. This may explain that relatives of female patients are at higher risk to develop aortic aneurysms than relatives of males patients, and underlines the importance of including families of female patients and female relatives in screening programs.[37] The male preponderance in familial AAA may reflect that similar gender related risk factors contribute to familial AAA and sporadic AAA. Alternatively, it may indicate the occurrence of phenocopies, ie, the occurrence of a sporadic form of AAA in families with familial AAA.

For the purpose of targeted family screening, we searched for distinguishing clinical characteristics of patients with a high familial risk for AAA. This showed that the prevalence of familial AAA was fourfold higher in AAA cases were diagnosed below the age of 65, without diabetes, and without hypertension. These characteristics, however, lack specificity and although relatives of these patients may have a higher risk of developing AAA, the absence of biological markers presently precludes the use of clinical features for prediction of risk for relatives.

We did not include offspring of AAA patients in the results of our study because these relatives were generally too young to develop aortic aneurysms yet.[19] No conclusions can therefore be drawn about risk for children from our data. It is unknown when to start screening, but a threshold to start screening from the age of 50 years seems adequate and is in line with the current European guidelines.[19] This threshold is based on the finding that 7% of AAA patients worldwide are younger than 60 years,[38] and an increase in abdominal aortic diameter is expected to start several years prior to the average age of presentation.[39] Since it is unknown whether familial abdominal aneurysms have different expansion rates than the average of 2-3 mm annually,[19] we may consider to keep relatives with a small aneurysm under surveillance as proposed in the current international guidelines.

Another question remains whether relatives with a negative ultrasound should be re-screened. The results from the Gloucestershire Aneurysm Screening Programme showed development of an AAA >5.4 cm in 15% of individuals with an initial aortic diameter of 2.6 to 2.9 cm after ten years and the MASS trial showed an increase in aortic ruptures in the screened group after eight years.[28, 30] Based on these data repeated aortic screening after five years for relatives of AAA patients with an initial aortic diameter <3.0 cm seems necessary.

Since screening for AAA involves presymptomatic testing of relatives, it is necessary that relatives at risk are provided with relevant information on genetic risk and treatment, and have the opportunity to discuss issues concerning possible adverse physical, psychological and socioeconomic consequences of participating in a screening program. Care for AAA patients should in our view extend to informing patients and their relatives about genetic risk and offering appropriate family screening.

Our study had several limitations. The data were a based on family history and not on aortic imaging of complete families. The validation of our family history data showed however that no relatives were reported incorrectly as affected, indicating that risk in relatives was not overestimated. On the other hand, underreporting of familial disease may have happened, in particular for a disease like AAA, where aneurysms in relatives may go unnoticed and relatives could have undiagnosed aneurysms or may have died before age of onset. It is therefore important to bear in mind that familial AAA cannot be excluded when family history of aneurysm is missing or uninformative. Underreporting in this study may also have influenced the outcome of our prediction model of clinical characteristics. The predominance of individuals of Caucasian descent in our population may limit the generalizability of our results to other ethnicities and other parts of the world

CONCLUSIONS

Male and female relatives have high risk for AAA, in particular the relatives of female AAA cases. The risk for relatives is much higher in familial AAA than in the general population. Families of female AAA patients should have similar access to screening as the families of male patients, and female relatives need to be included in the family screening as well. Relatives of AAA patients are for now the easiest identifiable high-risk population available for targeted AAA screening. The new insights in familial risk and family screening for AAA are important in primary health care in order to inform AAA patients and allow their relatives to benefit from screening.

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SUPPLEMENTAL TABLES

Supplemental Table S1 – Family history studies of abdominal aortic aneurysm

First author	Year	AAA index cases (N)		Positive family history (N)	Familial AAA (%)
Norrgard et al.[40]	1984	All	87	16	18.4
		Male	na	na	na
		Female	na	na	na
Johansen et al.[41]	1986	All	250	48	19.2
		Male	208	na	na
		Female	42	na	na
Powell et al.[31]	1987	All	60	25	41.7
		Male	53	19	30.0
		Female	7	6	83.3
Johnston et al.[42]	1988	All	666	41	6.2
		Male	533	na	na
		Female	133	na	na
Cole et al.[43]	1989	All	305	34	11.1
		Male	na	na	na
		Female	na	na	na
Darling et al.[32]	1989	All	542	82	15.1
		Male	449	53	11.8
		Female	93	29	31.2
Webster et al.[33]& Majumder et al.[44]	1991	All	91	14	15.4
		Male	79	11	14.1
		Female	12	3	25.0
Verloes et al.[45]	1995	All	313	39	12.5
		Male	na	na	na
		Female	na	na	na
Lawrence et al.[46]	1998	All	86	19	22.1
		Male	***73***	*na*	*na*
		Female	***13***	*na*	*na*
Rossaak et al.[34]	2001	All	248	48	19.4
		Male	194	36	18.6
		Female	54	12	22.2
Badger et al.[36]	2007	All	132	8	6.1
		Male	na	na	na
		Female	na	na	na
Sakalihasan et al.[47]	2014	All	618	62	10.0
		Male	563	na	na
		Female	55	na	na
Van de Luijtgaarden et al.[48]	2014	All	461	103	22.3
		Male	390	82	21.0
		Female	71	21	29.6
Total		All	3859	539	14.0
		Male	1165	197	17.3
		Female	237	71	30.0

Abbreviations: na, not applicable

Supplemental Table S2 – Family screening studies for abdominal aortic aneurysm

First author	Year	AAA index cases (N)	Screened relatives (N)			Affected relatives (N)			Familial AAA (%)		
			total	male	female	**total**	male	female	**total**	male	female
Bentgsson et al.[49]	1989	84	**87**	35	52	**13**	10	3	**14.9**	28.6	5.8
Collin et al.[50]	1989	108	**31**	16	15	**4**	4	0	**12.9**	25.0	0.0
Webster et al.[51]	1991	43	**103**	na	na	**4**	3	1	**3.9**	na	na
Adamson et al.[52]	1992	28	**53**	25	28	**6**	5	1	**11.3**	20.0	3.6
Bengtsson et al.[53]	1992	155	**62**	39	23	**9**	8	1	**14.5**	20.5	4.3
Van der Lugt et al.[54]	1992	37	**108**	56	52	**19**	16	3	**17.6**	28.6	5.8
Adams et al.[55]	1993	92	**74**	38	36	**9**	8	1	**12.2**	21.1	2.8
Fitzgerald et al.[56]	1995	52	**125**	60	65	**15**	13	2	**12.0**	21.7	3.1
Larcos et al.[57]	1995	38	**52**	26	26	**0**	0	0	**0.0**	0.0	0.0
Baird et al.[58]	1995	126	**54**	26	28	**10**	7	3	**18.5**	26.9	10.7
Jaakkola et al.[59]	1996	71	**123**	45	78	**5**	4	1	**4.1**	8.9	1.3
Van der Graaf et al.[60]	1998	291	**210**	210	0	**26**	26	0	**12.4**	12.4	0.0
Salo et al.[61]	1999	150	**238**	98	140	**8**	8	0	**3.4**	8.2	0.0
Rossaak et al.[34]	2001	248	**49**	na	na	**4**	3	1	**8.2**	na	na
Frydman et al.[35 a]	2003	400	**276**	150	126	**84**	64	20	**30.4**	42.7	15.9
		316 male	***221***	*121*	*100*	***65***	*51*	*14*	***29.4***	*42.1*	*14.0*
		84 female	***55***	*29*	*26*	***19***	*13*	*6*	***34.5***	*44.8*	*23.1*
Ogata et al.[62]	2005	132	**245**	98	150	**15**	11	4	**6.1**	11.2	2.7
Badger et al.[36]	2007	132	**300**	157	143	**10**	8	2	**3.3**	5.1	1.4
		(-) male	***229***	*125*	*104*	***7***	*5*	*2*	***3.1***	*4.0*	*1.9*
		(-) female	***71***	*32*	*39*	***3***	*3*	*0*	***4.2***	*9.4*	*0.0*
Linne et al.[63]	2012	412	**150**	66	84	**16**	11	5	**10.6**	16.7	6.0
Sakalihasan et al.[47]	2013	144	**186**	86	100	**24**	19	5	**12.9**	22.1	5.0
	Total	2743	2526	1231	1146	281	228	53	11.1	18.5	4.6
		male	**450**	**246**	**204**	**72**	**56**	**16**	**16.0**	**22.8**	**7.8**
		female	**126**	**61**	**65**	**22**	**16**	**6**	**17.5**	**26.2**	**9.2**

Abbreviations: na, not applicable

[a] In the study from Frydman *et al.* abdominal aortic aneurysms (AAA) were defined as ≥3.0 cm , or a ratio of > 1.5 of the suprarenal aortic diameter to the infrarenal diameter and included cases previously diagnosed with AAA.

