Sex matters to the arteries

studies into the (epi)genetic background and clinical outcome of atherosclerotic disease in women and men

Saskia Haitjema

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Sekse doet ertoe voor slagaders

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van aderverkalking in vrouwen en mannen

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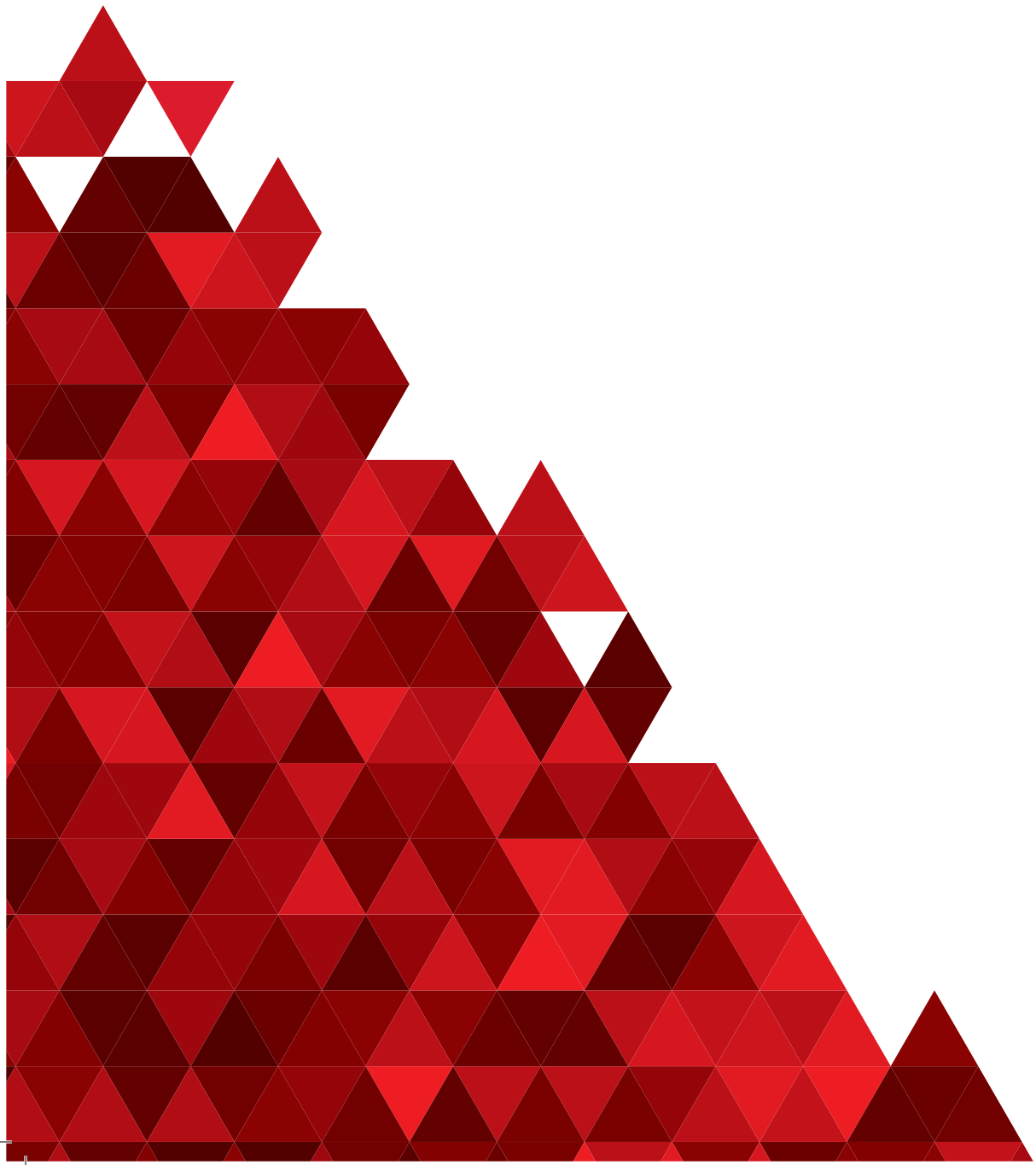

CHAPTER 1

Introduction and thesis outline

Part of this text has appeared in the editorial "Sex matters to the heart", published in Atherosclerosis (2015)
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Atherosclerosis

Atherosclerosis is a lipid-driven inflammatory disease of the arterial vessel wall[1]. It starts out during childhood, when endothelial cell damage affects mainly arterial branch points, and monocytes follow signal molecules into the subendothelial layer to help clean up lipid deposits. These "fatty streaks" are the first stage of atherosclerosis and are reversible. From the moment the inflammatory process in the arterial wall is no longer reversible, the lesion is called an atherosclerotic plaque[2]. The development and progression of the lumen-narrowing plaque takes many years. During this time, smooth muscle cells infiltrate into the plaque, forming a stabilizing cap. When inflammation progresses and a pool of dead cells gives rise to a lipid-rich necrotic core, new microvessels enter the plaque to supply oxygen to the inflammatory process. Some of them may leak and give rise to an intraplaque haemorrhage. The extracellular matrix, among others formed by collagen, may be weakened by the inflammation and by degrading enzymes, compromising the integrity of the plaque.

Atherothrombosis

Atherothrombosis can occur via two different mechanisms. The first mechanism, plaque rupture, entails cracks within the overlying fibrous cap, and exposure of the highly coagulable cellular debris of the necrotic core to the blood stream. Adherence of platelets to the debris occurs immediately, causing thrombosis. The second mechanism, plaque erosion, entails an abrasion of the endothelial layer covering the plaque, exposing the underlying extracellular matrix to the bloodstream. This alternative thrombotic process more heavily relies on the involvement of coagulation factors such as fibrin. Both mechanisms of atherothrombosis may lead to the formation of one or more emboli that block (smaller) vessels downstream of the plaque. In particular in plaque erosion, the downstream microvasculature is frequently affected by microemboli.

Cardiovascular disease

Cardiovascular diseases (CVD), more specifically atherosclerotic cardiovascular diseases, are symptoms that are caused by atherosclerosis and atherothrombosis. First of all, narrowing of the arterial lumen by atherosclerosis may cause insufficient blood supply to the organ, particularly when it needs it the most. Angina pectoris is caused by coronary atherosclerosis and oxygen deprivation of the heart and causes chest pain upon exercise, when the heart needs the most oxygen. Intermittent claudication occurs due to ilio-femoral atherosclerosis, leading to oxygen deprivation and cramping of the leg muscles while walking.

Second, atherothrombosis can lead to sudden ischemia of the downstream organ, causing immediate tissue death. This way, embolization of carotid artery thrombosis may lead to brain ischemia (stroke), and coronary artery thrombosis (myocardial infarction) to heart ischemia or even sudden death. Treatment of CVD is aimed at (1) prevention by eliminating risk factors: lowering blood lipid levels, inhibiting platelet adhesion, lowering blood pressure, lowering glucose levels and smoking cessation to prevent (further)

endothelial damage, and (2) restoring blood flow: either by opening blocked vessels through transluminal angioplasty or endarterectomy surgery or by bypass surgery. Despite these interventions, CVD compete each year with all cancers combined for the top spot in worldwide mortality and morbidity statistics[3,4]. Moreover, CVD can cause both cognitive and physical disabilities, leading to lost work years and loss of quality of life.

Genetic variation

Traditionally, characteristics of people that have the disease are compared to those of people that do not. This way, CVD risk factors such as smoking and diabetes have been identified. Moreover, a lot has been learned from people with an obvious genetic predisposition to CVD risk factors, for example patients with familial hypercholesterolemia; a disease that is caused by a mutation in one gene (a "monogenic" disease) and in virtually every patient leads to high blood lipid levels and premature atherosclerosis[5].

A relatively novel approach is to study single nucleotide genetic variation, or single-nucleotide polymorphisms (SNPs), across the genome, comparing people with and without the disease (first published by Narayanan et al.[6]). The human genome consists of three billion pairs of nucleotides where A (arginine) always pairs with T (thymine) and C (cytosine) always pairs with G (guanine). The order of these nucleotides encodes genes, or protein recipes, as it subsequently gets transcribed into RNA and translated into protein by an intricate transcription and translation machinery within the cell. However, the majority of the genome is not coding for any protein. Among others, these base pairs contain parts that regulate transcription of genes ("enhancers") or they encode parts that are only transcribed into RNA and not translated into protein (e.g. micro RNAs).

The human genome contains a lot of variation at the scale of nucleotides. It is thought that diseases where many genes influence the disease susceptibility ("polygenic diseases", such as most cases of atherosclerotic disease) are influenced by common SNPs in the genome that all exert a much weaker effect than the mutation of familial hypercholesterolemia[7]. By comparing these single nucleotide variants between people with and without the disease in so-called "genome-wide association studies (GWAS)", the complex nature of the disease can be appreciated[8]. An advantage of this approach is that it is hypothesis-free. For GWAS, large sample sizes are needed to identify the variants that are associated with the disease as they come with small effect sizes. Large initiatives have been started to gather cohorts with information on genetic variants to study atherosclerotic diseases such as coronary artery disease (CARDIoGRAMplusC4D) and stroke (METASTROKE)[9,10]. Such efforts yielded dozens of susceptibility loci with associating genetic variants throughout the genome. The biggest problem with these genetic variants is that they are spread across the genome, and thus not only within genes but also in areas without known function. It might thus not be immediately clear how the variant influences disease[11]. Indeed, few of the known loci for atherosclerotic diseases are located within a gene that has been associated with the disease.

Epigenetic causes or consequences

The human body contains multiple cell types, which look different because transcription and translation of the genome are differentially regulated. This regulation also enables the cell to react to environmental stimuli. Regulation of gene expression can occur because of folding of the DNA itself. If the DNA is tightly wound around the proteins that form a set of spools, the genetic code is not accessible for the transcription machinery and the genes cannot be read. Sometimes, the DNA needs to be bended a certain way to bring an enhancer motif in physical contact with a gene, upregulating its expression. Besides the folding, chemical groups can be attached to the DNA which alter its transcription. Probably the most studied chemical alteration of the DNA is methylation of a pair of C-G nucleotides. All these epigenetic effects regulate gene expression without changing the three billion base pairs. Epigenetic characteristics are heritable, just as the genetic code itself. However, as environmental stimuli can cause changes in epigenetic signature, one can never be sure if the observed difference between people with and without the disease is a cause or consequence. Indeed, lipid levels and diabetes mellitus are known to be associated with differences in DNA methylation status of CpG dinucleotides[12,13]. DNA methylation has also been associated with smoking, for which a causal effect may be expected by which smoking leads to DNA (de)methylation[14]. DNA methylation can be measured on a chip, just like genetic variation. Folding of the DNA, or its physical interactions require more sophisticated methods, where extraction of the regions of interest is key. Some regions that are of interest in cardiovascular disease have been investigated this way, but no systematic effort has been undertaken yet.

Sex- and gender-differences

The biological difference between XX and XY individuals is denoted as sex, whereas the social construct of an XX or XY individual within a society is considered gender. Gender-differences are relevant to study, however as the research in this thesis focuses on biology, only sex-differences will be discussed further. Women are different from men: during embryogenesis, the biological default of a homo sapiens is to become female, and the sex-determining region on the Y chromosome is needed for masculinization. These differences give rise to different hormone levels and consequently a different appearance of women and men.

Because of the fluctuating hormone levels that do not only characterize the child-bearing potential of a woman, but also influence cell metabolism and consequently physiological functioning of her body throughout the menstrual cycle, the female body can be considered a less convenient model to study health and disease. Moreover, the possibility that a woman might be pregnant and the child might be exposed to an experimental drug or procedure hampers inclusion of women in clinical trials. In addition, postmenopausal women lack these cycling hormones and represent a completely different physiological system from younger women. These phenomena have led to the extrapolation of research findings in the easy-to-study men to the fundamentally different bodies of women and the under-appreciation of sex-differences in health and disease[15].

For atherosclerotic disease, predominantly the higher incidence of the disease at a younger age in men has directed most cardiovascular research since the early 1980s towards men[16]. However, if studied, sex-differences are found in etiology, diagnostics, therapy and prognosis of CVD (Figure 1). Regarding clinical presentation, more women than men present with a myocardial infarction but non-obstructed arteries[17]. Cardiac ischemia due to coronary microvascular obstruction is more prevalent in women as compared to men, gives rise to chest pain in women, and is subject to underdiagnosis. Next to differences in the microvasculature, differences in the pathological substrate of macrovascular obstruction are also quite prominent between men and women. Women have more stable atherosclerotic plaques, and more plaque erosion as underlying cause for sudden cardiac death as compared to men in whom more vulnerable plaques and more plaque rupture are reported under similar conditions[18,19].

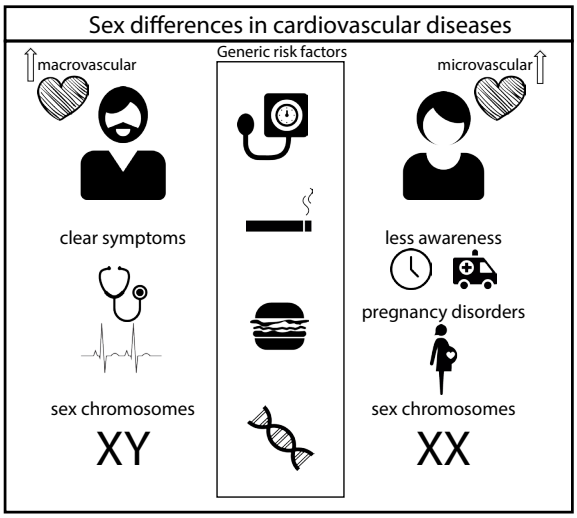

Figure 1. Schematic overview of sex differences in cardiovascular disease. Although risk factors for CVD overlap between sexes, some sex-specific risk factors have been identified such as pregnancy disorders, awareness of being at risk for CVD and the regulatory role of sex chromosomes.

It has been suggested that sex hormone status explains the differences between men and women and their progression to CVD. Indeed, estrogens in women appear to protect against CVD as loss of estrogens during and after menopause goes hand in hand with an increased cardiovascular risk. However, whether hormone replacement therapy after menopause confers cardiovascular benefit or harm remains controversial and appears to depend on time since menopause at the initiation of hormone replacement therapy[20–24]. Early menopause is an established sex-specific risk factor for CVD, alongside risk factors that are related to pregnancy, such as gestational diabetes, hypertension and preeclampsia. Some risk factors have a different effect in women compared to men. The risk factor that appears to be most sex-specific for CVD is prolonged smoking which appears to be more harmful for women than for men[25].

Sex chromosomes are beginning to be recognized as important players in sex-differences in disease development, independent of sex hormones[26,27]. The Y chromosome has mostly been excluded from the larger genome-wide association studies, due to technical difficulties (all other chromosomes come in pairs) and also due to the thought that the Y chromosome could be considered genetic wasteland[28]. Just recently this idea was contradicted as many Y chromosomal genes were found to be haplo-insufficient ("one is not enough", the other located on the X chromosome) regulatory genes, and genetic variation on the Y chromosome was associated with CVD risk factors such as blood pressure and inheritance of coronary artery disease, independent of sex hormones and aggression[29–32].

Also, the X chromosome is frequently excluded from GWAS[33]. This under-representation of X in GWAS is particularly striking as the X chromosome contains ≈5% of the human protein-coding genome. Inactivation of the X chromosome in women entails the random silencing of one of the two X chromosomes to compensate for the fact that men have only one, so-called dosage compensation. However, the mechanism of X chromosome inactivation is incomplete and flexible so it regulates gene expression between sexes, individuals and tissues[34]. The X chromosome contains information involved in inflammation, and contributes to autoimmune diseases that are highly female-specific. Indeed, it has been suggested that X-chromosome-genomic background contributes to the enhanced immune response in women[35,36].

Cardiovascular research angles

Of mice and (wo)men

CVD can be approached from many different angles. First, disease susceptibility can be studied. As atherosclerosis in humans can take a lifetime to develop, model organisms are used to study the disease development and progression. Getting these organisms to develop atherosclerotic disease is difficult, as atherosclerosis is a multifactorial process involving both lipid metabolism and inflammatory pathways against a background of endothelial damage. The most used organism to study atherosclerotic disease is the atherosclerotic mouse model. These mice are genetically modified and fed a high-fat diet to develop atherosclerosis in weeks[37]. Another way to study disease susceptibility is by using the human situation as a starting point. This can be achieved for example by using evidence from human genetics, such as GWAS results.