11

Arterial Elongation and Tortuosity Leads to Detection of a *de novo TGFBR2* Mutation in a Young Patient with Complex Aortic Pathology

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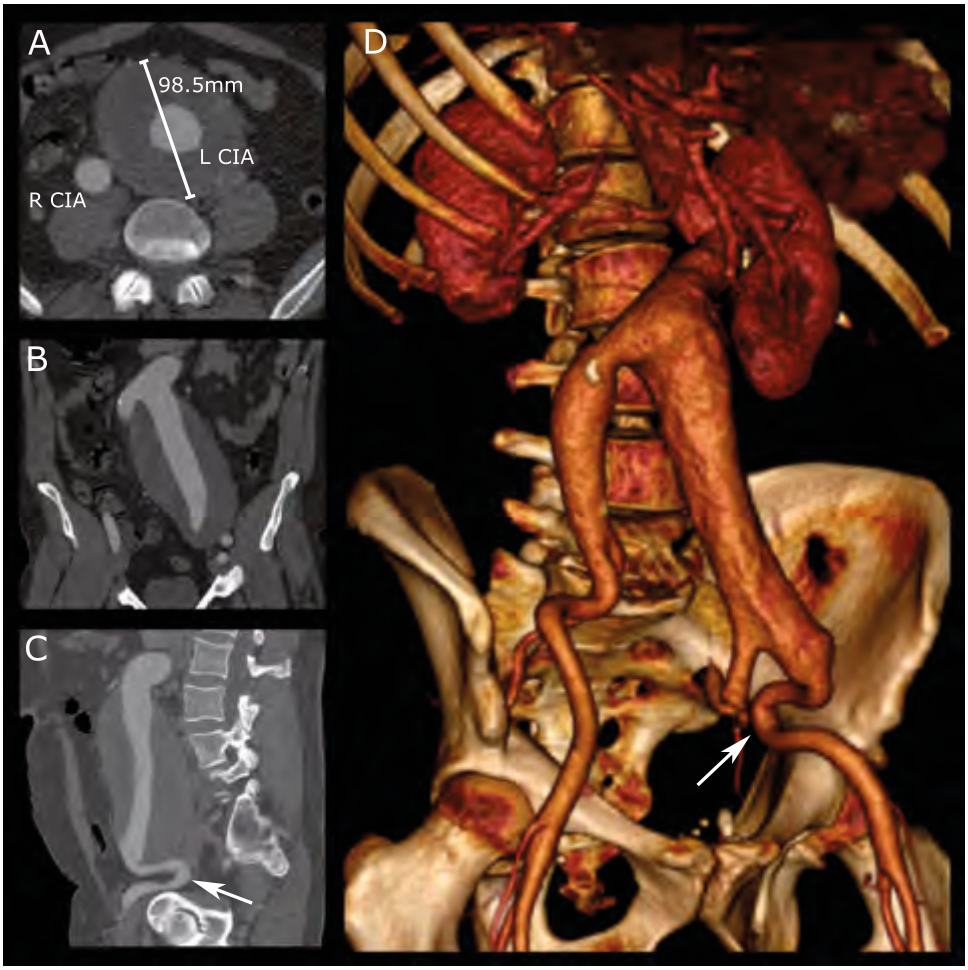
Hence J.M. Verhagen

Eur Heart J. 2013;34(15):1133.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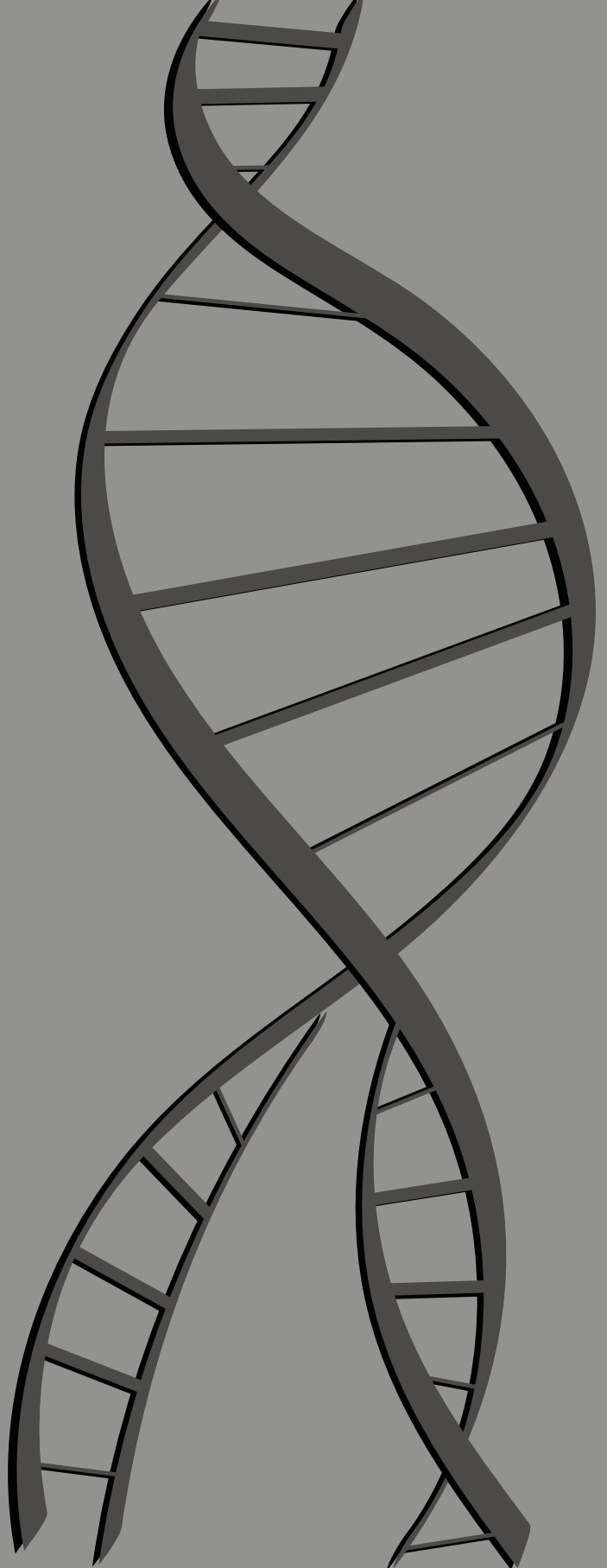
In a 47-year-old muscular build male, admitted with acute abdominal pain, imaging revealed a Stanford type-B aortic dissection associated with a pre-existing large aorto-iliac aneurysm (*Panel* A and B) and marked iliac-artery elongation and tortuosity (*Panel* C and D, arrows). The patient underwent uneventful elective open repair of the aorto-iliac aneurysm. The marked aortic elongation and tortuosity at young age in this patient prompted referral for genetic counselling after surgery. No characteristic facial or musculo-skeletal signs of Loeys-Dietz or Marfan syndrome were present and there was no family history of vascular disease. Nevertheless, DNA analysis showed a "*de-novo*" *TGFBR2* mutation.

Arterial elongation and tortuosity is a main feature in patients with characteristic facial and musculoskeletal appearance of the TGF-β pathway related genetic aneurysm syndromes. Therefore, our observation expands the phenotypic spectrum of TGF-β pathway related pathology to patients with severe abdominal and iliacal arterial disease without major dysmorphologic characteristics.

We demonstrate the importance of genetic testing in younger patients presenting with complex aortic pathology with marked arterial elongation and tortuosity, even in the absence of characteristic phenotypic features of a genetic aneurysm syndrome or a family history of aortic aneurysms. Correct genetic diagnosis of *TGFBR2* related aortic pathology is important for clinical management of the patients and for genetic counselling of the family. Since *TGFBR2* linked genetic aneurysms have an autosomal dominant inheritance, relatives at risk should be offered genetic counselling and presymptomatic testing for *TGFBR2* mutations. In this way, carriers of the *TGFBR2* mutation can benefit from screening and timely intervention.

Summary



SUMMARY

The aim of this thesis was to investigate the differences between aneurysmal and occlusive arterial disease and to delineate the clinical and genetic features of familial abdominal aortic aneurysm (AAA).

In **PART I**, the authors addressed the clinical and genetic features of patients with aneurysmal disease as compared to patients with occlusive arterial disease. **Chapter 1** revealed that the common carotid artery intima-media thickness (CIMT) was significantly lower in patients with aneurysmal disease than in those with occlusive arterial disease. This indicates that the atherosclerotic burden in aneurysmal disease is lower than in occlusive arterial disease and endorses the idea that other pathogenic mechanisms are involved in aortic aneurysm formation. **Chapter 2** showed a high prevalence of vitamin D deficiency in patients with arterial disease. There was a strong association between low vitamin D status and higher CIMT, lower ankle-brachial index, and higher high-sensitivity C-reactive protein, which are all markers for the severity of arterial disease. These associations were independent of traditional cardiovascular risk factors such as smoking, gender, and age, but also irrespective of type of arterial disease, suggesting a direct effect of vitamin D deficiency on the arterial wall in both occlusive and aneurysmal arterial disease. **Chapter 3** described gene expression profiles from abdominal aortic wall samples from patients with aneurysmal and occlusive arterial disease. Several genes were significantly upregulated in the aneurysmal disease samples, including *COL11A1*, *APIPOQ,* and *LPL*, which have previously been associated with aneurysmal disease. Upregulations of novel genes in aneurysmal disease samples were also discovered and included *CXCL13*, *SLC7A5,* and *FDC-SP,* amongst others. In addition, the study showed that simultaneous inhibition of bone morphogenetic protein and activation of TGF-beta signaling was present in the aneurysmal disease samples as compared to the occlusive arterial disease samples, suggesting that the TGF-beta pathway could also play a role in the formation of abdominal aneurysmal disease. **Chapter 4** demonstrated that chronic obstructive pulmonary disease (COPD) was more common in patients with aneurysmal disease than in patients with occlusive arterial disease. The association between COPD and aneurysmal disease was independent of traditional cardiovascular risk factors such as smoking and inflammation. It was concluded that factors other than cardiovascular risk profiles or systemic inflammation contributed to the association between COPD and aneurysmal disease. The results from the experimental part of the study showed that homozygous Fibulin-4, a known extracellular matrix glycoprotein, deficient mice developed also severe lung emphysema besides aneurysms whereas heterozygous Fibulin-4 deficient mice acquired alveolar breakdown with age and stress. The authors concluded that a genetic susceptibility to extracellular matrix degeneration and secondary inflammation were the common mechanisms in both COPD and aneurysm formation.

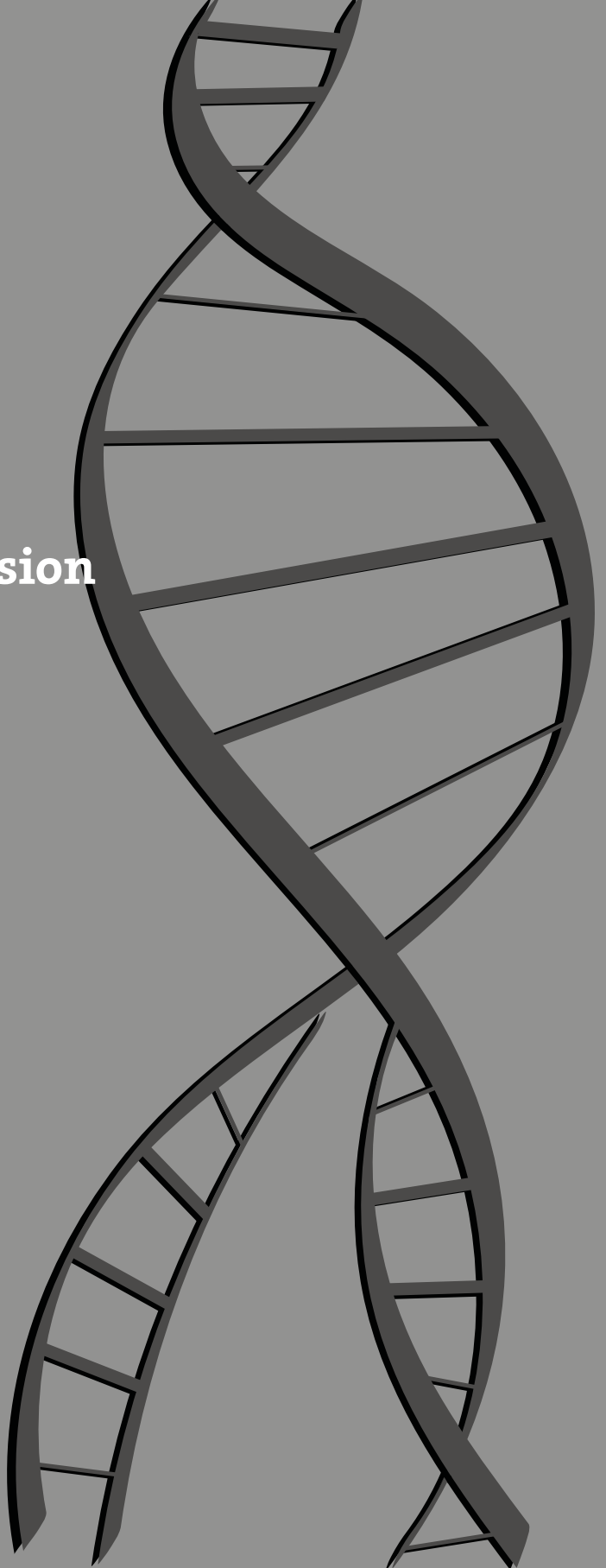
In **PART II**, the clinical and genetic features of familial abdominal aortic aneurysm were explored. In **Chapter 5** the authors described that the atherosclerotic burden, as reflected by the CIMT, was lower in patients with familial AAA as compared to patients with sporadic AAA. This difference was independent of common atherosclerotic risk factors. Although patients with familial AAA still had high CIMT, the authors concluded that it supported the view that atherosclerosis is not the primary driving factor in the development of familial AAA. **Chapter 6** reported about the first genetic analysis of aneurysm genes including the TGF-beta pathway genes *EFEMP2*, *FBN1*, *SMAD3*, *TGFB2*, *TGFBR1*, *TGFBR2* and the smooth muscle cell genes *ACTA2*, *MYH11*, and *MYLK*, in patients with familial and sporadic AAA. Pathogenic variants were observed in two percent of the AAA patients and implies that genetic testing may help to identify the cause of aneurysm formation in familial and sporadic AAA patients.

In **PART III,** the clinical outcome of patients with familial abdominal aortic aneurysm was described. **Chapter 7** showed that patients with familial AAA developed more aneurysm-related complications after endovascular aneurysm repair (EVAR) despite a similar AAA morphology. The twofold higher aneurysms-related complication rate after EVAR should create awareness of this possible incremental postoperative risk in familial AAA patients amongst clinicians. **Chapter 8** presented the results from a worldwide EVAR registry (ENGAGE) of patients treated with a late generation stent graft. No significant difference in clinical success, a composite of technical success and freedom from postoperative complications, was identified between familial and sporadic AAA. However, a higher aneurysm-related mortality and a trend towards more secondary procedures were observed in patients with familial AAA. The authors concluded that familial AAA patients have an increased risk after EVAR and should be offered a close follow-up regime.

PART IV addressed the clinical management of patients with familial abdominal aortic aneurysm. **Chapter 9** summarized the current evidence of familial AAA and described the clinical implications in daily practice for vascular surgeons. The most important conclusion was that the evidence of a genetic susceptibility for AAA is increasing, leading to new approaches for personalized treatment and prevention strategies, involving targeted screening. The authors also concluded that until adequate markers for familial AAA are available, family history remains the preferred method to identify familial AAA. **Chapter 10** investigated risk for relatives of AAA patients by gender and demonstrated that the risk for male and female relatives of AAA patients is much higher than the risk in the general population. Although male relatives were still more often affected, the increase in risk was relatively larger for female relatives than for male relatives. Relatives of female AAA patients also had a higher risk as compared to relatives of male AAA patients. The authors concluded therefore that families of female AAA patients should also have access

to screening programs similar as families of male patients do and that female relatives should be included in family screening as well. Furthermore, the study indicated that young age, female gender, complex vasculopathy, no hypertension, no hyperlipideamia, and no history of smoking indicate familial AAA and may help distinguish these patients from sporadic AAA. Finally, **Chapter 11** showed a case of a young patient with an Stanford type B dissection with a pre-existing large aorto-iliac aneurysm. Genetic testing was performed due to aneurysm morphology and revealed a *de novo TGFBR2* mutation. The authors concluded that the case warrants genetic testing in young patients presenting with complex aortic pathology with marked arterial elongation and tortuosity, even in the absence of characteristic phenotypic features of a genetic aneurysm syndrome or a positive family history.

General Discussion



GENERAL DISCUSSION

PART I: Clinical and genetic features of aneurysmal and occlusive arterial disease

Shared risk factors for atherosclerosis resulted in the belief that occlusive and aneurysmal arterial disease were both expressions of advanced atherosclerotic disease.[1] While it is undebated that atherosclerosis is the cause of occlusive arterial disease,[2] in aneurysmal disease, this view is questioned since the severity of aortic atherosclerosis does not correlate with the severity of aneurysmal disease.[3] And, more importantly, does not explain the weakening of the aortic wall. Histologic investigations of the aortic wall in occlusive arterial disease showed that mainly the intima and media are affected, while in aneurysmal disease all layers including the adventitia are affected.[4] In other words, there are differences between the two entities besides the opposite disease outcome, i.e. aneurysmal and occlusive, alone. One thing is clear however; both diseases share many atherosclerotic risk factors. We therefore performed several investigations to find evidence for a difference in etiology between both entities. First, we investigated whether there was a difference in the atherosclerotic burden in patients with aortic occlusive disease and aortic aneurysmal disease (*Chapter 1*). We measured the common carotid artery intima-media thickness (CIMT) to quantify the amount of atherosclerosis in patients with occlusive and aneurysmal disease and found that the CIMT was lower in patients in aneurysmal disease.[5] This indicates a lower atherosclerotic burden in patients with aneurysmal disease. Since all patients were "end stage" disease no difference in atherosclerotic burden was expected if both diseases were caused by atherosclerosis. Nevertheless, aneurysmal disease patients still had higher CIMT values as compared to the age-matched general population and are therefore not completely free of atherosclerosis.[6,7] This might be explained by the fact that atherosclerosis develops in parallel or as a consequence of inflammation, since it is known that both occlusive and aneurysmal disease are associated with arterial inflammation.[3] Another interesting factor associated with arterial inflammation is vitamin D. The exact role of vitamin D in arterial disease is unknown but vitamin D receptors have a wide tissue distribution including the vascular tree, suggesting it may play a role in the pathogenesis of arterial disease.[8,9] We showed a high percentage of vitamin D deficiency in patients with occlusive and aneurysmal disease (*Chapter 2*).[10] In addition, the severity of vitamin D deficiency was highly correlated with the severity of atherosclerosis, independently of atherosclerotic risk factors, suggesting a direct effect of vitamin D deficiency on the arterial wall in both occlusive and aneurysmal disease. Since the physiologic actions of vitamin D on the vascular wall include smooth muscle cell proliferation as well as reducing vascular inflammation through several different pathways,[10] vitamin D deficiency may lead to increased vascular inflammation and promoting atherosclerosis. This is confirmed by several studies, which showed atherosclerotic effects on the aortic wall of vitamin D