Standing on the shoulders of giants

A second way of studying atherosclerotic disease is looking at the atherosclerotic plaque itself. To study the cellular content of the atherosclerotic plaque, tissue specimens are needed. During endarterectomy surgery, atherosclerotic plaques are removed and can thus be studied. In a biobank, tissue characteristics are linked to patient characteristics and associations can give rise to hypotheses about the underlying biological mechanism. Fifteen years ago, on March 24th 2002, the first patient was included in the Athero-Express

Table 1. Overview of the Athero-Express cohort

	Athero-Express carotids n = 2377	**Athero-Express femorals n = 1056**
Age (mean, SD)	69.2 (9.3)	68 (9.1)
Male sex	1632/2377 (68.7)	754/1056 (71.4)
BMI (mean, SD)	26.5 (4.0)	26.1 (4.1)
GFR MDRD (mean, SD)	72.9 (21.3)	78.7 (30.6)
Current smoking (yes)	784/2311 (33.9)	404/1032(39.1)
Diabetes (yes)	558/2364 (23.6)	340/1052(32.3)
Hypertension (yes)	1722/2295 (75.0)	765/1020(75.0)
Hypercholesterolaemia (yes)	1468/2130 (68.9)	632/920 (68.7)
Inclusion diagnosis	Asymptomatic: 312/2341 (13.3) TIA: 996/2341 (42.5) Stroke: 381/2341 (27.9) Ocular: 381/2341 (16.3)	Fontaine II: 475/905 (52.5) Fontaine III: 242/905 (26.7) Fontaine IV: 188/905 (20.8)
CAD history (yes)	734/2360(31.1)	443/1050(42.2)
Stroke history (yes)	-	60/981(6.1)
PAOD (yes)	492/2360 (20.8)	-
Composite endpoint within 3 years of follow-up	537/2171 (22.6)	473/984 (48.1)

Categorical variables are presented as numbers/total (percentage). Continuous variables are presented as mean (SD).SD: standard deviation; BMI: body mass index; GFR: glomerular filtration index; MDRD: modification of diet in renal disease; TIA: transient ischemic attack; CAD: coronary artery disease; PAOD: peripheral arterial occlusive disease

Biobank Study (AE)[38]. The AE includes patients undergoing endarterectomy surgery of the carotid or ilio-femoral arteries. First patients were included in the St. Antonius Ziekenhuis Nieuwegein and University Medical Center Utrecht, from 2014 onwards only patients from the University Medical Center Utrecht are included. Atherosclerotic plaque and blood (if available) are stored for each patient. Moreover, patients are asked to fill out an extensive questionnaire, containing questions regarding family history, disease history, cardiovascular disease risk factors, atherosclerotic disease in cardiac, cerebral and femoral vascular beds, and quality of life. Follow-up information regarding secondary cardiovascular events is collected during a three-year postoperative period. To date (November 2016), 3433 patients (2377 carotid endarterectomy, 1056 femoral endarterectomy) are included in the AE (table 1). For every plaque the culprit lesion (location with the largest degree of luminal narrowing) is identified, embedded in paraffin and transversally cut using a cryomicrotome to obtain histological slides that can be stained. The rest of the plaque is processed into 5mm pieces. These pieces are snap frozen and stored in -80° to be used for protein, DNA or RNA isolation. Routine staining is performed to identify calcification, collagen, fat content, intraplaque haemorrhage, macrophages, smooth muscle cells and density of microvessels. Genotyping information is available for 1526 AE patients, for 1217 AE patients a Y chromosomal haplogroup is

available, and DNA methylation of the atherosclerotic plaque is studied in 488 AE patients. Over 100 publications feature clinical characteristics or material from the Athero-Express Biobank. It is the largest atherosclerotic plaque biobank in the world.

What matters to the patient

Studies that aim to identify CVD risk factors and disease initiation mechanisms may seem to have limited value for patients who already suffer from disease. It is therefore also important to study the outcomes that matter directly to the patient, such as mortality and quality of life measures. There are many more research angles in CVD research, but these three research angles are the ones being explored in this thesis.

Thesis outline

PART I

Models of disease susceptibility

In Part I, disease susceptibility is studied from both a murine and from a human point of view. Mice are not humans: mice display different lipid subfractions in their blood and have a different inflammatory response from humans[39]. It is therefore not surprising that the atherosclerotic plaque within atherosclerotic mouse models looks different from the human atherosclerotic plaque and that mice generally do not develop any symptoms. The question is to what extent the atherosclerotic mouse model represents human disease. In **Chapter Two** the translatability of the atherosclerotic mouse model is discussed by comparing the results of several decades of research of murine atherosclerosis with gene-based studies in humans. Instead of starting at model organisms, human atherosclerotic disease susceptibility can also be used as a starting point to study atherosclerotic disease mechanisms. The CARDIoGRAMplusC4D and METASTROKE initiatives have resulted in around 60 susceptibility loci for coronary artery disease and stroke respectively. However, when interpreting these disease susceptibility loci, annotation to the genes that exert the actual disease-associated effect can be difficult. In **Chapter Three**, a novel way of annotating human GWAS susceptibility loci is presented, namely by looking at bending of the DNA and the physical interactions it makes with susceptibility loci within regulatory regions in human cells.

PART II

Atherosclerotic plaque studies in the Athero-Express Biobank

Part II describes studies about atherosclerotic plaque characteristics in the Athero-Express Biobank. In **Chapter Four** the association of estradiol and plaque characteristics in female carotid plaques is studied, aiming at refinement of the knowledge on the association between female sex hormones and atherosclerotic disease. In **Chapter Five**, another subgroup of patients is studied: patients with diabetes mellitus, a risk factor of which the prevalence is dramatically increasing. The associations between diabetes

mellitus and plaque characteristics of ilio-femoral arteries are looked at. Furthermore, the clinical outcome in these patients is assessed, hypothesizing a dramatic effect of diabetes mellitus. **Chapter Six** deals with these same ilio-femoral arteries, and investigates a time-dependent effect in plaque composition that has been observed before in carotid artery specimens.

PART III

(Epi)genetic studies in the Athero-Express Biobank

Part III explores (epi)genetics in the Athero-Express Biobank. First, in **Chapter Seven** the association between Y chromosomal variation and characteristics of the diseased vessel wall is studied, following up on the earlier findings of Y chromosomal involvement in the indicence of CVD in literature. In **Chapter Eight**, continuing on the Y chromosome, the effects of Y chromosomal loss on clinical outcome after endarterectomy are explored, hypothesizing that part of the observed mortality increase in these patients can be attributed to cardiovascular disease. In **Chapter Nine** the differences between women and men on the level of DNA methylation in patients undergoing carotid endarterectomy are studied, scrutinizing diseased tissue for epigenetic differences that are known from healthy tissues.

PART IV

Studies on clinical outcome

In Part IV, recurrence of symptoms, mortality risk and health-related quality of life are studied in patients with atherosclerotic disease. In **Chapter Ten**, sex-differences in mortality of patients undergoing coronary artery bypass grafting surgery are studied, as preliminary evidence in literature shows a higher mortality risk in women. In **Chapter Eleven** long-term mortality risk of patients undergoing carotid and ilio-femoral endarterectomy is investigated in the Athero-Express Biobank, looking specifically at sex-differences. **Chapter Twelve** deals with another form of clinical outcome: health-related quality of life is analyzed in the Athero-Express Biobank, studying the association of cardiovascular disease burden in patients undergoing carotid and ilio-femoral endarterectomy.

The **Chapters Thirteen** and **Fourteen** contain the general discussion and Dutch summary.

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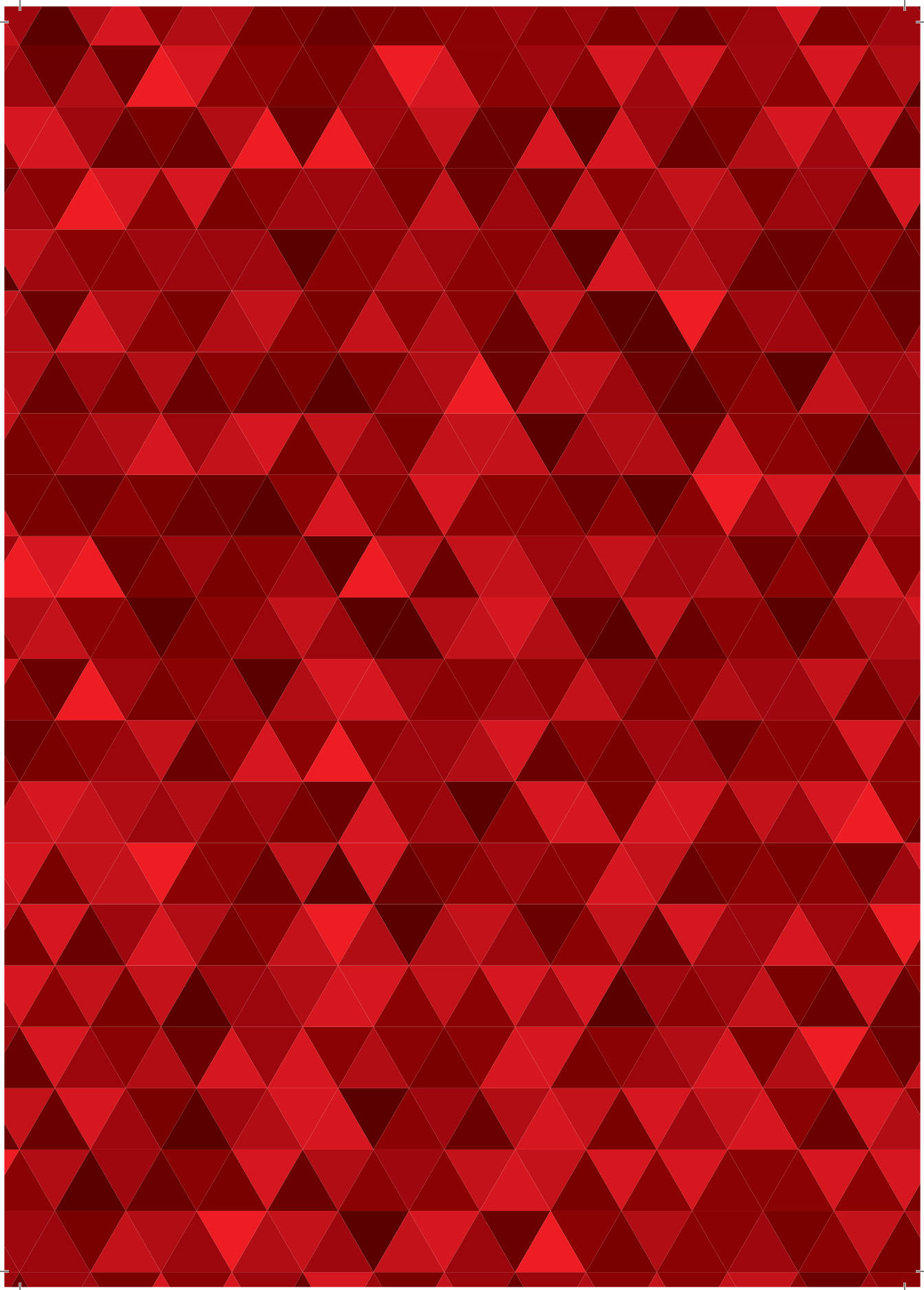
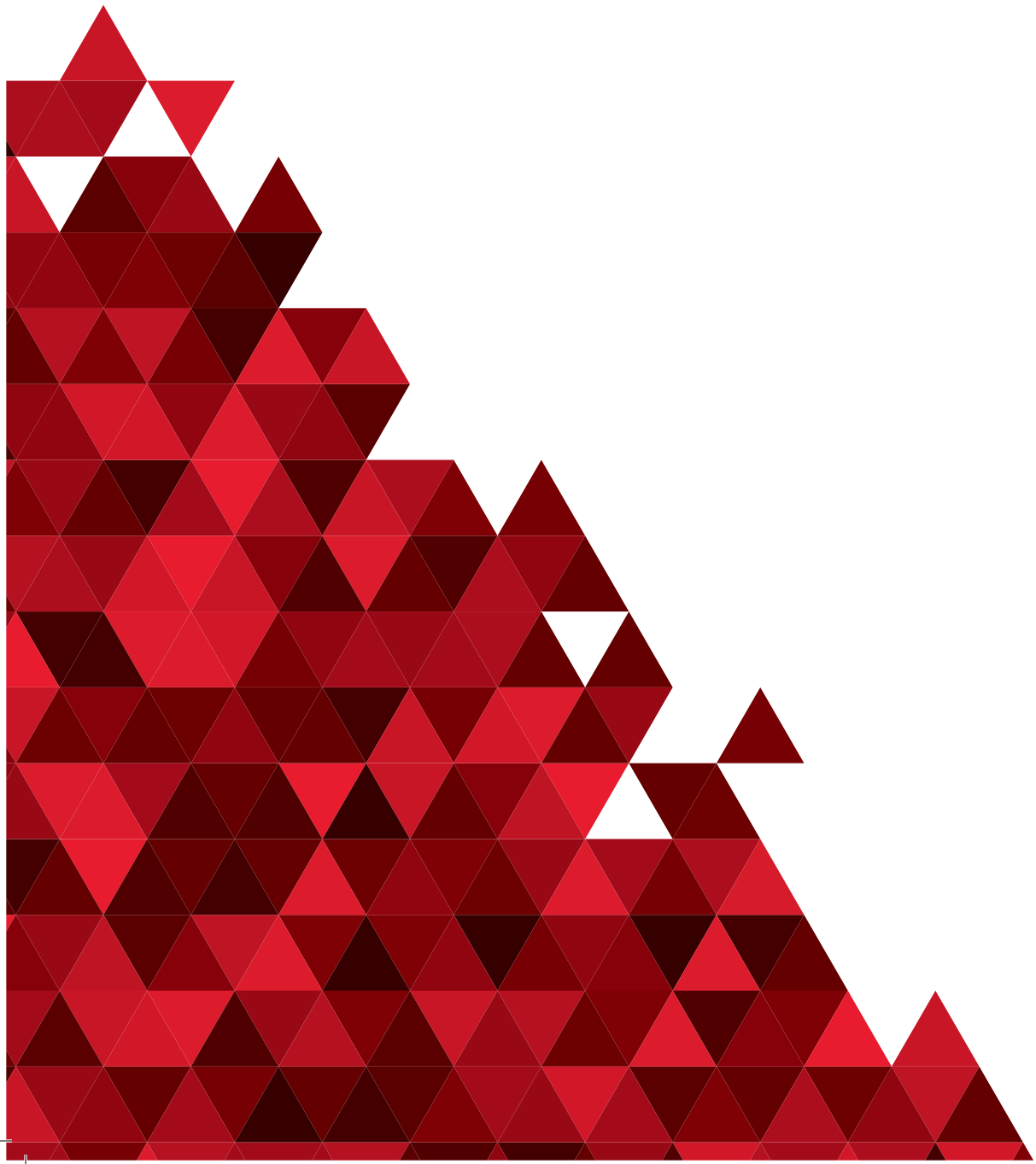


PART I

Models of atherosclerotic disease susceptibility



CHAPTER 2

Human validation of genes associated with a murine atherosclerotic phenotype

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Abstract

Background

The genetically modified mouse is the most commonly used animal model for studying the pathogenesis of atherosclerotic disease. We aimed to assess if mice atherosclerosis related genes could be validated in human disease through examination of results from genome wide association studies.

Methods and Results

We performed a systematic review to identify atherosclerosis-causing genes in mice and carried out gene-based association tests of their human orthologues for an association with human coronary artery disease (CAD) and human large artery ischemic stroke (LAS). Moreover, we investigated the association of these genes with human atherosclerotic plaque characteristics. Additionally, we assessed the presence of tissue-specific *cis*-acting expression quantitative trait loci (eQTLs) for these genes in humans. Lastly, using pathway analyses we show that the putative atherosclerosis-causing genes revealed few associations with human CAD, LAS or atherosclerotic plaque characteristics, despite the fact that the majority of these genes have *cis*-acting eQTLs.