deficiency in several experimental and clinical studies.[11-13] The most important question to answer is whether vitamin D deficiency is a cause or a confounder in arterial disease. Several randomized controlled trials showed however a beneficial effect of vitamin D supplements on blood-pressure regulation,[14] supporting the hypothesis that vitamin D deficiency have a causative effect in arterial disease. We continued our investigation between aneurysmal and occlusive arterial disease with analyses of genetic factors and molecular processes in aortic tissue from patients with abdominal aortic aneurysm (AAA) and aortic occlusive disease (*Chapter 3*). The experimental study showed that several genes previously associated with aneurysmal disease were significantly upregulated in the aneurysmal samples, including the genes *COL11A1* (encoding for components of type XI collagen), *APIPOQ (*associated with Kawasaki syndrome), and *LPL (*encoding for lipoprotein lipase*)*. We also identified novel genes upregulated in the aneurysmal samples, including the genes *CXCL13*, *FDC-SP*, and *POU2AF1*. The function of these genes are mainly associated with the immune and inflammation systems. The clinical data of the study supported these findings as we observed increased inflammation, as reflected by a higher high-sensitivity C-reactive protein (hs-CRP) values, in patients with aneurysmal disease. It is unknown whether increased inflammation is a confounder, but the clear overrepresentation of inflammation and immune pathways certainly points towards this direction and cannot be left unnoticed. In this study, we also observed dysregulation of the TGF-beta and bone morphogenetic protein pathways in the aneurysmal samples, known to be associated with thoracic aortic aneurysm (TAA),[15] suggesting also a role of the TGF-beta pathway in abdominal aneurysms.[16, 17] Another possible cause for aneurysm formation besides inflammation, are defects in the connective tissue of the aortic wall. Because chronic obstructive pulmonary disease (COPD) and AAA are both associated with extracellular matrix (ECM) degeneration, we tested the association between COPD and aneurysmal disease in a clinical and experimental setting (*Chapter 4*). We found an association between COPD and aneurysmal disease, independently of traditional atherosclerotic risk factors and inflammation status, suggesting that factors other than cardiovascular risk factors or systemic inflammation contribute to the association between COPD and aneurysmal disease.[18] In addition, we tested the association between COPD and AAA in an animal model. Since both COPD and aneurysmal disease are characterized by breakdown of ECM, we investigated whether a primary ECM defect provides the pathogenic link between these two diseases. Homozygous Fibulin-4 downregulated mice, a known aneurysmal animal model, showed alveolar breakdown characteristic for lung emphysema. Additional analyses showed besides ECM degeneration also inflammation in the alveoli and an increased TGF-beta signaling in the Fibulin-4 deficient mice. Since we did not observe any difference in inflammation between patients with and without COPD, as reflected by serum hs-CRP, we believe that inflammation is unlikely to account for the observed relation between COPD and aneurysmal disease. Our data questions

therefore the traditional view that smoking leads to inflammation resulting in loss of ECM in the alveoli and leading to lung emphysema in patients. Instead we believe that genetic ECM defects initiate a heightened inflammatory reaction in response to environmental triggers, such as smoking, leading to alveolar degradation.

In conclusion, our studies endorse that factors, such as ECM degeneration, inflammatory and immune disturbances, together with atherosclerosis have a role in the pathophysiology of AAA. We belief that future research should focus on these new findings in order to elucidate the pathophysiologic mechnisms leading to aneurysmal and occlusive arterial disease.

PART II: Clinical and genetic features of familial abdominal aortic aneurysm

We collected detailed family histories and DNA from a large number of AAA patients and investigated the clinical and genetic features of familial AAA. The lower CIMT measured in familial AAA compared to sporadic AAA in our study is, as far as we know, the first study to address a difference in atherosclerotic burden between familial and sporadic AAA (*Chapter 5*). We showed that atherosclerosis is unlikely to be the primary factor leading to aneurysm formation in familial AAA and supports the heterozygous and complex etiology in familial AAA.[19] Patients with familial AAA still had a marginally higher CIMT compared to the healthy age-matched general population, which showed that patients with familial AAA were not completely free of atherosclerosis and that atherosclerosis could still play a role in familial AAA.[6, 20] The high prevalence of a positive family history in AAA patients in approximately 20% suggests a genetic predisposition to abdominal aneurysm in at least 1:5 AAA patients.[19] Although a genetic role in the pathogenesis of AAA is clear, the genetic defects causing AAA remain largely elusive. Several inherited connective tissue disorders associated with aneurysmal disease have been described so far, including Loeys-Dietz (with defects in one of the following genes: *TGFBR1*, *TGFBR2*, *SMAD3*, *TGFB2*), Marfan (*FBN1*) and the vascular type of Ehlers-Danlos (*COL3A1*) syndrome. Most of these aneurysm syndromes are associated with TAA, but isolated AAA is occacionally observed in families with familial syndromic and non-syndromic thoracic aneurysms,[21] suggesting that there may be a greater overlap between TAA and AAA than currently believed. Besides some clinical overlap between abdominal and thoracic aneurysms, including age and gender, differences have also been described as they originate from a different embryologic origin. The majority of syndromic forms of TAA are associated with disorders in the TGF-beta pathway leading to uncontrolled release of TGF-beta.[22] Interestingly, recent studies found also an upregulation of TGF-beta in patients with non-syndromic TAA, suggesting a common pathogenic pathway between syndromic and non-syndromic TAA.[22] The role of TGF-beta in abdominal aneurysmal disease is controversial and remains

to be elucidated.[16,17,23,24] Due to the possible overlap between TAA and AAA we performed a molecular analysis of aneurysm genes in our AAA patient cohort (*Chapter 6*). Interestingly, we discovered that two percent of the AAA patients had a pathogenic variant in one of the genes involved in the TAA. Although this seems a low percentage, one must bear in mind that this is almost similar as the amount of patients affected with a mutation in breast cancer.[25] The mutations found in our cohort originated from several different pathways, including the TGF-beta pathway (*TGFBR2*), smooth muscle cells (*MYH11*) and connective tissue (*COL3A1*). The identification of an additional 44 variants of unknown significance in the analysed genes suggests that the role of genes associated with TAA are much larger in AAA than currently known. Importantly, the observed defects and variants were not only found in familial AAA patients but also in sporadic AAA patients. These results confirm therefore that genetic testing may help to find the cause of aneurysm formation not only in familial AAA. New genetic causes for aneurysms are continuously discovered and in the years to come the opportunity of next generation sequencing at reduced costs will enhance unraveling the genetics of AAA.

PART III: Clinical outcome of familial abdominal aortic aneurysm

Insight into the natural history and specific clinical features of familial AAA are needed to offer appropriate care. If familial AAA is caused by an unknown genetic defect associated with impaired extracellular matrix modelling, one may hypothesize that familial AAA patients may develop seal and fixation difficulties and subsequent sac growth. Endovascular therapy is for this reason unadvised in patients with connective tissue disorders, such as Marfan, Loeys-Dietz and Ehlers-Danlos syndrome.[26,27] The clinical outcome of patients with familial AAA is currently quite limited, only Brewster et al. reported several years ago a trend towards increased mortality in patients with a positive family history, as observed in a subanalysis of long-term follow-up after endovascular aneurysm repair (EVAR).[28] In order to investigate whether familiar AAA is associated with an increased risk of postoperative events, we evaluated the clinical outcome in our patient cohort (*Chapter 7*). We found a twofold higher risk for aneurysm-related complications after EVAR in patients with familial AAA.[29] We investigated aneurysm morphology using aortic imaging, since some genetic aneurysm syndromes are associated with specific anatomic features and with the knowledge that adverse aneurysm morphology may result in adjunctive procedures.[30-33] No differences in aneurysm morphology could be established between the groups, suggesting that disparities in complication rate could not be attributed to any morphologic differences. Secondary interventions and aneurysm sac growth were the most important elements accounting for the difference in complications after EVAR. Although this could be caused by a higher number of type I endoleaks, it failed to reach statistical significance. Our study is the first to describe

this phenomenon and needs verification from larger multicenter studies from different prospective patient cohorts. Recently a single center retrospective study from Ryer et al. showed an increased rate of endoleaks and secondary interventions in patients with familial AAA as well.[34] The authors concluded that patients with familial AAA might benefit from closer postoperative surveillance. We investigated the clinical outcome in a large worldwide single graft EVAR registry who reported a positive family history in 7% of the patients (*Chapter 8*). It is known that the prevalence of familial AAA is lower in populations where family history is not systematically ascentained. Clinical success showed not to be significantly different during follow-up in patients with familial AAA. But we did find a 5% difference in clinical success between familial and sporadic AAA however, suggesting a relevant clinical difference between the groups. We also observed a higher aneurysm-related mortality in patients with a positive family history (6% versus 1%), mostly due to higher 30-days mortality. There was a trend towards more secondary procedures in familial AAA, similar to our previous study, but we could not establish a difference in aneurysm sac growth or endoleaks.[29] Instead, familial AAA patients in this study suffered from more stent graft occlusion as compared to sporadic AAA patients. A possible explanation for this finding may be the overrepresentation of females in familial AAA. It is known that women have more perioperative complications after EVAR and require more adjunctive procedures.[35, 36] It is also known that women are associated with increased aneurysm-related mortality.[37-39]

In conclusion, patients with familial AAA have worse outcome after EVAR. Clinicians should be aware of the higher complication rate after endovascular treatment. Nevertheless, we cannot conclude that EVAR is contraindicated in familial AAA on the basis our results, because most patients with complications after EVAR could be treated with minimally invasive techniques with low morbidity.[29] Nevertheless, longer term follow-up and data from other patient cohorts are needed to answer whether familial AAA patients can be safely treated with EVAR and to establish the need for adaptation of the follow-up regime in these patients. For the time being, patients with familial forms of AAA should be offered a more rigorous follow-up regime.