Conclusions

A role for genes that has been observed in mice for atherosclerotic lesion development could scarcely be confirmed by studying associations of disease development with common human genetic variants. The value of murine atherosclerotic models for selection of therapeutic targets in human disease remains unclear.

Introduction

Atherosclerosis is a multifactorial process that develops over decades, underlying the majority of cardiovascular diseases. Due to its slow progression, studying the natural history of atherosclerosis requires serial examinations, thus complicating the design of studies in humans. Most research on biological mechanisms of atherosclerosis has been performed in genetically modified mice, eliminating these challenges faced in human studies. Thus genetically modified mice elegantly allow the study of atherosclerosis in, arguably, the best-controlled model system possible. The most commonly used atherosclerotic murine models are Apolipoprotein E (*ApoE*) or Low-Density Lipoprotein Receptor (*LDLR*) gene knockouts. These mice clearly display an accelerated atherosclerotic phenotype with human-like vascular lesions[1,2]. Experimental modifications of these murine models may accelerate vascular plaque development resulting in advanced lesions within several weeks[3,4]. Consequently such models have been crucial in understanding the murine molecular and cellular basis of atherosclerosis. Yet, the relevance in human atherosclerotic disease remains elusive for a number of reasons. Primarily, the morphology of atherosclerotic plaque in mice differs from that of humans and acute events due to luminal thrombosis and evident plaque rupture are rarely observed. Secondly, clinical disease manifestations such as coronary artery disease (CAD) or ischemic stroke in mice are rare or lacking. Thirdly, it is arguable whether complete knockouts in mice correspond with expression-changing mutations in humans. Lastly, despite the knockout of individual genes, genetic redundancy on the pathway level further complicates the interpretation of results from animal models[5].

In recent years, millions of common single-nucleotide polymorphisms (SNPs) in the human genome were identified, and our understanding of these variants with respect to the genomic architecture has increased significantly[6]. This has opened up the possibility to agnostically assess the effects of genome-wide variation on human traits and disease[7]. Indeed, meta-analyses of human genome-wide association studies (GWAS) have identified many risk loci for CAD[8] and large artery ischemic stroke (LAS)[9]. These GWAS provide the unique opportunity to validate the putative disease-causing genes identified through murine models in humans.

We performed a systematic review to identify atherosclerosis-causing genes in mice and carried out gene-based association tests of their human orthologues for CAD and LAS. Moreover, we investigated the association of these genes with human atherosclerotic plaque characteristics. Furthermore, we assessed whether there are tissue-specific *cis*-acting expression quantitative trait loci (eQTLs) for the genes in humans. We report that putative atherosclerosis-causing genes reveal little association with human CAD, LAS or atherosclerotic plaque characteristics, despite the fact that the majority of these genes have *cis*-acting eQTLs.

Materials and Methods

Literature search

A systematic search was performed at PubMed MEDLINE (http://www.ncbi.nlm.nih.gov) and Embase (http://www.embase.com) until July 1st 2014 using the key words (or synonyms and thereof): "ApoE$^{-/-}$", "LDLR$^{-/-}$, "atherosclerosis", "plaque", and "mice". After removal of duplicates we identified 11,219 publications (figure SI).

Based on title and abstract, publications were manually selected that met the following criteria:

- Murine knockout model on an atherosclerotic background (either ApoE$^{-/-}$ or LDLR$^{-/-}$) that resulted in altered atherosclerotic plaque characteristics or altered plaque volume.
- Murine transgenic model on atherosclerotic background that resulted in altered atherosclerotic plaque characteristics or altered plaque volume
- Murine model on atherosclerotic background with a targeted intervention that resulted in altered plaque characteristics or plaque volume, either treated with a chemical compound or a biological entity (e.g. an antibody, hormone, siRNA or morpholino) that is recognized for protein or gene specificity.

Gene selection for human extrapolation

In total 2,076 papers met our predefined criteria (figure SI) and we distilled 703 unique murine genes (table SI). Subsequently these genes were mapped to their human orthologues using an automatic script together with the search function of *GeneCards* (http://genecards.org). Results were manually checked for accuracy.

When a single murine gene mapped to multiple human orthologous genes, all human orthologues were included. We grouped each gene into one of three hierarchical categories (knockout, transgenic or compound) if any of the associated articles reported the gene in a knockout model, a transgenic model, or as a specific target of a chemical compound or biological entity (table SI). We excluded genes that could not be mapped by the gene-based association analysis software (n=21) or mapped to the X-chromosome genes (n=23). A total of 659 murine genes could be mapped to a human orthologue on genome build 36 and was thus available for downstream analyses (table SI).

Athero-Express Biobank Study: plaque collection and phenotyping

The details of the study-design and the plaque phenotyping have been described elsewhere[10]. In short, carotid plaque specimens were obtained from carotid endarterectomy (CEA) patients during surgery. Plaques were immediately processed in the laboratory, where the culprit lesion with a length of 5 mm was fixed in 4% formaldehyde, subsequently followed by decalcification and embedding in paraffin. Cross-sections (5 μm) were sliced and routinely stained for different characteristics: atheroma size (based on interpretation of hematoxylin and eosin (HE), elastica von Gieson and collagen staining (picrosirius red)), macrophages (CD68), smooth muscle cells (α smooth muscle actin (SMA)), collagen, calcification (assessed using HE), intraplaque

haemorrhage (HE and fibrin), and intraplaque vessel density (CD34)[11]. Collagen and calcified regions were semi-quantitatively scored as absent/minor vs. moderate/ heavy staining. Atheroma size was semi-quantitatively analyzed as <40% vs. >40% intraplaque fat content. CD68 and SMA were visualized with DAB (3,3'-diaminobenzidine), and were quantitatively analyzed using AnalySIS 3.2 software (Soft Imaging Systems GmbH, Münster, Germany) and expressed as % of plaque area. Likewise CD34 was visualized using DAB and the number of vessels per 3-4 hotspots per plaque was determined. Intraplaque haemorrhage was semi-quantitatively scored as no vs. yes.

Genome-wide association study summary statistics of CAD and LAS

To test the association of genes with atherosclerotic disease, we obtained summary statistics from GWAS on the traits of interest as follows. Data for CAD were downloaded from the CARDIoGRAMplusC4D website (http://www.cardiogramplusc4d.org). These data are the results from CARDIoGRAM, a meta-analysis of 14 GWAS on CAD comprising of 22,233 cases and 64,762 controls of European descent[8]. Data for LAS were obtained from METASTROKE[9] (http://www.strokegenetics.com/members-area/meta-stroke), a meta-analysis of data from 15 ischemic stroke cohorts with a total of 12,389 cases and 62,004 controls, all of European ancestry. In METASTROKE 2,167 cases were determined to be of the "large artery stroke" subtype according to the TOAST classification system[12], with matching 49,159 controls. More details on genotyping, imputation, and study inclusion of the CARDIoGRAM and METASTROKE meta-analyses of GWAS can be found in the respective publications[8,9].

Athero-Express Biobank Study: genotyping

The targeted SNP based analyses *in-silico* analyses were performed using data from two imputed genome-wide genotyping experiments carried out in 1,858 consecutive patients from the AE. For these experiments DNA was extracted from blood or plaque samples (when no blood was available) following standardized in-house validated protocols. The first dataset (Athero-Express Genomics Study 1, AEGS1) was genotyped using Affymetrix Genome-Wide Human SNP Array 5.0, the second dataset (Athero-Express Genomics Study 2, AEGS2) was genotyped using the Affymetrix Axiom® GW CEU 1 Array. We adhered to community standard quality control and assurance (QCA) procedures to clean the whole-genome data obtained in AEGS1 and AEGS2[13]. We used the HapMap 2 CEU release encompassing over 2.5 million SNPs as the reference panel for imputation for autosomal missing genotypes in the 1,443 individuals that passed the QC. Depending on the phenotype we applied linear or logistic regression models adjusting for age, sex, year of surgery, chip-type, and 10 principal components. We assumed an additive genetic model. We obtained summary level data from targeted SNP-based analyses (focused on SNPs in and around [$\pm$50kb] the 659 genes) on 7 plaque characteristics in the Athero-Express Biobank Study (AE). The local ethical committee approved the study and all patients gave written informed consent after the nature and possible consequences of the study were explained.

eQTL of human orthologues of selected murine genes

There is no human analogue for murine genetically modified models, other than naturally occurring genetic variation that affects gene expression (known as expression quantitative trait locus, eQTL). Therefore, we identified eQTLs through two approaches. First, we queried three online resources (table SVI) for eQTLs in lymphoblastoid cells[14,15], monocytes[16], subcutaneous adipose tissue[14], and skin tissue[14]. Secondly, we performed eQTL analyses in 7 tissue types that could be relevant for disease development in the STAGE study[17].

The Stockholm Atherosclerosis Gene Expression (STAGE) Study

In the STAGE study, seven vascular and metabolic tissues of well-characterized coronary artery disease (CAD) patients were sampled during coronary artery bypass grafting (CABG)[17]. The samples from atherosclerotic arterial wall (AAW), internal mammary artery (IMA), liver, skeletal muscle (SM), subcutaneous fat (SF), visceral fat (VF), and fasting whole blood (WB), were obtained during CABG for DNA and RNA isolation. Patients were included if they were eligible for CABG and had no other severe systemic diseases (e.g. widespread cancer or active systemic inflammatory disease).

In order to prepare inferred genotypes for STAGE for genotype imputation, SNPs were quality controlled for minor allele frequency MAF < 5%, Hardy-Weinberg equilibrium (HWE) p-value < $1x10^{-6}$, and call rate of 100%. Thereafter, genotypes for the STAGE study were imputed using IMPUTE2 using 1000 Genomes EUR as the reference[18]. Quality control measures for imputed genotypes used an additional filter of IMPUTE2 INFO score of < 0.3. This yielded a total of 5,473,585 SNPs. The Ethical committee of the Karolinska Hospital approved the study, and all patients gave written informed consent after the nature and possible consequences of the study were explained.

As previously described[17], an expression trait was tested for association with each genotyped and imputed SNP using Kruskal-Wallis test and false discovery rate to correct for multiple testing. First, all *cis*-pairs of SNPs within 50kb of the transcription start or end site for each gene were identified. Next, *cis* SNP-gene pairs were tested for association in all seven STAGE tissues using kruX[19]. The p-value for eQTL inclusion in kruX was set at 0.05. Finally, an empirical FDR estimate for each eQTL-gene pair was calculated using ten permutations by shuffling patient IDs on genotype data. As a result, the most significant eQTL-gene association in each tissue was reported.

Gene based association study of target genes

We used "a versatile gene-based association study" (VEGAS)[20] to calculate gene-based association statistics from the summary statistics of each target gene for each trait. The details of the methods applied by VEGAS have been described elsewhere[20]. In short, SNPs are mapped to the gene (in and around ±50kb from 5' and 3' gene borders), and using the GWAS p-value a gene-based test statistic is calculated corrected for the underlying population linkage disequilibrium structure (based on HapMap 2 CEU). Finally using simulations an empirical gene-based *p*-value of association with the phenotype is calculated per gene[20].

Pathway analysis

The 659 genes were analyzed through the use of QIAGEN's Ingenuity Pathway Analysis (IPA, 2014 winter version, QIAGEN Redwood City, www.qiagen.com/ingenuity)[21]. We used IPA to identify canonical biological pathways within the Ingenuity Knowledge Base to which the murine gene targets were mapped. Only direct relationships that were experimentally observed in humans were included in the analysis.

Statistical analyses

This study focused on 659 human orthologous genes for the atherosclerotic murine genes and thus, in our gene-based analyses of the atherosclerotic disease traits (CAD, LAS, and the 7 plaque characteristics), we corrected conservatively for multiple testing ($p\text{-value}_{gene} = 0.05/659 = 7.59 \times 10^{-5}$). However, to allow optimal description of relevant biological pathways (in IPA) with the trait we also report data using a nominal p-value < 0.05. In the public eQTL sources there is no information on the number of SNPs mapped in and around ±50kb of the genes, we only were able to obtain the total number of eQTLs. STAGE uses 1000G imputed data and thus has denser SNP coverage per gene. In STAGE we mapped a total of 68,402 independent common SNPs in and around ±50kb of the genes for the *cis*-eQTL analysis. Therefore based on these numbers, we conservatively set the threshold at $p\text{-value}_{eQTL} = 0.05/68{,}402 = 7.31 \times 10^{-7}$. We queried the 4 eQTL studies and noted the total number of nominally associated (p<0.05) eQTLs (within the ±50kb range) with the target gene expression. We also report the most significantly associated eQTL for each gene. The three mouse model groups (knock-out, transgenic and compound) were compared using chi-square tests.

Results

Of the 659 murine genes (table SI) a total of 486 genes (73.75%) were studied in knockout mice, 57 genes (8.65%) were studied in transgenic mice, and 116 genes (17.60%) were targeted by specific compounds (table SI). For 185 genes (28.07%) there are (pre-clinical) drugs available (table SI).

Validation within GWAS of CAD and LAS

We obtained summary statistics from GWAS on CAD[8] and large artery stroke[9] for SNPs ±50kb from the 5' and 3' gene borders of the 659 studied murine genes (Supplemental Material). We used these as input for a gene-based analysis using VEGAS which assigned an empirical p-value (after permutations) to each of the 659 genes based on the p-value of the SNPs in and ±50 kb around the genes of interest[20]. Thus, each gene was given a p-value of association to CAD or LAS based on the GWAS results while taking into account the correlation between SNPs that may exist. Out of the 659 studied genes that have been shown to affect atherosclerotic phenotypes in mice, 11 (1.7%) genes were associated with CAD after correction for multiple testing (p-value ≤ 0.05/659

≤ 7.59x10^{-5}, table SIII). In contrast, none of the genes were associated with LAS after correction for multiple testing. The top 10 most significant genes for CAD and LAS are shown in table 1. A total of 84 (12.7%) and 41 (6.2%) of the genes were associated with CAD and LAS respectively, at a nominal p-value ≤ 0.05. The overlap of associated genes between LAS and CAD is limited; only the locus at 9p21 (containing *CDKN2A/B*), significantly associated with CAD, was also nominally associated with LAS (p-value < 0.0062). We did not observe any further overlap between CAD and LAS top-associated genes. When looking at model groups to which the genes were assigned (knockout, transgenic or compound), we did not observe significant differences between groups for the 11 significant genes (p-value = 0.513 using a Chi^2 test).

Validation using human atherosclerotic plaque

Subsequently, we conducted a similar gene-based analyses using VEGAS on seven plaque characteristics in the Athero-Express Biobank Study[10]. These human plaque characteristics have previously been associated with clinical presentation[22] and have been examined in many atherosclerotic murine models. Overall, out of the 657 genes a low number of genes nominally associated with a human plaque characteristic and followed the expectation under the null (range 4.1%-6.1%, table SV). Only two genes were significantly associated with human intraplaque macrophages, *F10* on chromosome 13 (p-value = 1.00x10^{-6}), and *TNFAIP8L2* on chromosome 1 (p-value = 5.80x10^{-5}) after correction of multiple testing. All gene-based association results for the 659 target genes with plaque characteristics are provided in table SVI.

Validation using pathway analyses

The genes that reached nominal significance in the gene-based analysis of CAD and LAS, were further analyzed using Ingenuity to identify canonical pathways associated with CAD and LAS (table SIV). Table 2 provides the 25 most significant canonical pathways based on the 659 murine target genes and the translation of these genes to human CAD and LAS. The LXR/RXR activation pathway (involved in lipid metabolism) that has been extensively studied in atherosclerotic mice, was found to be significantly enriched for genes associated with CAD and LAS. In contrast, the NFκB inflammatory signaling pathway that has been extensively studied in murine models, revealed less genes that associated with CAD or LAS (table 2). Another example is the T-lymphocyte differentiation pathway that has been extensively studied in mice and associated with murine atherosclerosis, but for which we found little supportive evidence in our gene-based analysis associating with human CAD or LAS.

eQTL analysis of the murine genes

In murine models the 659 genes are clearly affected through knockout, transgenic techniques or targeted compound treatment. A close human analogue of such an effect would be *cis*-acting common genetic variants affecting gene expression through reducing or upregulating expression. Such variants are known as expression quantitative

Table 1. Top 10 gene-based association results of the 659 target genes for CAD and LAS

Gene	Chr	Start	End	CARDIoGRAM					METASTROKE				
				n SNPs	P Value	FDR	Best SNP	P Best SNP	n SNPs	P Value	FDR	Best SNP	P Best SNP
CDKN2A	9	21.957.750	21.984.490	100	$<2.00\times10^{-6}$*	$<2.00\times10^{-6}$*	rs10738604	2.27×10^{-16}	120	0.006†	0.380	rs634537	1.05×10^{-4}
CDKN2B	9	21.992.901	21.999.312	92	$<2.00\times10^{-6}$*	$<2.00\times10^{-6}$*	rs10738604	2.27×10^{-16}	107	0.003†	0.349	rs634537	1.05×10^{-4}
MRAS	3	139.549.314	139.607.067	81	$<2.00\times10^{-6}$*	$<2.00\times10^{-6}$*	rs2306374	3.34×10^{-8}	93	0.637	0.911	rs10513053	0.026
APOA5	11	116.165.295	116.167.794	77	2.00×10^{-6}*	3.29×10^{-4}*	rs964184	8.02×10^{-10}	114	0.533	0.891	rs5110	9.21×10^{-3}
LPL	8	19.840.861	19.869.050	177	5.00×10^{-6}*	6.57×10^{-4}*	rs3779788	2.40×10^{-7}	199	0.079	0.753	rs2410616	4.34×10^{-3}
TGFB1	19	46.528.490	46.551.656	54	7.00×10^{-6}*	7.67×10^{-4}*	rs12327659	3.93×10^{-6}	77	0.095	0.753	rs2217656	8.39×10^{-4}
LDLR	19	11.061.056	11.105.505	101	9.00×10^{-6}*	8.45×10^{-4}*	rs1122608	9.73×10^{-10}	119	0.044	0.749	rs1799898	3.14×10^{-3}
LIPA	10	90.963.305	91.001.640	154	1.30×10^{-5}*	1.07×10^{-3}*	rs7922269	7.56×10^{-6}	174	0.079	0.753	rs4933497	5.03×10^{-3}
GIP	17	44.390.916	44.400.954	59	1.50×10^{-5}*	1.10×10^{-3}*	rs46522	3.57×10^{-6}	69	0.121	0.794	rs4794015	0.020
APOA4	11	116.196.627	116.199.221	65	1.70×10^{-5}*	1.12×10^{-3}*	rs964184	8.02×10^{-10}	101	0.353	0.891	rs5110	9.21×10^{-3}
IL25	14	22.911.857	22.915.452	88	0.046†	0.370	rs10143597	2.75×10^{-3}	116	0.001†	0.171	rs12894524	4.52×10^{-5}
MMP12	11	102.238.673	102.250.922	114	0.055	0.408	rs7124926	4.25×10^{-3}	132	0.001†	0.171	rs660599	4.74×10^{-5}
MMP3	11	102.211.737	102.219.552	135	0.079	0.454	rs2408489	5.72×10^{-3}	158	0.001†	0.171	rs475007	4.05×10^{-5}
MAPK7	17	19.221.658	19.227.445	27	0.119	0.509	rs739830	1.53×10^{-3}	46	0.006†	0.380	rs1110467	1.27×10^{-4}
MMP9	20	44.070.953	44.078.607	82	0.126	0.509	rs7270354	1.94×10^{-4}	95	0.005†	0.380	rs17448653	1.06×10^{-4}
ALOX15B	17	7.883.082	7.893.176	90	0.202	0.635	rs4792214	6.92×10^{-3}	111	0.006†	0.380	rs4792203	9.36×10^{-5}
CD40	20	44.180.312	44.191.791	123	0.253	0.689	rs1321003	8.17×10^{-3}	142	0.000†	0.091	rs4239702	1.41×10^{-5}
TGFB3	14	75.494.194	75.517.845	67	0.902	0.965	rs8008060	0.090	82	0.000†	0.120	rs3917187	4.16×10^{-5}

CARDIoGRAM: meta-analysis of genome-wide association studies (GWAS) of CAD. METASTROKE: meta-analysis of GWAS of LAS. Results are given per gene with its chromosomal (chr) start and end base pair position. n single-nucleotide polymorphisms (SNPs): number of SNPs studied for that gene. Also given are the most significant (best) SNP for that gene and its P value. CAD indicates coronary artery disease; and LAS, large artery ischemic stroke.

* indicates P values for genes with Bonferroni corrected $P_{gene} \leq 7.59\times10^{-5}$, and † for genes with nominal $P<0.05$.

Table 2. Top 25 Canonical pathways for murine target genes

Rank	Ingenuity Canonical Pathways	Overlap Pathways (%)	CARDIoGRAM		METASTROKE		Murine Genes	
			n Genes P≤0.05	P Value	n Genes P≤0.05	P Value	n Genes P≤0.05	n Genes P>0.05
1	LXR/RXR activation	59/121 (49)	16	1.84×10^{-8}	5	0.098	19	40
2	FXR/RXR activation	50/125 (40)	12	4.22×10^{-6}	1	0.202	13	37
3	PPARα/RXRα activation	40/165 (24)	10	1.78×10^{-5}	2	0.278	12	28
4	Clathrin-mediated endocytosis signaling	27/184 (15)	8	3.27×10^{-5}	4	0.034	10	17
5	Acute phase response signaling	53/168 (32)	11	4.32×10^{-5}	2	0.252	13	40
6	Glioma invasiveness signaling	11/57 (19)	5	1.06×10^{-4}	1	0.329	6	5
7	Atherosclerosis signaling	58/120 (48)	11	1.17×10^{-4}	8	6.33×10^{-3}	17	42
8	Oncostatin M signaling	13/34 (38)	5	2.67×10^{-4}	3	0.021	7	6
9	IL-12 signaling and production in macrophages	47/131 (36)	9	3.79×10^{-4}	4	0.123	12	35
10	TR/RXR activation	21/85 (25)	6	3.93×10^{-4}	1	0.376	6	15
11	Hepatic fibrosis/hepatic stellate cell activation	70/196 (36)	11	5.12×10^{-4}	6	0.077	15	55
12	Adipogenesis pathway	22/124 (18)	6	5.13×10^{-4}	2	0.207	7	15
13	Cellular effects of Sildenafil (Viagra)	9/124 (7)	4	6.09×10^{-4}	0	0.630	4	5
14	VEGF signaling	23/89 (26)	6	6.59×10^{-4}	0	0.307	6	17
15	Hepatic cholestasis	51/158 (32)	9	6.89×10^{-4}	3	0.222	11	40
16	Production of nitric oxide and reactive oxygen species in macrophages	42/179 (23)	8	8.06×10^{-4}	3	0.194	10	32
17	p70S6K Signaling	18/118 (15)	5	1.37×10^{-3}	1	0.376	6	12
18	Relaxin signaling	19/132 (14)	5	1.77×10^{-3}	1	0.377	6	13
19	LPS/IL-1–mediated inhibition of RXR function	38/208 (18)	7	2.01×10^{-3}	2	0.277	9	29
20	Glioma signaling	20/94 (21)	5	2.24×10^{-3}	2	0.189	5	15
21	Nitric oxide signaling in the cardiovascular system	20/95 (21)	5	2.24×10^{-3}	1	0.377	6	14
22	Endothelin-1 signaling	30/167 (18)	6	2.71×10^{-3}	3	0.127	8	22
23	VEGF family ligand–receptor interactions	21/76 (28)	5	2.80×10^{-3}	0	0.341	5	16
24	eNOS signaling	21/135 (16)	5	2.80×10^{-3}	1	0.376	6	15
25	Renal cell carcinoma signaling	13/69 (19)	4	2.82×10^{-3}	0	0.513	4	9

For each ingenuity canonical pathway, the number and percentage of murine genes overlapping with the total number of pathways was determined (Overlap). For coronary artery disease (CARDIoGRAM) and large artery stroke (METASTROKE) the number of murine genes reaching $P\leq0.05$ (n genes $P\leq0.05$) for association with the respective disease is given. *P* indicates the binomial *P* value and represents the proportion of murine genes associated with the disease compare with the total number of murine genes. For all murine genes the total n genes $P\leq0.05$ and n genes $P>0.05$ for association with either disease is given. eNOS indicates endothelial nitric oxide synthase; IL, interleukin; LPS, lipopolysaccharide; LXR, liver X receptor; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; TR, thyroid hormone receptor; and VEGF, vascular endothelial growth factor.

trait loci (eQTLs). We conducted *cis*-eQTL analyses using the STAGE study and queried three online public resources (table SVII) to identify common variants modulating the expression of the 659 genes in humans. Across the four datasets and 11 cell/tissue types we found eQTLs (p-value $\leq 7.31 \times 10^{-7}$ after correction for 68,402 variants in and around these genes) for 411 out of the 659 genes (these genes are marked orange in table SVIII). For all genes we found SNPs in *cis* affecting expression (p-value < 0.05) in any of the four datasets queried (table SVIII). In a representative example of a canonical pathway (NFκB) we show that each target gene has a cis-eQTL, i.e. a common variant (SNP) in or around the gene that has a significant effect on tissue-specific gene expression in humans.

Not all genes will exert their effect on clinical outcome via gene expression, rather gene function. Thus, we compared the gene-based association results for CAD and LAS for the genes with a valid eQTL ($p\text{-value}_{eQTL} < 7.31 \times 10^{-7}$) and without ($p\text{-value}_{eQTL} \geq 7.31 \times 10^{-7}$), but found no significant difference in enrichment for disease association in either CAD (p-value = 0.882) or LAS (p-value = 0.634). Similarly, we compared the gene-based association results between the model groups for the 84 and 42 genes that associated with CAD and LAS (knockout, transgenic, or compounds) and found a statistical difference between groups for CAD (p-value = 0.0013, $Chi^2 = 13.291$). A pairwise comparison of the three model groups revealed that knockout and compounds do not have statistically different gene-based results (p-value = 0.718), whereas the results between knockouts and transgenic mice (p-value = 0.00049, $Chi^2 = 12.146$) or transgenic mice and compounds do (p-value = 0.0029, $Chi^2 = 8.852$). We found no statistical difference for LAS (p-value = 0.637) when comparing the three model groups. We also stratified our pathway analyses based on these three model groups, but this revealed no additional significant pathways.

Discussion

This study shows that putative atherosclerosis-causing genes identified in murine atherosclerosis models, reveal little association with human CAD, LAS or atherosclerotic plaque characteristics, despite the fact that the majority of these genes have *cis*-acting eQTLs.

Overall, the majority of genes associated with an atherosclerotic phenotype in mice do not carry variants that associate with human CAD, LAS or advanced plaque characteristics. In contrast, murine genes involved in lipid metabolism significantly associated with human CAD which is consistent with the known role of lipids in CVD risk. Indeed, lipid-lowering drugs, such as statins, act through *HMGCR* (chromosome 5q13.3) to lower circulating lipids[23]. A recent GWAS showing that variants in the *HMGCR* locus are associated with an increase of 2.84 mg/dL total cholesterol[24], effectively confirmed this drug action retrospectively. However, for most murine genes and pathways of innate and adaptive immunity, there was no association with human CAD, LAS or plaque characteristics.

ApoE$^{-/-}$ and LDLR$^{-/-}$ models are widely used to study the initiation and progression of atherosclerosis. To study the effect on plaque development[25] or therapeutic strategies[26], additional (double) knockout or transgenic atherosclerotic models have been developed. Yet, they lack plaque rupture thrombosis and subsequent cardiac or cerebral ischemia. Although therapeutic strategies have been developed based on animal models, failures in clinical utilization underscores the need for human verification and translation before initiating targeted drug development programs[27,28]. Indeed, a *post hoc* analysis by deCODE genetics and Amgen assessed the validity of results from human genetic studies as positive predictors of successful clinical trials[29]. Essentially all failed clinical trials targeting a gene (locus) lack any evidence from genetic association studies[29].

There are several potential explanations for the observed discrepancies of genes involved in atherosclerotic murine studies and human cardiovascular disease. First, they may be explained by differences in effect sizes. Common genetic variants associated with human disease often have a modest effect, in contrast to experimental gene manipulation (i.e. knockout) in animal models to study atherosclerotic disease. These genetic modifications in mice limit inferences regarding dose dependent effects which is relevant for predicting drug effects in human disease. Furthermore, the combined effect of human population history and selection may have yielded very little functional genetic variation and thus no association with CAD or LAS, even in a large sample. The low number of murine genes associated with plaque characteristics in the Athero-Express study may be explained by limited sample size and therefore should be interpreted with caution. However, for our gene-based analysis of CAD and LAS, we had access to GWAS results based on large mete-analyses, providing substantial statistical power to detect genes associated with disease. In addition, we studied the enrichment of murine derived atherosclerosis-related gene sets within canonical pathways. We then tested whether their human orthologues (that were nominally associated with disease based on a gene-based test) were also observed in these pathways. For the majority of pathways, weak or even absent evidence was found in humans. Of note is the LXR pathway which is proven to be linked with human cardiovascular disease, and for which we found ample evidence using our gene-based and our pathway analysis. Furthermore, in atherosclerotic mouse models, plasma lipid levels are the main determinant of lesion development. The associations between mice and human GWAS studies may improve when human individuals are studied with (genetic) susceptibility to abnormal lipid metabolism. Such interactions have not been explored in the present study. However, such an association would imply that the atherosclerotic mouse models cannot represent disease development in the general population.

Second, one might argue that the SNP to gene mapping done by VEGAS is over-conservative and consequently excludes (regulatory) variants of greater effect that may lie as far as 1Mb. However, most variants significantly affecting gene expression are found within 50 kb of the gene body[30], and regulatory elements are usually found in intergenic regions. Nevertheless, it is unlikely that erroneous annotations alone can explain the strong discrepancy between murine genes and their human orthologues within pathways.

Third, undoubtedly the Ingenuity Knowledge Base is a comprehensive summary of the current knowledge from literature on gene networks and pathways. While it is constantly updated and manually curated, our Ingenuity based pathway analysis is biased. Indeed, genes (and thereby networks and pathways) that are not studied (in humans or any model system) would simply not exist in Ingenuity, thus partly explaining an apparent discrepancy between associated pathways in humans and mice. In addition, pathways are often cell and organ specific and a selective approach towards cell types that are considered to play a dominant role in atherogenesis may affect the readout of our pathway analyses.
Fourth, the annotation of candidate genes to GWAS loci is usually based on literature and proximity to the genome-wide significant SNP, but whether such a gene actually influences disease is still unknown. For our gene-based approach we mapped the mouse genes to their human orthologue and tested their association with CAD or LAS. Therefore, the genes that have been annotated to GWAS loci may not appear in our results even when the SNPs in their proximity meet the genome-wide significance threshold.
Lastly, a transgenic or compound-treated mouse may not be comparable to a knockout model, thus explaining the lack of association of these genes with human disease. Given the statistically different gene-based results, we stratified our pathway analyses based on these three different model types, and found no difference. Hence, it is unlikely that the type of murine intervention model explains the lack of association with human disease.
Previous studies have raised doubts upon the validity of translating murine models to human pathophysiology in studying immune responses[31–33] although conflicting results have been obtained[34]. Our study adds to the debate on the relevance of murine models for human atherosclerotic disease, and supports the view that the direction of the scientific process matters. The lack of association of murine atherosclerotic genes with human CAD, LAS or human plaque characteristics may be due to diverged gene expression patterns among mice and humans resulting in different phenotypic effects. Indeed, results from the recently published Mouse ENCODE project show that gene expression patterns diverge between mice and humans[35,36]. Moreover, phenotypic effects of orthologous genes frequently differ between species. This suggests that a sensible approach would be to group genes based on mouse-human orthology in order to improve the translational power of putative murine genes. Genome-wide association studies agnostically provide human evidence for the involvement of genetic loci in the underlying mechanisms of atherosclerotic disease, including CAD and LAS. Therefore, from the outset, the rationale to initiate an examination of the role of genes in these loci in murine models of atherosclerosis could be supported by human data.
Our study may have several limitations. Individuals of non-European descent are underrepresented in the GWAS we examined. It remains to be investigated if results will differ for GWAS in non-European cohorts.
The genetic association study on human plaque characteristics was executed in the Athero-Express study and included 1,443 patients with significant atherosclerosis. Although this study represents the largest collection of histologically investigated plaques, this study may suffer from limited power when examining the genetics of plaque characteristics.