PART IV: Clinical management of familial abdominal aortic aneurysm

The results of the studies presented in this thesis show that familial AAA matters and has implications for daily practice with regard to identifying patients, screening relatives and treatment strategies. Up to date knowledge of this matter is needed for all clinicians involved in AAA management, especially for the vascular surgeon who often has a leading role in the treatment of patients with AAA.

The first step in clinical management should be to accurately identify patients with familial AAA. As no marker or genetic test for familial AAA is presently available, family

history is still the best way to identify patients with a higher genetic susceptibility. Because familial occurrence of disease may easily be missed we advise -in order to increase the validity of the family history- to obtain family history data from semi-structured questionnaires. The best definition of familial AAA in our view is when at least one first-degree relative (parent, sibling, offspring) is diagnosed with an aortic aneurysm.[19, 40] Preferably, the family history is evaluated by a clinical geneticist, in this way family history can be confirmed by requesting medical records of affected relatives, and the patient can also be informed about the risk for relatives and provide the family with appropriate information regarding family screening. Another advantage of involving the clinical geneticist in the management of AAA is that patients can be adequately tested for genetic defects in aneurysm genes and new genetic techniques may lead to identification of causative genes in the near future. It is important to realize that informed consent is required for DNA storage, testing and performing research. In our family history study we observed increased risk for all relatives of AAA patients, including female relatives who had a relative larger increase in risk than male relatives. The increase in risk for relatives was most prominent in the families of female AAA patients. Current guidelines on family screening show large differences and fail to address the increased risk for families of female patients appropriately.[2, 41-44] Based on the results of our study, we advise to perform family screening in all relatives over the age of 50 of all AAA patients. In this way, we hope to identify more affected AAA patients, since population screening has been shown to be less successful over the last years.[45, 46] In families with only abdominal aneurysms, an abdominal ultrasound will be the method of choice for family screening. While in families where abdominal and thoracic aneurysm co-occur, we would refer relatives at risk for CT or MRI imaging, in order to evaluate the diameter of the entire aorta. The need for re-screening of negatively screened relatives in familial AAA is still matter of debate. For now, it seems sufficient to perform re-screening in about 5 years.[45, 47] Although we understand that it is not possible to evaluate all AAA patients by a clinical geneticist in the years to come, it is important that all vascular surgeons have knowledge on this matter in order to refer patients with possible genetic defects, as in our case of a young patient with complex aortic pathology showed.[32] Currently, unconventional aneurysm morphology, positive family history and characteristic facial or musculoskeletal signs of Loeys-Dietz or Marfan syndrome are all clear indications to refer to a clinical geneticist. In addition, this study showed that a number of clinical characteristics may help distinguish familial form sporadic AAA, including young age, female gender, complex vasculopathy, no hypertension, no hyperlipideamia, and no history of smoking. These characteristics may indicate familial AAA and relatives of these patients have an increased risk for aneurysm formation. Adequate diagnosis of genetic defects is needed to optimize patient treatment and to perform counseling to their relatives at risk. As described in Part III, the observed adverse clinical outcome in patients with familial AAA suggests the need

for an altered treatment strategy and intensified post-operative surveillance after EVAR. Since no data on familial AAA and open repair is available, no recommendations can be provided for patients treated with open repair.

Taken together, evidence on a genetic origin in AAA formation is accumulating and warrants up to date knowledge for all clinicians involved in AAA care. Until new guidelines are available, we recommend altered management for all patients with abdominal aortic aneurysm, as presented in Figure 1.

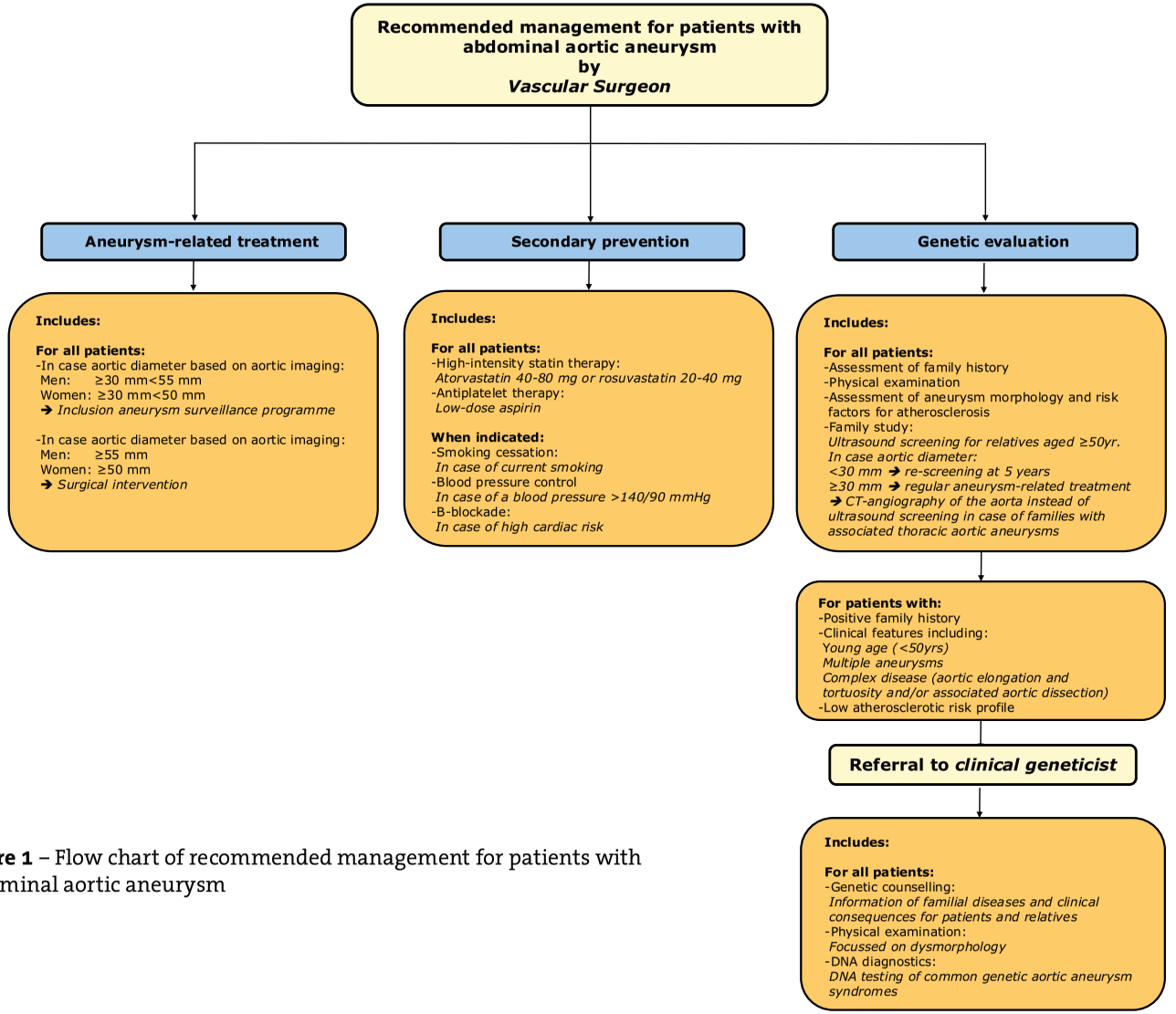


Figure 1 – Flow chart of recommended management for patients with abdominal aortic aneurysm

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Nederlandse Samenvatting

NEDERLANDSE SAMENVATTING

Het doel van dit proefschrift was om de verschillen tussen aneurysmatisch en occlusief arterieel vaatlijden aan te tonen en de klinische en genetische kenmerken van familiair abdominaal aneurysmatisch vaatlijden (AAA) te onderzoeken.