We studied all published papers that applied the ApoE$^{-/-}$ and LDLR$^{-/-}$ as a model for atherosclerosis. However, alternative murine models have been studied in atherosclerotic disease that we did not include in our search and we cannot exclude that these reveal better associations with the human genetic outcome studies.
In conclusion, a role for genes that has been observed in mice for atherosclerotic lesion development could scarcely be confirmed studying associations of disease development with common human genetic variants indicating that knockout models of atherosclerosis are not a good reflection of the variation that underlies common forms of atherosclerosis. The value of murine atherosclerotic models for selection of therapeutic targets in human disease remains unclear.

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Supplemental material

Figure SI. Flow-chart showing the filtering and selection of relevant papers and the identification of murine atherosclerotic genes mapped to human orthologues.

Table SI. All 703 human orthologous genes identified in murine models.

Table SII. All gene-based association results of the 659 target genes for CAD and LAS.

Table SIII. Canonical pathways to which any of the 659 genes were mapped.

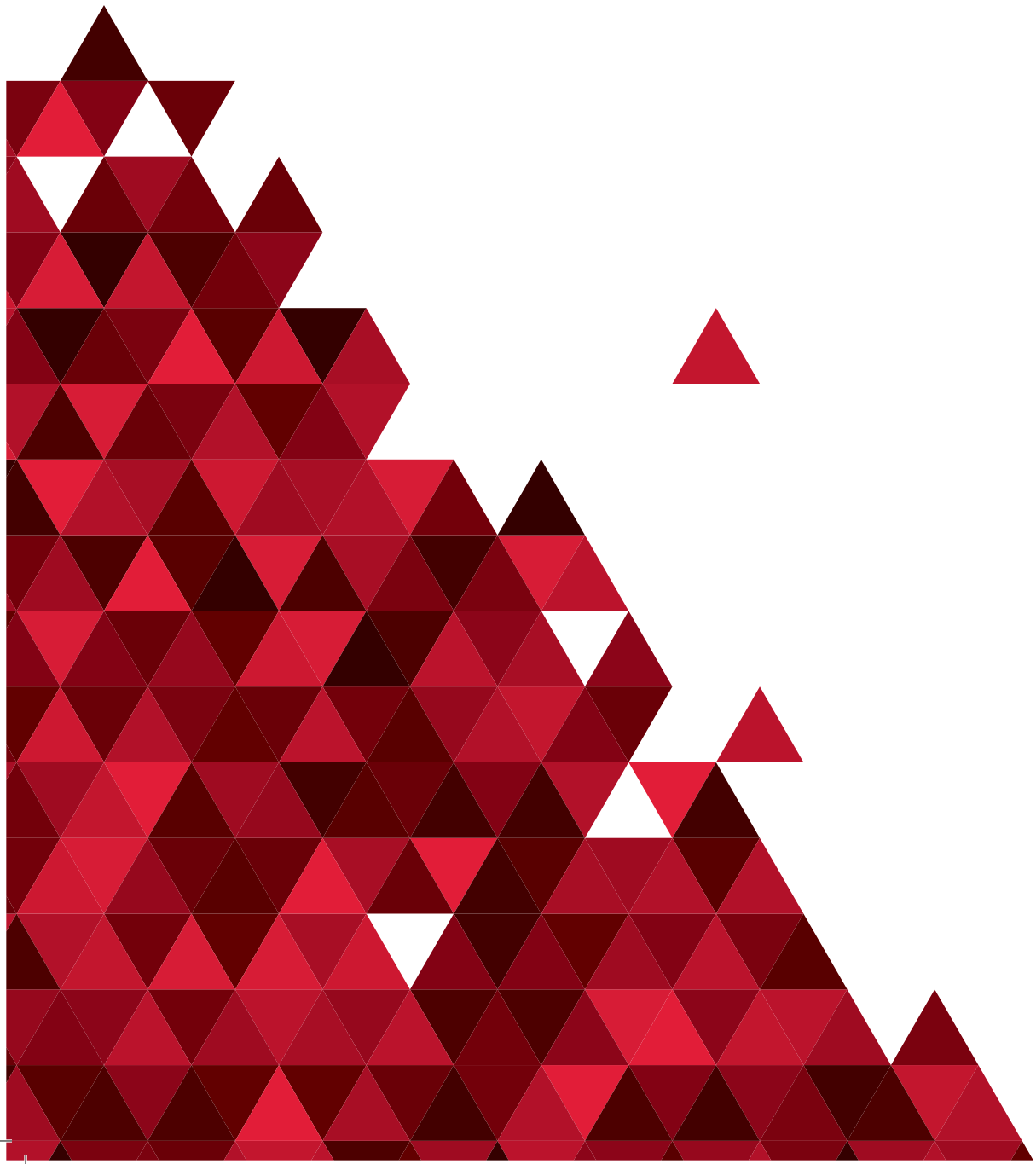
Table SIV. Number of genes with $p<0.05$ in the gene-based association study of plaque phenotypes.

Table SV. Gene-based association results for the atherosclerotic plaque characteristics in the Athero-Express Biobank Study.

Table SVI. Background information of the publically, online available eQTL datasets queried.

Table SVII. Cis-eQTL results from the analysis in STAGE and the online query of various datasets.

Supplemental material is omitted because of space limitations



CHAPTER 3

Additional candidate genes for human atherosclerotic disease identified through annotation based on chromatin organization

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Abstract

As genome-wide association efforts, such as CARDIoGRAM and METASTROKE, are ongoing to reveal susceptibility loci for their underlying disease: atherosclerotic disease, identification of candidate genes explaining the associations of these loci has proven the main challenge. Many disease susceptibility loci co-localize with DNA regulatory elements, which influence gene expression through chromatin interactions. Therefore, the target genes of these regulatory elements can be considered candidate genes. Applying these biological principles, we used an alternative approach to annotate susceptibility loci and identify candidate genes for human atherosclerotic disease based on circular chromosome conformation capture followed by sequencing (4C-seq). In human monocytes and coronary endothelial cells we generated 63 chromatin interaction datasets for 37 active DNA regulatory elements that co-localize with known susceptibility loci for coronary artery disease (CARDIoGRAMplusC4D) and large artery stroke (METASTROKE). By 4C-seq we identified a physical 3D interaction with 326 candidate genes expressed in at least one of these cell types, of which 294 have not been reported before. We highlight 16 genes based on expression quantitative trait loci. Our findings provide additional candidate-gene annotation for 37 disease susceptibility loci for human atherosclerotic disease that are of potential interest to better understand the complex pathophysiology of cardiovascular diseases.

Background

Atherosclerosis is a chronic inflammatory disease of the lipid-rich vascular wall that underlies many cardiovascular diseases (CVD)[1]. A large part of the disease burden of atherosclerosis can be traced back to coronary artery disease (CAD) and large artery stroke (LAS). Genome-wide association studies (GWAS) have helped to unravel the complex genomic background of these diseases, currently explaining about 10% of heritability[2,3]. The current approach is to annotate a novel susceptibility locus with the gene at the nearest genomic position. Some alternative strategies also take into account gene expression or protein-protein interactions[4,5]. A recent effort employing these bioinformatics-based approaches resulted in 98 new candidate genes for CAD[6]. In the last few years, the evidence that variants identified by GWAS also contribute to the disease pathogenesis by affecting the regulatory DNA sequences they reside in is growing[7–9]. These genetic variants may affect the activity of the DNA regulatory elements (DRE) and, under specific circumstances, lead to dysregulation of gene expression. This is mediated by long range 3D chromatin-chromatin interactions where the regulated candidate genes can be located up to ~1 MB away[10–12] – a distance much larger than is normally used to annotate candidate genes in GWAS. These candidate genes can be identified by capturing the physical chromatin-chromatin interaction between a known disease susceptibility locus and the promoter of the gene(s) it presumably regulates[13]. Here we systematically apply this principle (study design is summarized in Figure 1) to variants identified by large meta-analyses of GWAS for CAD and LAS; altogether assaying 47 previously identifiedsusceptibility loci[2,3]. Atherosclerotic disease starts in the endothelial lining of the affected arteries and involves attraction and proliferation of monocytes[14]. Therefore, we studied 37 loci that co-localize with active DRE in human monocytes and/or in cardiac endothelial cells. We used circular chromosome conformation capture sequencing (4C-seq) to identify candidate genes based on their physical interaction with one of the active DRE.

Methods

Cell culture

Primary commercially available human cardiac endothelial cells (CEC) that were isolated by enzymatic detachment (Lonza *Clonetics*™) were cultured in RPMI-1640 with 10% FCS and standard supplements. Cells were harvested for 4C template preparation by trypsinisation at 60-80% confluence.

Monocyte isolation

Human peripheral blood was collected from a healthy donor in sodium-heparin tubes. Peripheral Blood Mononuclear Cells (PBMCs) were isolated by Ficoll-Paque gradient centrifugation. PMBCs were incubated with magnetic CD14+-microbeads (Milteny, order nr. 130-050-201) according to manufacturer's manual. Thereafter cells were magnetically separated by the AutoMACS™ Separator, the positive fraction (monocytes) was used for 4C template preparation.

Circular Chromosome Conformation Capture-Template preparation

The 4C template was prepared as previously described[13]. Summarized, 10x10^6 cells were used per cell type (monocytes and CEC). Cells were crosslinked in 2% formaldehyde. After chromatin isolation, the chromatin was digested with *DpnII* (NEB, #R0543L). Digestion was stopped through heat inactivation of the restriction enzyme. Samples were diluted and ligated by T4 DNA ligase. The second digestion was carried out with CviQI (NEB, #R069S) and inactivated by phenol:chloroform extraction. The chromatin was diluted, for the final T4 ligation and the chromatin was purified. The quality of digestion and ligation was assessed on agarose gels.

Viewpoint selection and primer design

All SNPs from table 1 and 2 and the young-CAD SNP (rs16986953) from the CARDIoGRAMplusC4D paper[2] (n=47) and the two replicated SNPs (rs2383207 and rs2107595) from METASTROKE[3] were considered for viewpoint design (Supplemental table 8). When the SNPs within the susceptibility locus were less than 15,000 bp apart (e.g. rs12740374, rs602633 and rs599839 in the *SORT1* region), only one SNP was selected as a viewpoint. Susceptibility loci that overlapped with active DRE were identified through FAIRE, the presence of H3K4Me1, H3K4Me3, H3K27Ac, H3K4Me2 or H3K9Ac, EP300 or CTCF binding sites or DNase hypersensitivity sites (Supplemental table 9). DRE falling within the susceptibility locus coordinates were considered overlapping with the susceptibility locus. The primers were designed as was described previously[13]. Primer sequences are listed in Supplemental table 8. In summary, primers were designed in a window of 5 kb up- and downstream from the associated SNP. Forward and reverse primers were designed at least 300 bp apart. Forward (reading) primers were designed on top of the first restriction enzyme site. The reverse (non-reading) primer was designed close to (max 100bp away from) the second restriction enzyme site. In case no primer pair could be designed within the initial window, the window was extended 5 kb up- and downstream (n=8). In the case of rs1561198 this did not result in a suitable primer, so a primer pair that was 299bp apart was selected for this viewpoint.

Circular Chromosome Conformation Capture- Sequencing (4C-seq) library preparation

4C-sequencing library preparation was performed as described previously[13], with minor adaptations in order to make the protocol compatible with the large number of viewpoints: the PCR of 4C template was performed with 600 ng (monocytes) or 1,6 µg (coronary endothelial cells) of 4C template per reaction. 8 to 10 primer pairs were multiplexed in the initial PCR reaction (primer sequences are listed in Supplemental table 8). Primers pairs were pooled according to primer efficiency (based on intensity on gel electrophoresis signal after PCR on test template). PCR products were purified after an initial PCR reaction of 6 cycles (reaction volume = 200 µL) and divided among 8-10 PCR reactions containing single primer pairs for another 26 cycles (reaction volume = 25 µL). Thereafter, PCR products derived from the same cells were pooled in equimolar amounts and a final 6 cycle PCR reaction containing 20 ng of pooled PCR product (reaction

volume = 100 µL) was performed with primers that contained sequencing adaptor sequences. All fragments >700bp were removed using size selection on a 1% agarose gel follow by gel extraction of the selected products (Qiagen, #28704). Quality measures for the 4C library preparation and sequencing can be found in Supplemental figure 1.

Sequencing

Libraries were sequenced using the HiSeq2500 platform (Illumina), according to the manufacturer's protocol, producing 50 bp single end reads.

Data analysis

The raw sequencing reads were de-multiplexed based on viewpoint specific primer sequences. Reads were then trimmed to 16 bases and mapped to an *in silico* generated library of fragends (fragment ends) neighboring all *DpnII* sites in human genome (NCBI37/hg19), using the custom Perl scripts. No mismatches were allowed during the mapping and the reads mapping to only one possible fragend were used for further analysis.

Identification of the interacting genes

First, we calculated the number of covered fragends within a running window of *k* fragends throughout the whole chromosome where the viewpoint is located. The *k* was set separately for every viewpoint so it contains on average 20 covered fragends in the area around the viewpoint (+/- 100kb). Next, we compared the number of covered fragends in each running window to the random distribution. The windows with significantly higher number of covered fragends compared to random distribution ($p<10^{-8}$ based on binominal cumulative distribution function; R *pbinom*) were considered as significant 4C-seq signal. The following criteria were defined for the identification of the candidate genes; i) the Transcriptional Start Site (TSS) co-localizes with a significant 4C-seq signal ($P<10^{-8}$) within 5 kbp; ii) the susceptibility variant or other variant in linkage disequilibrium (LD) co-localizes with a DNA regulatory element identified though FAIRE, the presence of H3K4Me1, H3K4Me3, H3K27Ac, H3K4Me2 or H3K9Ac, EP300 or CTCF binding sites or DNase hypersensitivity sites (Supplemental table 9) in the cell type from which the 4C-seq signal originated and iii) the gene is expressed (RPKM > 0.5) in the assayed cell type.

Identification of gene expression

For monocyte expression, data from the ENCODE database were used (Supplemental table 10)[30]. For coronary endothelial cell expression, HMVECs (Lonza) were cultured on gelatine coated plates in EGM2-MV (Lonza) supplemented with penicillin and streptomycin. Subsequently, HMVECs were cultured for 20 hours in low serum medium (EBM + 0.5% FCS), followed by cell lysis and RNA isolation using the RNeasy isolation kit (Qiagen). Polyadenylated mRNA was isolated using Poly(A) Beads (NEXTflex). Sequencing libraries were made using the Rapid Directional RNA-Seq Kit (NEXTflex) and sequenced on Illumina NextSeq500 to produce single-end 75 base long reads (Utrecht Sequencing Facility). Reads were aligned to the human reference genome GRCh37 using STAR

version2.4.2a[31]. Read groups were added to the BAM files with Picard's AddOrReplaceReadGroups (v1.98). The BAM files are sorted with Sambamba v0.4.5 and transcript abundances are quantified with HTSeq-count version 0.6.1p1[32] using the union mode. Subsequently, reads per kilobase of transcript per million reads sequenced (RPKM's) are calculated with edgeR's rpkm() function[33].

Pathway analysis

The interacting genes (with and without expressed CARDIoGRAMplusC4D/METASTROKE genes) were analyzed using QIAGEN's Ingenuity Pathway Analysis (IPA, 2015 winter version, QIAGEN Redwood City, www.qiagen.com/ingenuity). We used IPA to identify canonical biological pathways within the Ingenuity Knowledge Base to which the interacting genes were mapped. Limits were set to only direct relationships that were experimentally observed in humans. We performed six rounds of pathway analysis, three in each of the cell types: one with only CARDIoGRAMplusC4D/METASTROKE genes that were expressed in the cell type, one with the CARDIoGRAMplusC4D/METASTROKE genes supplemented by the newly identified genes and one with the novel genes only.

Tracks and plots

All tracks were accessed from the UCSC browser (hg19) (http://genome.ucsc.edu/). Regional plots were generated using LocusZoom version 1.3[34].