DEEL I van dit proefschrift richt zich op de klinische en genetische kenmerken van patiënten met aneurysmatisch vaatlijden in vergelijking met patiënten met occlusief arterieel vaatlijden. **Hoofdstuk 1** toonde aan dat de intima-media dikte (CIMT) van de halsslagader significant lager is bij patiënten met aneurysmatisch vaatlijden dan bij patiënten met occlusief vaatlijden. Dit geeft aan dat patiënten met aneurysmatisch vaatlijden minder atherosclerose hebben dan patiënten met occlusief vaatlijden en ondersteunt de hypothese dat andere pathologische mechanismen betrokken zijn bij vorming van aneurysma`s. **Hoofdstuk 2** liet een hoge prevalentie van vitamine D deficiëntie zien bij patiënten met arterieel vaatlijden. We toonden aan dat er een sterke associatie is tussen een lage vitamine D status en een hoge CIMT alsook een lage enkel-arm index en een hoge ultra-sensitief C-reactief proteïne, welke allen markers zijn voor de mate van ernst van arterieel vaatlijden. Deze associaties waren onafhankelijk van de traditionele cardiovasculaire risicofactoren en ongeacht occlusief of aneurysmatisch vaatlijden, en duidt op een direct effect van vitamine D deficiëntie op de vaatwand in zowel occlusief als aneurysmatisch vaatlijden. In **Hoofdstuk 3** werden de RNA expressie profielen beschreven tussen het abdominaal aorta weefsel van patiënten met aneurysmatisch en occlusief vaatlijden. We identificeerden meerdere up-regulaties in genen tussen het aneurysmatisch en occlusief aorta weefsel, waaronder de genen *COL11A1* (coderend voor componenten van type XI collageen), *APIPOQ* (geassocieerd met Kawasaki syndroom), en *LPL* (coderend voor lipoproteïnelipase), welke eerder al geassocieerd werden met aneurysmatisch vaatlijden. Tevens werden er nieuwe genen beschreven welke nog niet eerder geassocieerd werden met aneurysma's, waaronder *CXCL13*, *SLC7A5*, en *FDC-SP*, en die een up-regulatie van immuun en inflammatie signalering toonden. Het aneurysma weefsel toonde ook een simultane remming van het bot morfogenetisch proteïne en een activering van de transformerende groeifactor (TGF)-beta pathway ten opzichte van het occlusief aorta weefsel, wat suggereert dat de TGF-beta signalering ook een rol speelt bij de formatie van abdominale aneurysma's. **Hoofdstuk 4** toonde dat chronisch obstructieve longziekte (COPD) meer voorkomt bij patiënten met aneurysmatisch vaatlijden dan bij patiënten met occlusief arterieel vaatlijden. De associatie tussen COPD en aneurysmatisch vaatlijden was onafhankelijk van cardiovasculaire risicofactoren als roken en inflammatie en suggereert dat andere factoren, naast het cardiovasculaire risicoprofiel en ontsteking, bijdragen aan de associatie tussen COPD en aneurysmatisch vaatlijden. De resultaten van de experimentele studie toonden dat homozygote Fibulin-4, een extracellulaire matrix glycoproteïne, deficiënte muizen naast aneurysma vorming ook ernstig longemfyseem

ontwikkelde, terwijl heterozygote Fibulin-4 deficiënte muizen alleen alveolaire afbraak toonden bij toegenomen leeftijd en stress. Er werd geconcludeerd dat de experimentele data een genetische aanleg suggereren voor extracellulaire matrix degeneratie en secundair daaraan ontsteking als de gemeenschappelijke mechanismen in zowel COPD als aneurysmatisch vaatlijden.

In **DEEL II** van dit proefschrift werden de klinische en genetische kenmerken van familiair abdominaal aneurysmatisch vaatlijden onderzocht. In **Hoofstuk 5** beschreven we dat de "atherosclerotische last", gemeten middels de CIMT, lager was bij patiënten met familiair AAA in vergelijking met patiënten met sporadisch AAA, onafhankelijk van gemeenschappelijke atherosclerotische risicofactoren. Patiënten met familiair AAA hadden echter nog steeds een hoge CIMT, wat de hypothese ondersteunt dat atherosclerose wel aanwezig is maar niet de primair drijvende factor is in de ontwikkeling van familiair AAA. **Hoofdstuk 6** toonde de eerste genetische analyse van aneurysma genen in patiënten met familiair en sporadisch AAA. Genen geïncludeerd in de studie waren de TGF-beta pathway genen *EFEMP2*, *FBN1*, *SMAD3*, *TGFB2*, *TGFBR1*, *TGFBR2,* en de gladde spiercel genen *ACTA2*, *MYH11* en *MYLK*. Het merendeel van deze genen is geassocieerd met thoracale aneurysma`s van de aorta (TAA). In onze studie werden pathogene varianten ontdekt bij twee procent van de AAA populatie wat impliceert dat genetische testen kunnen helpen om de oorzaak van aneurysma vorming bij zowel familiair als sporadisch AAA te vinden. De data impliceert tevens dat de rol van TGF-beta in abdominale aneurysma`s wellicht groter is dan aanvankelijk werd gedacht. Het is de verwachting dat in de komende jaren nieuwe genetische testen, zoals next generation sequencing, zullen leiden tot de ontdekking van nieuwe genen en dat de kennis over genetica van het abdominaal aneurysma zal toenemen.

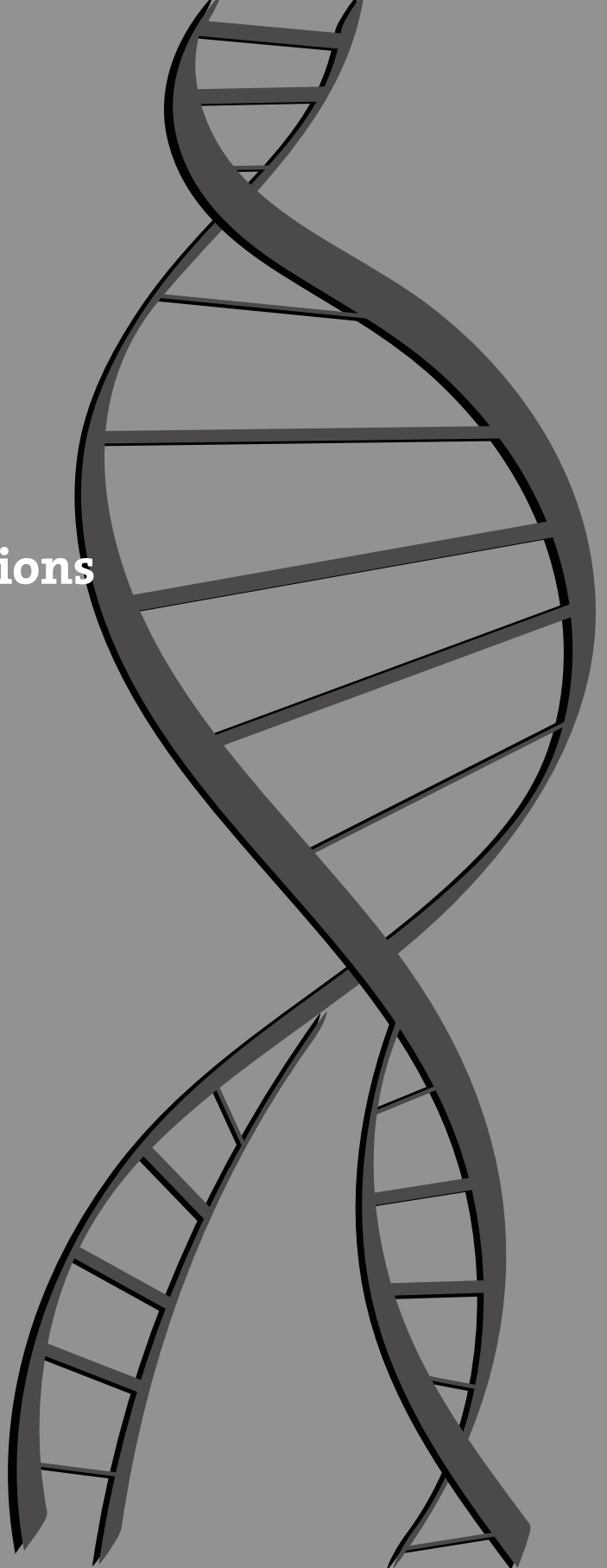
In **DEEL III** werden de klinische resultaten van patiënten met familiair abdominaal aneurysmatisch vaatlijden beschreven. In **Hoofdstuk 7** beschreven we dat patiënten met familiair AAA tweemaal zoveel aneuryma-gerelateerde complicaties na een endovasculaire procedure (EVAR) ontwikkelden dan patiënten met sporadisch AAA, ondanks vergelijkbare morfologie van het aneurysma. Secundaire interventies en groei van de aneurysmazak waren de belangrijkste oorzaken voor het verschil in complicaties. Dit verschil werd mogelijk veroorzaakt door een toename van type I endoleaks maar bereikte geen statistische significantie. Onze studie was de eerste die deze associatie beschreef en toont dat familiair AAA een subgroep vormt van de patiënten met AAA en suggereert dat deze patiënten een aangepaste follow-up regime nodig hebben. **Hoofdstuk 8** beschreef de resultaten uit een wereldwijde EVAR registry (ENGAGE) van de patiënten die behandeld werden met een late generatie stent graft. Er werd geen significant verschil in klinische uitkomst, gedefinieerd als technisch succes en vrijheid van postoperatieve complicaties zoals secundaire interventies en endoleaks, tussen beide groepen gevonden.

Patiënten met familiair AAA hadden wel een hogere aneurysma-gerelateerde mortaliteit en een trend naar meer secundaire procedures. Er kon echter geen verschil in endoleaks of groei van de aneurysmazak worden vastgesteld, wat de exacte oorzaak van het hogere complicatie risico na een EVAR procedure vooralsnog onbekend laat. Resumerend werd geconcludeerd dat familiair AAA patiënten een beperkte maar significante toename van het risico op complicaties na EVAR hebben en dat toekomstig onderzoek moet aantonen of een aangepast follow-up regime nodig is. Tot die tijd adviseren wij familiair AAA patiënten een aangepast schema aan te bieden door middel van frequentere controle.