Gene-based tests

Data for CAD were downloaded from the CARDIoGRAMplusC4D website (http://www.cardiogramplusc4d.org). We obtained summary statistics from GWAS on body mass index (BMI), blood lipids including LDL, HDL, total cholesterol and triglycerides, systolic and diastolic blood pressure, coronary calcification, fasting glucose, smoking behavior, and type 2 diabetes from public online resources and data on intima-media thickness and plaque-presence via data request (Supplemental Table 11). We used a VErsatile Gene-based Association Study (VEGAS) to calculate gene-based association statistics from the summary statistics of each interacting gene for each trait. The details of the methods applied by VEGAS have been described elsewhere[35]. In short, SNPs are mapped to the gene (in and around $\pm$50kb from 5' and 3' gene borders), and using the GWAS p-value a gene-based test statistic is calculated corrected for the underlying population linkage disequilibrium structure. Finally using simulations an empirical gene-based *p*-value of association with the phenotype is calculated per gene. VEGAS results were considered multiple testing significant if they were $P < 6.97 \times 10^{-6}$ (0.05/22 phenotypes x 326 available genes in VEGAS).

eQTL analysis in STAGE

Within the STAGE study, patients undergoing coronary artery bypass grafting (CABG) surgery were sampled for seven different tissues, namely atherosclerotic arterial wall (AAW), internal mammary artery (IMA), liver, skeletal muscle (SM), subcutaneous fat (SF), visceral fat (VF), and fasting whole blood (WB) for RNA and DNA isolation[36]. Patients that

were eligible for CABG and had no other severe systemic diseases (e.g. widespread cancer or active systemic inflammatory disease) were included. For quality control in genotyping, SNPs filtered for minor allele frequency MAF < 5%, Hardy-Weinberg equilibrium (HWE) p-value < $1x10^{-6}$, and call rate of 100%. Imputation was carried out using IMPUTE2 with 1000 Genomes EUR as the reference[37]. Quality control for imputed genotypes used additionally an IMPUTE2 INFO score filter < 0.3. After QC a total of 5,473,585 SNPs remained. The Ethical committee of the Karolinska Hospital approved the study, and all patients gave written informed consent after the nature and possible consequences of the study were explained. An expression trait was tested for association with each genotyped and imputed SNP using Kruskal-Wallis test and false discovery rate to correct for multiple testing as described before. First, all *cis*-pairs of SNPs within 50kb of the transcription start or end site for each gene were identified. Next, *cis* SNP-gene pairs were tested for association in all seven STAGE tissues using kruX[38]. The p-value for eQTL inclusion in kruX was set at 0.05. Finally, an empirical FDR estimate for each eQTL-gene pair was calculated using ten permutations by shuffling patient IDs on genotype data. As a result, the most significant eQTL-gene association in each tissue was reported.

eQTL analysis in Haploreg

Data on eQTL in healthy individuals were extracted from Haploreg version 4.1 (http://www.broadinstitute.org/mammals/haploreg/haploreg.php). The viewpoint SNPs and all SNPs in LD ($r^2 > 0.8$) were used as input. From the output, for each interacting gene, the most significant eQTL within each tissue was extracted.

eQTL analysis in CTMM circulating cells

CTMM circulating cells is a Dutch cohort from four different hospitals comprising of 714 patients undergoing coronary angiography of whom blood was stored. Monocytes were isolated by density centrifugation followed by positive magnetic bead isolation (CD14) and expression was measured using the Illumina humanHT-12 v3 Gene Expression BeadChip Array. After removal of samples with a median intensity of <50, 370 patients were included in the analysis. The data were quantile normalized and and log2 transformed using the lumi R package[39].

Genotyping was performed using a customized Affymetrix Axiom Tx array containing 767,203 genetic markers. Community standard quality control was performed, filtering out samples with missingness >5%, outlying heterozygosity ($\pm$ 4SD from the cohort mean) or inconsistent sex. Samples of non-European descent or those that were out of Hardy-Weinberg equilibrium ($p<5x10^{-5}$) were removed. In total, 622 were used in the current analysis. Untyped variants were imputed using a combined reference panel of the 1000 Genomes Project[40] and Genome of the Netherlands[41] totaling more than 90 million genetic variants across the genome. We used the software packages SHAPEIT[42] for phasing and IMPUTE2[37] for imputation. Prior to *cis*-eQTL analysis we filtered the imputed genotype data from CTMM based on MAF > 0.5%, HWE $P > 1x10^{-6}$, Info-metric > 0.9, and only focused on those variants in LD (r2 >= 0.8) with the CAD associated variants.

We then used fastQTL (v2.184)[43] to perform I-eQTL analyses using a fixed range (based on the 4C interactions) around each probeID available on the expression array.

Mouse knockout models

Murine gene names were mapped to the genes as follows. First, a custom data file was downloaded from the HUGO Gene Nomenclature Committee (http://www.genenames.org/cgi-bin/download) including the Approved gene name and the Mouse Genome Database ID from the Mouse Genome Informatics database and a file containing all available phenotypic information for all knockout mice was downloaded from MGI (ftp://ftp.informatics.jax.org/pub/reports/index.html#pheno). Next, for all approved gene names of genes identified through 4C-seq, the mouse phenotypes were looked up by linking the MGI IDs. If no linkage could be made for the MGI ID, this was coded as no available mouse model. If a mouse model was available, but no phenotype was found, this was coded as no available phenotype. If a mouse model was specifically coded as not showing any phenotype upon knockout, this was coded as a gene not resulting in any phenotype. Murine cardiovascular phenotypes were defined as a phenotype resulting in any of the following: impaired blood coagulation or abnormal platelets, abnormal glucose levels or homeostasis, abnormal vascular morphology, vascular remodelling or arterial differentiation, abnormal blood pressure, abnormal vasoconstriction, vasodilatation or vascular permeability, abnormal stress response of the heart, myocardial infarction, abnormal (circulating) lipid levels, abnormal fat morphology or amount, abnormal body weight, abnormal lipid droplet or fat cell size, abnormal macrophage response or inflammation, abnormal wound healing, arteritis, vasculitis, vascular occlusion or atherosclerosis.

Human knockout models

The interacting genes were extracted from the supplementary tables of the studies of Sulem *et al.* and MacArthur *et al.*[26,27]. For each of the interacting genes, all SNPs and indels resulting in human functional knockouts were reported.

Drug targets

For the lookup of existing drugs that target any of the candidate genes, we used a custom built drug pipeline that searches for drug-gene interactions using DGIdb[44] , which merged the most known drug-gene interaction databases, such as DrugBank[45] and PharmGKB[46]. We removed redundant results using STITCH[47] and WHO's INN[48]. We tested overrepresentation of drug groups according to ATC codes[49] using Fisher exact tests.

Results

4C-seq identifies additional candidate genes

We identified 37 active DNA regulatory elements that co-localize with susceptibility loci for CAD or LAS. Twenty-six were active in both monocytes and coronary endothelial

cells, 5 were only active in monocytes and 6 were only active in coronary endothelial cells (Supplemental table 1). To identify the target genes of these active DRE, we generated 63 4C-seq interaction datasets. We applied the following criteria for the identification of candidate genes: I) the transcriptional start site (TSS) co-localizes with a significant 4C-seq signal ($P<10^{-8}$) within 5kb; II) the susceptibility variant or any other variant in LD ($r^2 \geq 0.8$) co-localizes with an active DRE signal in the cell type from with the 4C-seq signal was obtained and III) the gene is expressed (RPKM > 0.5) in the studied cell type. With this approach, we identified 326 candidate genes (Supplemental table 1), 77 in human male coronary endothelial cells, 84 in human male monocytes and 165 in both cell types (Figure 1). In total, we identified 294 candidate genes that were not previously reported by the CAD and LAS GWAS (Supplemental table 1). We replicated 235/242 (97.1%) of the chromatin interactions with expressed genes that were identified in male coronary endothelial cells in female coronary endothelial cells (Supplemental table 1).

4C-seq identifies candidate genes in novel pathways

We performed cell-type specific pathway analysis of the candidate genes identified by 4C-seq combined with the candidate genes that were previously identified by the GWAS on CAD and LAS (Supplemental table 2). Notably, these analyses revealed the *Hypoxia signaling in the cardiovascular system* pathway in monocytes ($P = 0.01$) and the *NRF-mediated oxidative stress response* pathway ($P = 4.68*10^{-4}$ and $P = 0.026$ in coronary endothelial cells and monocytes respectively, Supplemental table 2). These pathways are both involved in the cellular response to oxidative stress. Additionally, the 4C-seq approach revealed *PTEN* (a player in the *Hypoxia signaling in the cardiovascular system* pathway) as a novel candidate gene (Supplemental table 1). Although this gene was never reported via previous GWAS annotation, *PTEN* (phosphatase and tensin analog) was found to be a likely candidate gene based on dose-dependently upregulation by statins through higher peroxisome proliferator-activated receptor-gamma (PPAR gamma) activity[15]. A mutation of *PTEN* led to inflammatory plaque characteristics in human atherosclerotic plaque[16] and increased stability of PTEN was found to ameliorate atherosclerosis[17]. Furthermore, *PTEN* shares its upstream transcription regulator *ZEB2* with *CDKN2A* and *CDKN2B* (enrichment *P* of overlap for ZEB2-regulated genes: $3.02x10^{-3}$ in monocytes and $3.06x10^{-3}$ in coronary endothelial cells). We reveal multiple novel pathways related to cardiovascular disease and we now show that *PTEN* physically interacts with a DRE at rs2246833 in monocytes (*P* interaction = $2.36x10^{-10}$).

Expression of identified genes is genotype dependent

DRE exert their function through regulation of gene expression. We explored this mechanism by studying expression quantitative trait loci: the GWAS SNPs (or a SNP in LD; $r^2>0.8$) that significantly affected the expression of the candidate genes identified by 4C-seq in the studied tissues (Table 1). For the candidate genes identified by 4C-seq in coronary endothelial cells, we performed lookups within eQTL data of atherosclerotic artery wall and internal mammary artery in the STAGE cohort of patients undergoing

cardiac bypass surgery. We identified two eQTL (FDR<0.1) in atherosclerotic artery wall (rs9818870: *MRAS* and rs2281727: *SRR*, Supplemental table 3a). The *SRR* gene, that has not been reported previously, encodes for the serine racemase enzyme that is an endogenous ligand of the glycine site of NDMA receptors in the brain. Blockage of this site was found to prevent stroke damage[18]. Interestingly, a set of twice the number of genes from the same genetic locus that were not identified by 4C-seq as a target gene resulted in no significant eQTL in STAGE. Using the HaploReg tool, we additionally examined expression in aorta, coronary artery and tibial artery tissue and identified another seven eQTL for genes that we identified in coronary endothelial cells (Supplemental table 3b), of which *ARL3* and *FAM117B* were not reported before. Both genes are poorly studied in the context of cardiovascular disease. Within the *VAMP5*-

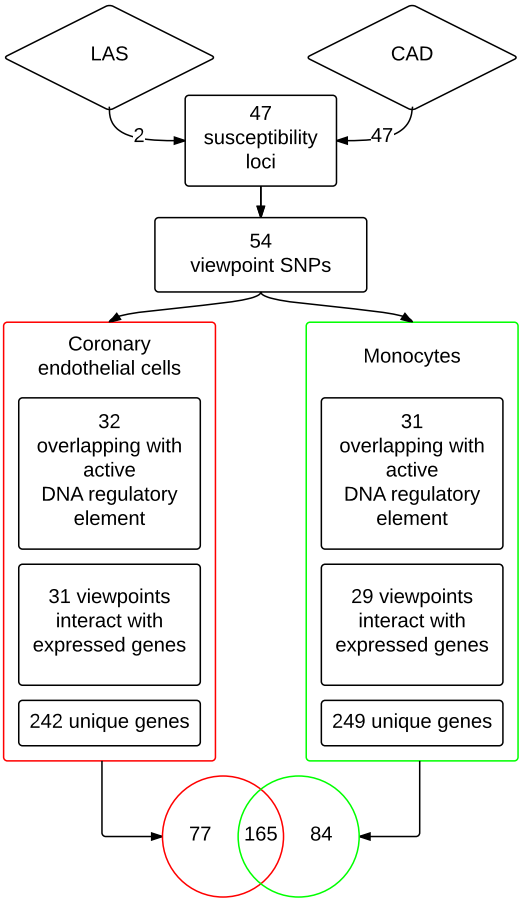

Figure 1. Flowchart of identification of candidate genes
Susceptibility loci: SNPs associated with risk of disease in METASTROKE and/or CARDIoGRAM. Viewpoint SNPs: SNPs used as the focus point for the primer design of the 4C experiment.

VAMP8-GGCX locus we replicate rs1561198, that was previously reported to be an eQTL for *GGCX* in mammary artery by the CARDIoGRAMplusC4D investigators in the ASAP study[19], as an eQTL for *GGCX* in aorta and tibial artery.

For genes identified by 4C-seq in monocytes, we performed *cis*-eQTL analysis in monocytes from 370 patients undergoing coronary angiography for coronary artery atherosclerosis in the CTMM (Center for Translational Molecular Medicine) Circulating Cells cohort[20]. We identified four eQTL (FDR<0.1) of which the genes overlap with genes identified by 4C-seq in monocytes of these patients (rs12740374: *PSRC1*, rs1561198: *VAMP8*, rs2246833: *LIPA*, rs12413409: *USMG5*, Supplemental table 3c). Previously, the CARDIoGRAMplusC4D investigators also identified rs1561198 as an eQTL for *VAMP8* in lymphoblastoid cells and skin in the MuTHER study[21]. Inclusion of the previously published cardiovascular cohort of Zeller et al.[22] revealed five additional genes (Supplemental table 3d). The SNP that revealed the strongest association with gene expression of *PSRC1* in monocytes of CTMM (rs7528419) is in perfect LD (1) with rs12740374 in the *SORT1* region. Interestingly, whereas the minor allele of the latter SNP is known to increase *SORT1* expression in liver, we found no such association between rs7528419 and *SORT1* expression in monocytes (nominal P = 0.87). In addition, we found an association between higher *PSRC1* expression in monocytes with a more severe atherosclerotic phenotype identified by a higher atherosclerotic burden, quantified using the SYNTAX score (P = 0.003). This association with high atherosclerosis burden could not solely be explained by LDL levels, the putative mechanism through which *SORT1* expression affects cardiovascular disease phenotypes (P when corrected for circulating LDL levels = 0.01). Expression of *PSRC1* in whole blood has previously been associated with cardiovascular disease in an Asian population[23]. Largely because the functional significance of the minor allele of rs12740374 as a transcription factor binding site that increases *SORT1* expression directly, no further attention has been given to alternative candidate genes in the *SORT1* region. With our 4C-seq approach in monocytes, we here show first evidence that the expression of *PSRC1*, a candidate gene in the *SORT1* locus, is genotype-dependent expressed in monocytes and related to the severity of atherosclerosis. This example further supports the implication of our additionally identified candidate genes in cardiovascular disease.

Additional genetic annotation

We further explored current genetic knowledge for the candidate genes identified through 4C-seq (Table 1, Supplemental table 4-7). First, if the candidate genes are effector genes of the DREs within CVD susceptibility loci, one would expect the genes to be enriched for (common) variants associated with CVD. Using the VEGAS algorithm, we concatenated GWAS p-values of all single-nucleotide polymorphisms (SNPs) in or within 50kb of a gene into a p-value for that particular gene. This way, we studied the genes identified by 4C-seq in published and unpublished GWAS data studying a total of 22 traits, either surrogate markers of atherosclerosis or known risk factors for cardiovascular disease (Supplemental table 4). Of all 326 candidate genes, 33 showed a significant association ($P < 0.05/(22x326)$

Table 1. Candidate genes identified by 4C-seq in human coronary endothelial cells and/or human monocytes

Chr	Susceptibility locus	4C-seq viewpoint(s)	Gene identified by 4C-seq	Cell type of identification		eQTL
				Coronary endothelial cells	Monocytes	
1	MIA3	rs17464857	*AIDA*	√	√	
			BROX	√	√	
			MARC1	√		
			MIA3	√	√	
			TAF1A	√	√	
1	SORT1	rs12740374	*AMPD2*	√	√	
			ATXN7L2	√	√	
			CYB561D1	√	√	
			GNAI3	√	√	
			GNAT2	√		
			GSTM2	√	√	
			GSTM4	√	√	
			PSMA5	√	√	
			PSRC1	√	√	Monocytes
			SARS	√	√	
			SORT1	√	√	
2	APOB	rs515135	*LDAH*	√	√	
2	VAMP5-VAMP8-GGCX	rs1561198	*GGCX*	√	√	CEC
			C2orf68	√	√	
			ELMOD3	√	√	
			MAT2A	√	√	
			RETSAT	√	√	
			RNF181	√	√	
			TGOLN2	√	√	
			TMEM150A	√	√	
			USP39	√	√	
			VAMP5	√	√	
			VAMP8	√	√	Monocytes
2	WDR12	rs6725887	*CARF*		√	
			FAM117B	√	√	CEC/Monocytes
			NBEAL1	√	√	CEC
			WDR12	√	√	
3	MRAS	rs9818870	*MRAS*	√	√	CEC
6	ANKS1A	rs12205331	*C6orf106*	√	√	
			RPS10	√	√	
			SNRPC	√	√	
			UHRF1BP1	√	√	
6	PHACTR1	rs9369640, rs12526453	*MYLIP*		√	
			PHACTR1	√	√	CEC
8	LPL	rs264	*INTS10*	√	√	
8	TRIB1	rs2954029	*TRIB1*	√	√	
9	CDKN2BAS	rs1333049, rs3217992, rs2383207	*CDKN2A*	√		
			CDKN2B	√	√	
10	CYP17A1-CNNM2-NT5C2	rs12413409	*ARL3*	√	√	CEC
			USMG5	√	√	Monocytes

Table 1. *Continued*

Chr	Susceptibility locus	4C-seq viewpoint(s)	Gene identified by 4C-seq	Cell type of identification		eQTL
10	CNNM2	rs12413409	*BORCS7*	√	√	
			WBP1L	√	√	
10	KIAA1462	rs2505083	*KIAA1462*	√		CEC
10	LIPA	rs11203042, rs2246833	*LIPA*	√	√	CEC/Monocytes
13	COL4A1-COL4A2	rs4773144	*COL4A1*	√		
			COL4A2	√		
17	RAI1-PEMT-RASD1	rs12936587	*PEMT*	√	√	Monocytes
			RASD1	√	√	Monocytes
17	SMG6	rs2281727	*SRR*	√	√	CEC
			SMG6	√	√	
17	UBE2Z	rs15563	*CALCOCO2*	√	√	Monocytes
			KPNB1		√	
			UBE2Z	√	√	Monocytes
19	LDLR	rs1122608	*C19orf52*		√	
			CARM1		√	
			LDLR		√	
			SMARCA4		√	
			TSPAN16		√	
			YIPF2		√	

* Only genes that have their interacting viewpoint as eQTL or genes with a significant gene-based *P* value ($P < 6.97 \times 10^{-6}$ by gene-based test using VEGAS (corrected for multiple-testing 0.05/22 phenotypes x 326 available genes) are depicted. The full list of genes identified by 4C-seq can be found in Supplemental table 1. Susceptibility locus: name of the locus as given by CARDIoGRAMplusC4D or METASTROKE; viewpoint: SNP used as the focus point for the primer design of the 4C experiment; Gene: gene physically interacting with viewpoint, determined by 4C-seq; Underlined genes: genes that have previously been reported by CARDIoGRAMplusC4D or METASTROKE; Chr: chromosome; eQTL: expression quantitative trait locus; GWAS: genome-wide association study CAD: coronary artery disease; BMI: body mass index; TC: total cholesterol; LDL: low-density lipoprotein; HDL: high-density lipoprotein; TG: triglycerides

= 6.97×10^{-6}) with coronary artery disease in the CARDIoGRAMplusC4D GWAS and 149 were nominally associated with coronary artery disease in the CARDIoGRAMplusC4D GWAS (significant enrichment, 149/326: binomial $P = 2.9 \times 10^{-102}$). Additionally, we found 7 genes that were significantly associated with BMI in GIANT and 29 genes that were significantly associated with at least one lipid trait in GLGC.

Second, we annotated the prioritized interacting genes from our 4C-seq experiment with phenotypic information of mouse models within the Mouse Genome Informatics database (MGI, www.informatics.jax.org). We found murine phenotypic information on 144 mouse homologues (Supplemental table 5). Knockout of 67 of them resulted in a phenotype related to cardiovascular disease (significant enrichment, 67/144: binomial $P = 1.36 \times 10^{-47}$), such as abnormal blood vessel morphology (*Col4a1, Cxcl12, Epor, Shc1, Tcf7l1*), altered circulating fatty acid levels (*Csf2, Kdm3a, Ldlr, Lipa, Pten*) and impaired vascular contractility (*Acta2*). Human variants in *ACTA2* are associated with early onset stroke and MI[24]. Knockout of two candidate gene murine homologues affected development of atherosclerotic lesions, namely *Ldlr* (accelerated development of atherosclerosis) and *Shc1* (resistance to diet-induced atherosclerosis). The p66 isoform of human SHC1 is implicated in reactive oxygen species generation and its knockdown

in endothelial cells of obese mice attenuated production of these radicals and of fatty acids oxidation[25]. Third, we investigated the biological effect of human knockout variation of the candidate genes to study druggability. We queried two datasets of available information on SNPs and insertion/deletion variants that cause human functional knockouts[26,27]. We found human knockouts, caused by nonsense, splice or frameshift variants, for 89 candidate genes (Supplemental table 6). Fourth, using a custom-built drug discovery pipeline, we found available compounds to target 50 of the candidate genes (Supplemental table 7a). These drugs showed a relative overrepresentation for usage as immunomodulating agents ($P = 0.012$ in coronary endothelial cells, $P < 0.001$ in monocytes) (Supplemental table 7b). Together, these findings provide further evidence that by using the 4C-seq method we identified additional candidate target genes for human atherosclerotic disease.

Discussion

Based on 3D chromatin-chromatin interactions with DNA regulatory elements that co-localize with previously identified susceptibility loci, we present 294 additional candidate genes for CAD and LAS that are of potential interest in the pathophysiology of human atherosclerotic disease. This study is the first to systematically study the human chromatin interactions of the CARDIoGRAMplusC4D and METASTROKE loci. Many of the additional genes have not been implicated in atherosclerosis before. Our approach, from a DNA regulatory point of view, complements conventional methods for candidate gene identification of GWAS susceptibility, can help further unravel diseases with a complex genetic background, and pave the way for cell-type specific drug development.

We have highlighted the 4C candidate genes that we could annotate via additional analyses and that therefore have known or foreseeable effects on cardiovascular disease. Based on tissue-specific pathway analyses, we highlighted *PTEN* that is known to be upregulated by statins and to possess effects on atherosclerosis[15–17]. Furthermore, based on eQTL studies, we identified *SRR*, the effect of which was previously implicated in stroke[18], and *USMG5*, that was previously associated with white matter hyperintensities in the brain[28]. Of special interest is the finding of an alternative mechanism by which the susceptibility locus that contains rs7528419 (*SORT1* region) may exert its effect. Using 4C-sequencing we identified a physical interaction between an active regulatory element that overlaps rs7528419 and *PSRC1* in monocytes. Moreover, we found an association between rs7528419 and the expression of *PSRC1* in monocytes and an association between *PSRC1* expression and atherosclerosis severity. This association was independent of LDL levels, which is the putative mechanism of rs12740374, a SNP in perfect LD (1.0) with rs7528419 that was previously found to increase *SORT1* expression in liver.

Mapping the SNPs that identify susceptibility loci in GWAS to genes that affect a complex disease, such as cardiovascular disease, is a challenging task. By annotating the locus with the linearly closest gene, the 3D conformation of chromatin is inadvertently not

taken into account. Many of the additional candidate genes we report are located outside the GWAS susceptibility loci. Using 5C (chromosome conformation capture carbon copy) the importance of studying 3D interactions has been highlighted previously; in a sample of 628 TSS from the ENCODE project only 7% of the over 1000 long-range looping interactions were with the nearest gene[10]. In a previous effort to identify candidate genes based on DNA regulatory mechanisms, 33 enhancers in the 9p21 locus were scrutinized[29]. Interestingly, the chromatin interaction between the enhancers identified by 3C (chromatin conformation capture) was found to be remodeled upon treatment with interferon-γ in HUVECs. In our 4C-seq experiment, we confirmed the physical chromatin-chromatin interaction between the 9p21 susceptibility locus and several candidate genes, among which interferons, in human coronary endothelial cells and monocytes. However, we found that these genes were not actively expressed in these cell types and therefore did not consider them any further.

There are some limitations to this study. First, there is no consensus about the gold standard approach to analysis of 4C-seq data. For example, we used a conservative cut-off for calling a chromatin-chromatin interaction ($P < 10^{-8}$). Altering this cut-off may result in more candidate genes. However, this likely leads to more false-positive results. We therefore report a quantitative measure for the p-value of the interaction of the DRE with the proposed candidate gene to enable the reader to take these considerations into account when interpreting the data. Second, while 4C-sequencing enables us to look at physical interactions, these interactions do not necessarily mean that the expression in the studied tissue is in fact regulated by the association locus or even expressed. We therefore decided to only report only genes that are actively expressed in the studied tissues. Furthermore, we found no eQTL association between the SNP of interest and any of the genes *in the vicinity* of the genes that were identified by 4C-sequencing, indicating that the resolution of the technique is sufficient to distinguish between candidate genes and less relevant genes within a genomic region.A more accurate cell type-specific mapping of susceptibility loci to candidate genes in humans is of paramount importance for the development of specific compounds in the pharmaceutical industry. The genes we identified display only partial overlap between coronary endothelial cells and monocytes. This finding stresses the importance of cell-specific approaches in order to grasp the complex biology of atherosclerotic disease. It also highlights the possibility to develop cell-specific compounds to target atherosclerotic disease. Our results therefore underline the need to investigate cell type-specific 3D chromatin conformation in future functional follow-up of GWAS data.

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Supplemental material

Supplemental figure 1. Quality of 4C datasets of coronary endothelial cells and monocytes.

Supplemental table 1. DNA regulatory elements and their interacting genes identified by 4C-seq for all gene transcripts.

Supplemental table 2. Tissue-specific Ingenuity Pathway Analysis, on genes identified by 4C-seq and/or genes previously identified by CARDIoGRAM/METASTROKE.

Supplemental table 3a. Significant eQTLs for the genes identified by 4C-seq and their viewpoint SNPs from the STAGE cohort.

Supplemental table 3b. Significant eQTLs for the genes identified by 4C and their viewpoint SNPs from HaploReg.

Supplemental table 3c. Significant eQTLs for the genes identified by 4C and their viewpoint SNPs in monocytes from CTMM.

Supplemental table 3d. Significant eQTLs for the genes identified by 4C and their viewpoint SNPs in monocytes from Zeller et al.

Supplemental table 4a. VEGAS analysis for all genes identified by 4C, disease phenotypes.

Supplemental table 4b. VEGAS analysis for all genes identified by 4C, risk factor phenotypes.

Supplemental table 5. All available murine phenotypes for all genes identified by 4C-seq.

Supplemental table 6. Genes identified by 4C-seq that carry human functional knockout variants.

Supplemental table 7a. Known compounds targeting genes identified by 4C-seq.

Supplemental table 7b. Overrepresentation tests for current use of known compounds targeting genes identified by 4C-seq.

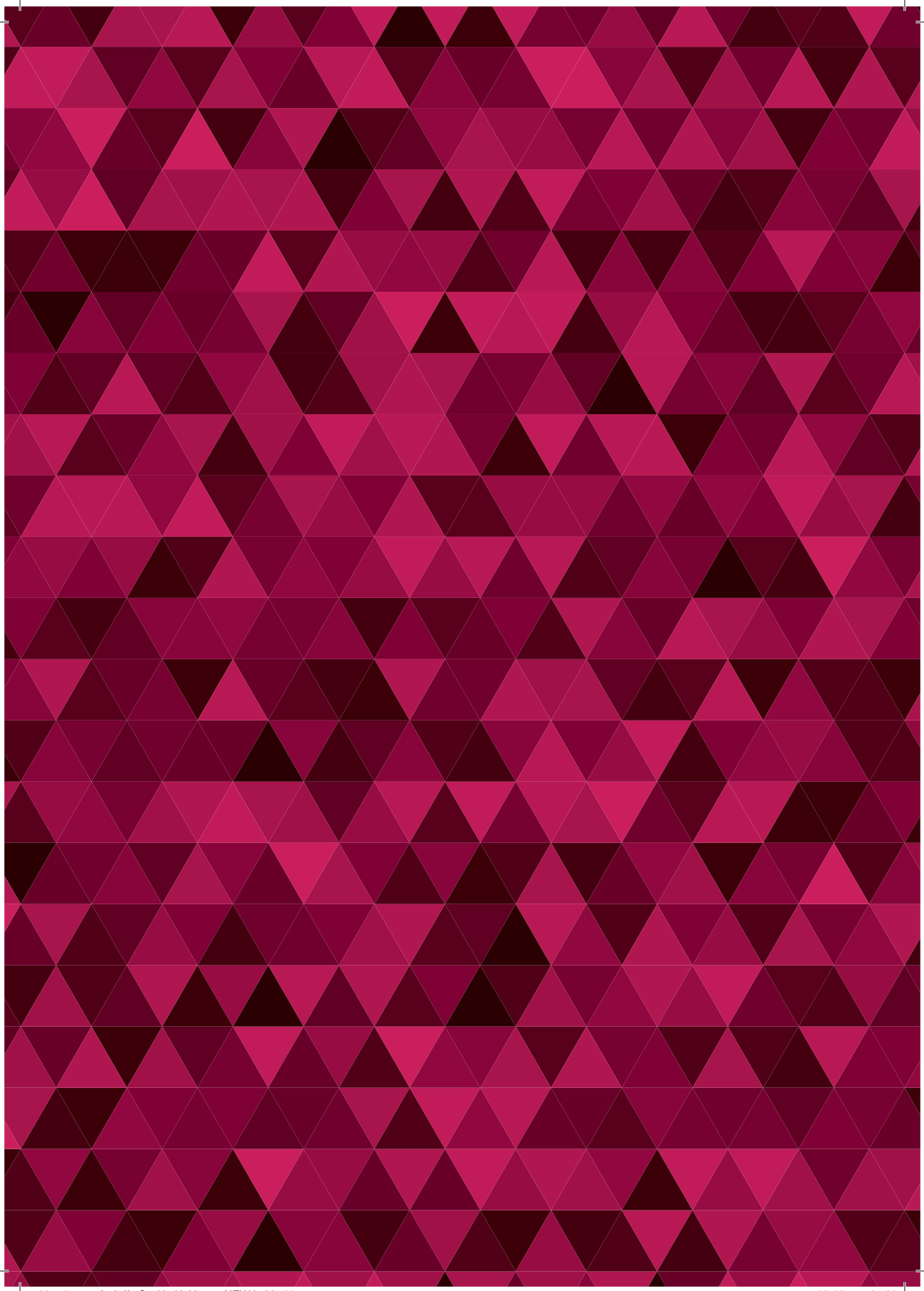
Supplemental table 8. Primer design.

Supplemental table 9. Datasets used in the identification of active DRE

Supplemental table 10. Datasets used in the identification of expressed genes.