DEEL IV van dit proefschrift is gericht op het klinische management van patiënten met familiair abdominaal aneurysmatisch vaatlijden. **Hoofdstuk 9** vat de huidige stand van familiair AAA samen en beschreef de klinische consequenties in de dagelijkse praktijk voor vaatchirurgen. De belangrijkste conclusie was dat er steeds meer bewijs bestaat over een genetische oorzaak van AAA en dat nieuwe genetische technieken waarschijnlijk zullen leiden tot de identificatie van genen die AAA veroorzaken. Er werd verder geconcludeerd dat familiegeschiedenis de beste manier blijft om patiënten met familiair AAA te identificeren totdat adequate klinische markers beschikbaar zijn. Als definitie van familiair AAA werd voorgesteld om iedere AAA patiënt met ten minste één eerstegraads familielid met een aneurysma van de aorta te classificeren als familiair AAA. **Hoofdstuk 10** beschreef het risico voor familieleden van AAA patiënten en toonde dat het risico op een abdominaal aneurysma voor zowel mannelijke en vrouwelijke familieleden veel hoger is dan het risico in de algemene bevolking. Ondanks dat mannelijke familieleden vaker waren aangedaan dan vrouwelijke familieleden was de relatieve toename in risico groter voor vrouwelijke familieleden. Tevens hebben familieleden van vrouwelijke familiair AAA patiënten een hoger risico vergeleken met die van mannelijke familiair AAA patiënten. Er werd daarom geconcludeerd dat families van de vrouwelijke AAA patiënten ook gescreend moeten worden inclusief de vrouwelijke familieleden. **Hoofdstuk 11** toonde tot slot een casus van een jonge patiënt met een Stanford type B dissectie met een reeds bestaand aorto-iliacaal aneurysma. Gezien de typische tortueuze morfologie werd genetische analyse verricht, welke een *de novo TGFBR2* mutatie toonde. Er werd geconcludeerd dat genetische analyse nodig is bij patiënten met complexe aneurysma`s met evidente tortuositeit en elongatie, ook bij afwezigheid van typische kernmerken van een genetisch aneurysma syndroom of een positieve familieanamnese.

Samenvattend neemt het bewijs voor een genetisch oorzaak van abdominale aneurysma`s toe waardoor alle artsen betrokken bij de behandeling van AAA patiënten op de hoogte moeten zijn van deze ontwikkelingen. Totdat nieuwe richtlijnen beschikbaar zijn raadden wij een alternatieve work-up voor alle AAA patiënten aan zoals gepresenteerd in Figuur 1 van de general discussion.

List of Publications



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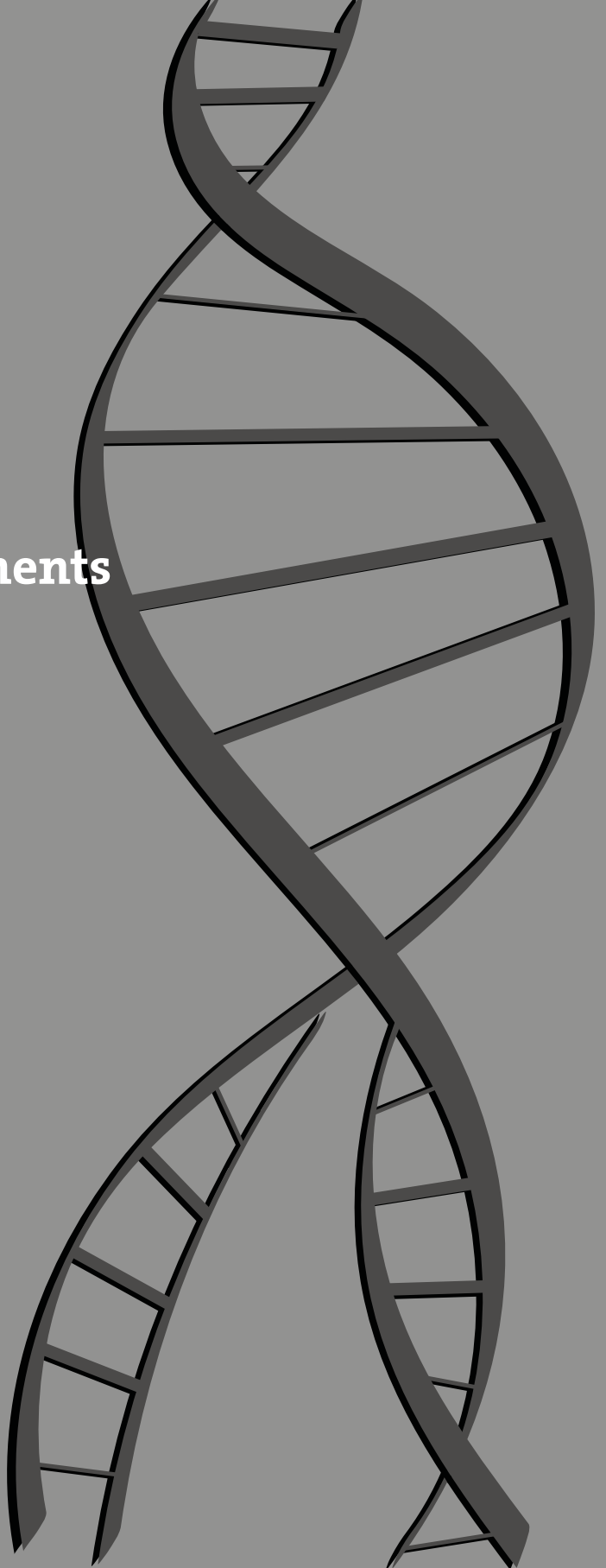

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Dr. D. Majoor-Krakauer, beste Danielle, je genetische kennis is van grote invloed geweest op mijn proefschrift. De combinatie van klinisch en genetisch onderzoek beschouw ik als zeer waardevol en hebben een wereld voor mij geopend. Jouw manier van onderzoek doen is bewonderingswaardig en ik wil je graag bedanken voor alle mooie en gezellige momenten die wij de afgelopen jaren hebben gehad!

Dr. E.V. Rouwet, beste Ellen, dank voor je hulp aan mijn proefschrift. Jouw manier van schrijven is werkelijk fantastisch en heeft mij zeer zeker geholpen bij het schrijven van dit proefschrift. Naast de wetenschappelijke bijdrage staan ook bij jou alle mooie en gezellige momenten mij zeer nader.

Dr. P.T. den Hoed, beste Ted, de woorden "Dus jij wilt chirurg worden?" *Antwoord (Koen): Eeeeh JA!* "Dan gaan we dat regelen!!" zal ik nooit vergeten. Het wetenschappelijke uitstapje duurde iets lager dan verwacht, maar na jaren van onderzoek is het proefschrift dan eindelijk af. Dank voor de fantastische opleiding die ik al jaren van je krijg!

Overige leden van de kleine en grote commissie, dank voor jullie wetenschappelijke bijdrage en kritische beoordeling van dit proefschrift.

Dr. D. Poldermans, beste Don, wat had ik graag met jou "dat ene artikel" geschreven. Helaas is het allemaal anders gelopen en we hebben de rit niet samen mogen afmaken. Desalniettemin zal ik jou wetenschappelijke drive nooit vergeten. Dank voor het vertrouwen dat je in mij had!

Dr. S.E. Hoeks, beste Sanne, dit proefschrift heeft echt alleen gemaakt kunnen worden met jouw hulp! Onze onderzoeksgroep mag zich zeer gelukkig prijzen met jou als persoon en als statisticus. Je hebt altijd tijd voor ons, voor onderzoek en anders wel voor de gezelligheid. Dank voor alles!

Mijn paranimfen, Frederico en Michiel aka *brothers in arms*. Guys, thanks for all the good memories, especially during the international conferences. Unforgettable!!

Carola, Isabel, Adrie en Virginie, dank voor alle logistieke hulp de afgelopen jaren.

Co-auteurs, graag wil ik alle co-auteurs bedanken voor hun bijdrage aan mijn wetenschappelijke artikelen. Met name Eric en Tabita, mijn trouwe kamergenoten in de kelder. Ik zal de hoogtepunten van een onderzoeksdag voor een promovendus, namelijk koffie drinken, nooit vergeten.

Gijs, het is klaar! Eindelijk, wat een mooi moment! De gedachte dat jij net zoals ik zich zo gelukkig prees met het doen van full-time wetenschappelijk onderzoek heeft me jarenlang op de been gehouden. Het feit dat we dit heugelijke moment samen mogen afmaken verguld mij met een blijdschap die niet in woorden is uit te drukken.

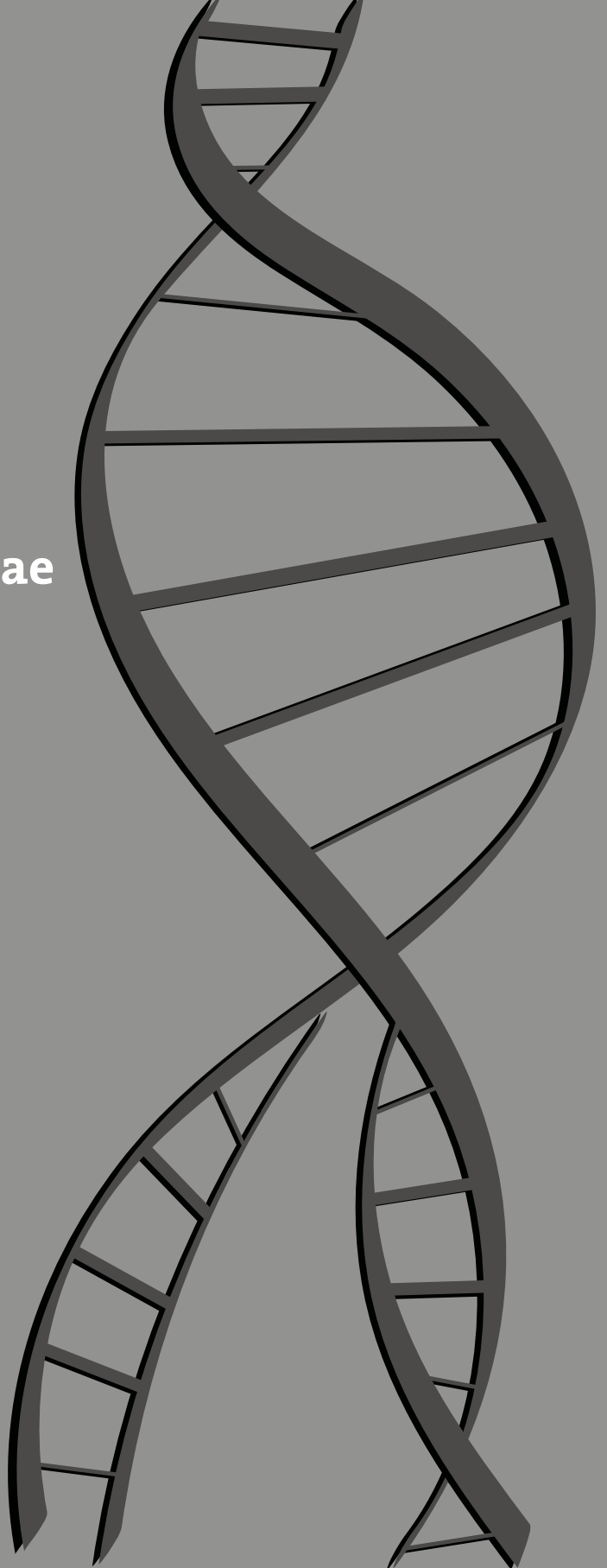
Uiteraard wil ik alle chirurgen en (oud-)arts-assistenten uit het Ikazia Ziekenhuis bedanken voor de leerzame en gezellige tijd de afgelopen jaren.

Verder wil ik alle vrienden, familie en collega`s danken voor alle interesse en steun de afgelopen tijd.

Lieve pap en mam en natuurlijk mijn lieve zus, dank voor jullie betrokkenheid en steun. Jullie zijn onmisbaar!

Tot slot, lieve Anika. Wat ben ik ongelofelijk blij met jou! Jouw steun en relativeringsvermogen, maar met name je gezelligheid hebben ervoor gezorgd dat de afgelopen jaren fantastisch waren en dat ik het boekje heb kunnen afmaken. Ik ben ervan overtuigd dat we nog vele mooie jaren voor ons hebben!

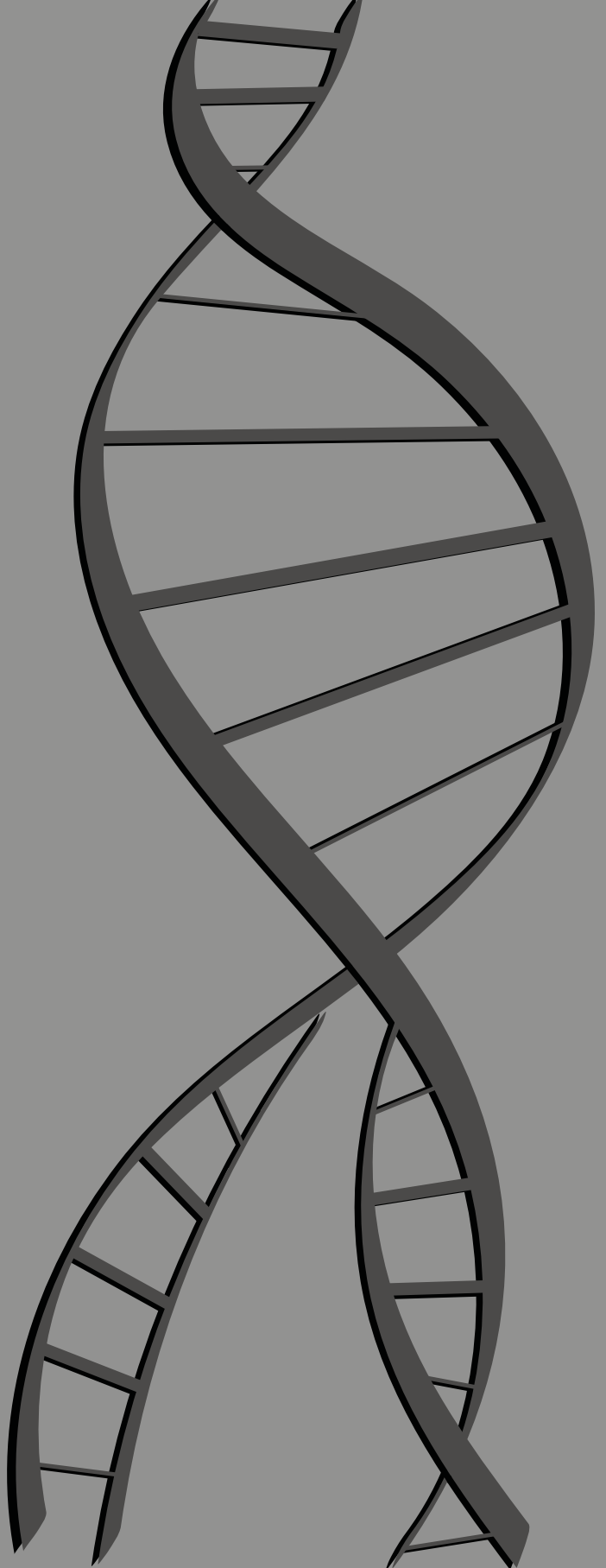
Curriculum Vitae



CURRICULUM VITAE

Koen Matthijs van de Luijtgaarden was born on the 24th of November 1982 in Hilversum, the Netherlands. He attended secondary school at the Willem de Zwijger College in Bussum from 1995 to 2001. Next he started Medicine at the Erasmus University Medical School in Rotterdam of which he graduated in 2009. During Medical School he joined the "Forgeron" student team of the Department of Emergency Medicine of the Ikazia Hospital in Rotterdam, where he became interested in the field of surgery. In 2006 he went to the Orthopeadic Surgery Hand and Upper Extremity Service for a research internship in the Massachusetts General Hospital in Boston, USA. In 2008 he visited Hospital Británnico for a clinical internship at the Department of General Surgery in Buenos Aires, Argentina. After graduating from Medical School, he started working as a senior house officer at the Ikazia Hospital under supervision of dr. P.T. den Hoed. In August of 2010 he started his research career as a PhD candidate at the Department of Vascular Surgery and Anesthesiology, under supervision of prof.dr. H.J.M. Verhagen and prof.dr. R.J. Stolker. In July 2013 he started his General Surgery residency at the Ikazia Hospital and at the Erasmus University Medical School, under supervision of dr. P.T. den Hoed and dr. B.P.L. Wijnhoven.

PhD Portfolio



PhD PORTFOLIO

Summary of PhD training and teaching activities

Name PhD student:	Koen Matthijs van de Luijtgaarden	**PhD period:**	2010-2015
Erasmus MC Department:	Vascular Surgery / Anesthesiology	**Promotors:**	Prof.dr. H.J.M. Verhagen
Research School:	Coeur		Prof.dr. R.J. Stolker

1. PhD training

	Year	ECTS
Courses and academic skills		
NIHES - Principles of Research in Medicine	2010	0.7
NIHES - Introduction to Data-analysis	2010	1.0
Coeur PhD course - Cardiovascular Pharmacology	2010	1.5
CPO - Good Clinical Practice	2011	1.5
NIHES – Regression Analysis for Clinicians	2011	1.4
MOLMED – Basic Concepts in Human Genetics	2011	0.5
MOLMED – SNP`s and Human Diseases	2011	2.0
Coeur PhD course – Atherosclerosis and Aneurysmal Disease	2012	1.5
Seminars and workshops		
Journal club and research meetings	2010-2013	1.5
Coeur PhD day	2011	0.8
Vascular rounds	2010-2015	1.5
Fundamental Endovascular Course	2012	0.5
Presentations (0.5 points/each)		
International scientific presentations	2011-2013	5.0
National scientific presentations	2011-2013	1.5
Symposia and meetings (0.3 points/day)		
International symposia and meetings	2010-2013	7.5
National symposia and meetings	2010-2015	3.6
Other activities		
Organising committee – Wound healing and Wound management	2011-2013	3.0

2. Teaching activities

	Year	ECTS
Supervising		
MSc medical students at the Erasmus Medical Center	2010-2013	1.0