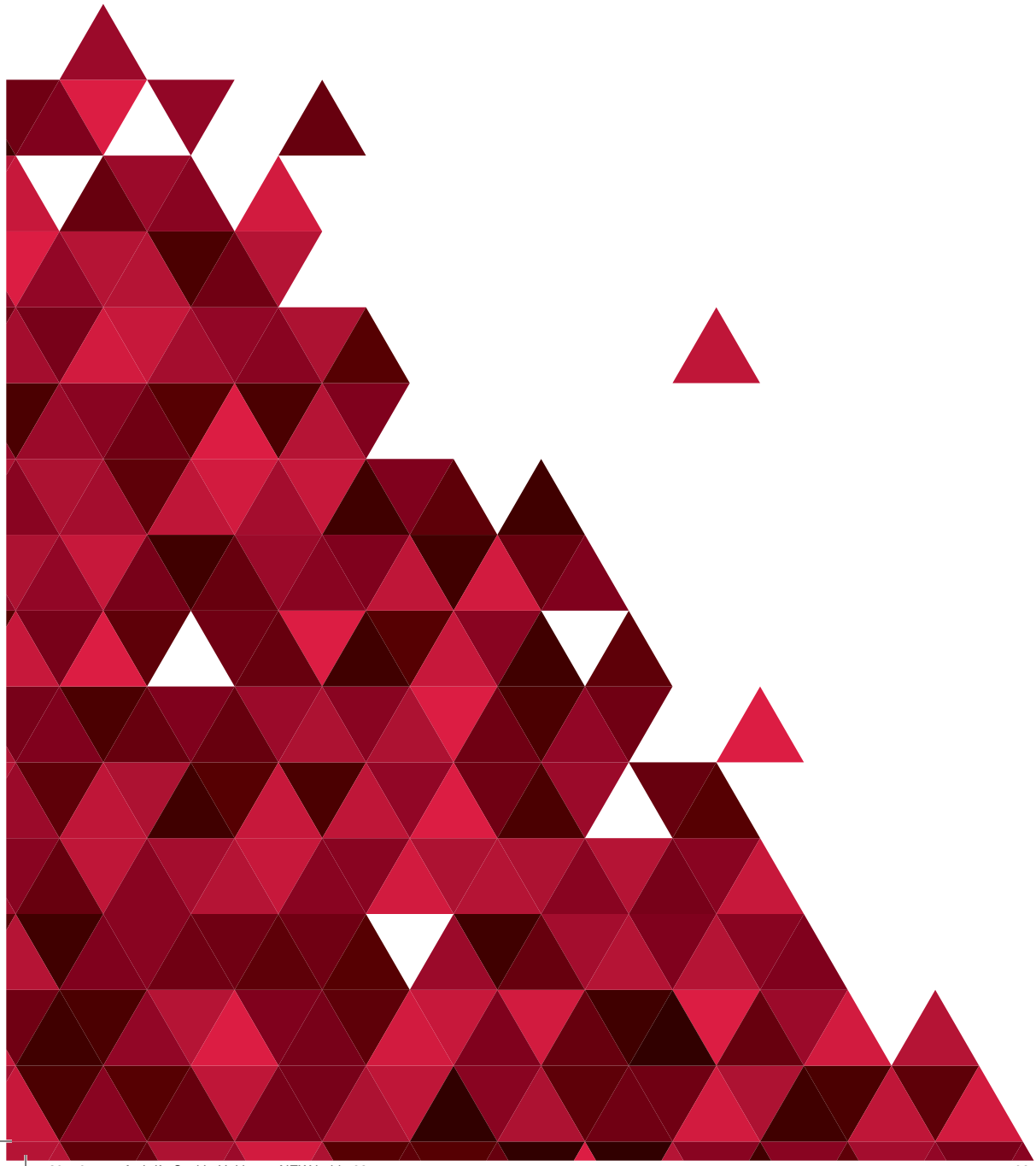
Supplemental table 11. Datasets used for VEGAS analysis.

Supplemental material is omitted because of space limitations



PART II

Atherosclerotic plaque studies in the Athero-Express Biobank



CHAPTER 4

Estrogen-associated atherosclerotic plaque characteristics in women with severe atherosclerotic disease around menopause

In preparation

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Abstract

Background

Women aged 55 years and younger show very small reductions in cardiovascular disease (CVD) mortality rates as compared to the rapidly declining rates seen in older aged women. The drivers of these major differences in CVD mortality trends merit urgent investigation. Estrogen levels reflect the biological process of aging and menopausal transition and their role in cardiovascular disease remains controversial. Therefore, we studied serum estradiol levels and their association with the composition of the atherosclerotic plaque in women with severe atherosclerotic disease around menopause.

Methods

Women (n = 374) aged between 35 and 70 who underwent carotid endarterectomy were analyzed. The extracted atherosclerotic plaques were histologically assessed; semi-quantitatively for calcification, collagen content, intraplaque haemorrhage, and lipid content, and quantitatively for macrophage content, microvessel density and smooth muscle cell content. Blood was drawn at the time of surgery. Serum estradiol levels, that were available for 171 women, were binned into four groups and associated with histological characteristics in a multivariable manner.

Results

Women of younger age showed the highest levels of estradiol (median age below 50 years of age: 172 pmol/L (IQR: 103-227.5 pmol/L), median age 60 to 70 years of age: 39 pmol/L (IQR: undetectable - 69 pmol/L), overall P <0.001). Overall, we observed high collagen content (79% of all analyzed plaques showed moderate to heavy collagen content) and low fat content (10.3% of all analyzed plaques showed a lipid core >40%) of the atherosclerotic plaque in these women. Adjusted for age, we observed a positive association between groups of estradiol levels and number of intraplaque microvessels (mean group 1: 5.04 (SEM: 0.81), mean group 4: 9.62 (SEM 0.99), overall P = 0.004), and a non-significant positive association between estradiol levels and percentage of macrophages of the total plaque area (mean group 1: 0.54 (SEM: 0.16), mean group 4: 1.29 (SEM:0.21), overall P = 0.053). No association was found with calcification, collagen, fat, intraplaque haemorrhage or amount of smooth muscle cells.

Conclusions

In women with atherosclerotic disease all plaques had features of stability, unrelated to serum estradiol levels. However, within these plaques, estradiol levels were associated with increased angiogenesis and a trend towards more inflammation. The implications of these associations warrant further investigation, but add to the controversy surrounding estrogens in women around menopause.

Introduction

Cardiovascular disease (CVD) is the number one cause of death in women all over the world. Despite a global decline in CVD death, women aged 55 years and younger show very small reductions in CVD mortality rates since 1990 as compared to the rapidly declining rates seen in older aged women[1]. This trend is especially of concern as women overall are understudied in cardiovascular research, and this subgroup is an important population to target for preventive treatment[2–4]. Prevention of CVD during this life stage can have great personal and societal health benefits.

From the moment the female ovaries are no longer able to maintain a regular menstrual cycle, women are in menopausal transition. Levels of estrogens fluctuate, finally stabilizing around two years after the final menstrual period. In this post-menopausal phase, estradiol levels have dropped significantly. In premenopausal women, endogenous estrogens are associated with reduced prevalence of CVD risk factors and CVD itself.

Not much is known about endogenous exposure to estrogens and the composition of the atherosclerotic plaque. Although evidence of a high prevalence of plaques with features associated with stability in young women provided from autopsy studies has been compelling, associations with endogenous plasma estrogens have not been studied[5–8]. Therefore, we examined histological features of the carotid atherosclerotic plaque and their association with serum estradiol levels in women before, during and after menopause undergoing carotid endarterectomy within the Athero-Express Biobank Study.

Methods

Patient population

The Athero-Express Biobank Study includes all patients undergoing carotid endarterectomy at the University Medical Center Utrecht and St. Antonius Hospital Nieuwegein, The Netherlands, without exclusion criteria. The study design has been described before[9]. In brief, patient characteristics are obtained through standardized preoperative questionnaires and from patient files. Surgery was performed in conformation with international guidelines[10]. The study protocol has been approved by the medical ethics boards of both hospitals. The study is conducted in accordance with the declaration of Helsinki. Patients gave written informed consent. Of the total of 2331 carotid endarterectomy patients in the Athero-Express Biobank, 374 women and 870 men (aged 35-70) were included in the present analysis. As men were not the main focus of our study, data on male atherosclerotic plaques can be found in the Supplemental material.

Definitions of patient characteristics

Upon inclusion, all patients completed an extensive questionnaire. Height, weight and blood pressure were measured at admission. Creatinine levels were measured as part of

the clinical workup and used to calculate glomerular filtration rate (GFR). Hypertension and hypercholesterolemia were defined as self-reported hypertension and hypercholesterolemia. Diabetes mellitus consisted of either one of the following: 1) history of diabetes extracted from the patient file, 2) self-reported diabetes, 3) use of antidiabetic drugs. Smokers were considered current smokers if they had smoked in the past year. History of coronary artery disease (CAD) was present if the patient experienced a past myocardial infarction or underwent coronary artery bypass grafting surgery or percutaneous coronary intervention. Peripheral arterial occlusive disease was considered to be present if claudication could be extracted from the patient file, if the ankle-brachial index was below 0.70 for any of the lower limbs or if the patient reported a previous percutaneous intervention of the lower limbs. A positive family history for cardiovascular disease was considered to be present if any first-degree relative experienced either an abdominal aortic aneurysm, a myocardial infarction, a stroke or died from a cardiovascular disease before age of 60 years. Contralateral stenosis was scored as degree of stenosis in the contralateral carotid artery. Patients were scored for inclusion diagnosis according to the indication for surgery, either asymptomatic high-degree stenosis, transient ischemic attack (TIA), stroke or ocular symptoms (amaurosis fugax or retinal infarction). Medication use was extracted from the patient file and only scored positive if prescribed before surgery. None of the included patients used hormone-replacement therapy. Serum estradiol was measured by immunoassay on an ARCHITECT ci8200 system (Abbott Diagnostics, Abbott Laboratories, USA).

Histology of the atherosclerotic plaque

The atherosclerotic plaque was immediately processed after surgical removal. The plaque was transversely cut through the culprit lesion and subsequently sliced into pieces of 5mm that were stored at -80°C. The culprit lesion was stored in formaldehyde, decalcified for handling purposes and embedded in paraffin. (Immuno)histochemical staining was routinely performed for collagen (picro-sirius red and elastin von Gieson), macrophages (CD68), microvessels (CD34), smooth muscle cells (α-actin) and presence of intraplaque hemorrhage (IPH, consisting of the combination of luminal thrombi or intraplaque hemorrhages, hematoxylin-eosin and fibrin, assessed by Mallory's phosphotungstic acid-hematoxylin staining). The size of the lipid core was assessed using polarized light and cut off at 40% of the plaque area. Microvessels were identified morphologically in the CD34 immunostain, counted in three hotspots and averaged per slide. Collagen and calcification were semi-quantitatively scored at 40x magnification and dichotomized for the current analyses, where no/minor staining represents absent staining or staining in a few small areas and moderate/heavy staining represents larger areas of positive staining. Macrophages and smooth muscle cells were quantified using computerized analyses (AnalySiS version 3.2, Soft Imaging GmbH, Munster, Germany) as mean percentage of three representative regions of plaque area. The same dedicated experienced technician, blinded for patient characteristics, assessed all histological slides[11].

Statistical analyses

Continuous variables are reported as mean ± S.E.M. Age categories were defined as below 50 years of age, 50-60 years of age and 60-70 years of age for women as well as for men. Estradiol levels were available for 171 women and 385 men. Estradiol levels were compared between the age groups using a non-parametric Kruskall-Wallis test. Estradiol groups were split into a separate group for non-detectable values (<37 pmol/L, group 1, (n=74)) and the remaining group was split into tertiles (group 2: 37-56 pmol/L (n=33), group 3: 56-102 pmol/L (n=32), group 4: above 102 pmol/L (n=32)) in women. In men, the groups range from 0-68 pmol/L (n=101), 68-90 pmol/L (n=96), 90-111 pmol/L (n=93) and above 111 pmol/L (n=95) for groups 1-4 respectively. Plaque characteristics were available in a subset of patients in whom serum estradiol levels were measured (Supplemental table 1). Associations between estradiol and continuous plaque characteristics were calculated using UNIANOVA, correcting for age. For categorical plaque characteristics, associations with estradiol were calculated using logistic regression analyses adjusting for age. Odds ratios are displayed per standard deviation (SD) increase of estradiol with a 95% confidence interval (CI). Associations needed to show a consistent direction of effect over the estradiol groups to be considered relevant. All statistical tests were performed in SPSS version 21. A *P* value < 0.05 was considered significant.

Results

Baseline characteristics

We included 374 women between 35 and 70 years of age that underwent carotid endarterectomy surgery in the current analyses. Of these patients, 22 (5.8%) were 50 years of age or younger, 133 (35.6%) were between 50 and 60 years of age and 219 (58.6%) were between 60 and 70 years of age. Prevalence of hypertension and hypercholesterolemia, as well as the prevalence of diabetes, show a steep increase after the age of 50 years (59.1-80.3% for hypertension, 54.5-74% for hypercholesterolemia, 0-21.5% for diabetes mellitus). About half the women were current smokers, across all age groups. GFR declined with age (84.5 - 71.1), as well as the prevalence of a family history of CAD (60-45.6%). The overall lipid profiles did not differ between the age groups. All baseline characteristics can be found in table 1.

Estradiol levels

Estradiol levels within the Athero-Express Biobank, that were available in 171 women, were similar as reported in large population based cohorts[12,13] and showed a negative association with increasing age (median age below 50: 172 pmol/L (IQR: 103-227.5 pmol/L), median age 60-70: 39 pmol/L (IQR: undetectable – 69pmol/L), overall *P* <0.001, Figure 1). Baseline characteristics stratified by estradiol levels can be found in Supplemental table 2.

Table 1. Baseline characteristics

	Age <=50 n = 22	Age 50 - <=60 n = 133	Age 60 - <=70 n = 219
BMI, median (IQR) (kg/m^2)	26.1 (22.1-28.2)	26.0 (23-29.5)	26.0 (23.5-29.3)
GFR MDRD, median (IQR) (ml/min/1.73m^2)	84.5 (76.6-99.5)	74.8 (67.9-87.9)	71.1 (58.3-87.7)
Hypertension (%) (yes vs no)	13/22 (59.1)	94/129 (72.9)	171/213 (80.3)
Hypercholesterolemia (%) (yes vs no)	12/22 (54.5)	89/125 (71.2)	148/200 (74.0)
Diabetes mellitus (%) (yes vs no)	0/22 (0)	29/133 (21.8)	47/219 (21.5)
Current smoking (%) (yes vs no)	11/22 (50)	82/131 (62.6)	101/214 (47.2)
History of CAD (%) (yes vs no)	2/22 (9.1)	24/133 (18.0)	45/219 (20.5)
Presence of PAOD (%) (yes vs no)	4/22 (18.2)	34/133 (25.6)	44/219 (20.1)
Family history of CVD <60 years (%) (yes vs no)	12/20 (60)	65/120 (54.2)	89/195 (45.6)
Contralateral stenosis >50% (yes vs no)	8/17 (47.1)	51/115 (44.3)	87/198 (43.9)
Inclusion diagnosis (%)			
Asymptomatic	3/22 (13.6	21/132 (15.9)	24/218 (11.0)
TIA	8/22 (36.4)	53/132 (40.2)	94/218 (43.1)
Stroke	6/22 (27.3)	30/132 (22.7)	58/218 (26.6)
Ocular symptoms	5/22 (22.7)	28/132 (21.2)	42/218 (19.3)
Total cholesterol, median (IQR) (mmol/L)	4.7 (4.2-5.5)	5.4 (4.5-6.2)	4.9 (3.9-5.8)
LDL cholesterol, median (IQR) (mmol/L)	2.6 (2.3-3.3)	3.1 (2.5-4)	2.9 (2.1-3.7)
HDL cholesterol, median (IQR) (mmol/L)	1.4 (1.2-1.6)	1.3 (1.0-1.5)	1.2 (1.0-1.4)
Triglycerides, median (IQR) (mmol/L)	1.2 (1.0-1.9)	1.5 (1.1-2.2)	1.4 (1.0-1.9)
CRP, median (IQR) (ug/ml)	1.92 (0.68-5.97)	2.17 (1.16-4.05)	1.92 (0.99-4.04)
Statin use (%) (yes vs no)	17/22 (77.3)	112/133 (84.2)	181/218 (83.0)
Antiplatelet use (%) (yes vs no)	22/22 (100)	128/133 (96.2)	202/218 (92.7)

BMI: body mass index, CAD: coronary artery disease, CRP: C reactive protein, CVD: cardiovascular disease, GFR: glomerular filtration rate, IQR: interquartile range, MDRD: modification of diet in renal disease, PAOD: peripheral arterial occlusive disease, TIA: transient ischemic attack. Categorical variables are displayed as percentages, continuous variables are displayed as medians with interquartile ranges.

Histopathological characteristics

Women in the Athero-Express Biobank displayed in general atherosclerotic plaque characteristics that are associated with features of stability[14,15]. Among all female plaques for which estradiol levels were available, 79% showed moderate/high collagen content and only 10.3% contained a lipid core >40% (Figure 2A, Supplemental figure 1). To study the association between estradiol levels and plaque characteristics, estradiol levels were split into a separate group for non-detectable values (<37 pmol/L, group 1). The remaining group was split into tertiles (group 2: 37-56 pmol/L, group 3: 56-102 pmol/L, group 4: above 102 pmol/L). Corrected for age, we observed a positive association of estradiol levels with density of intraplaque microvessels (mean group 1: 5.04 (SEM: 0.81), mean group 4: 9.62 (SEM: 0.99), overall P = 0.004, Figure 2B) and a non-significant association

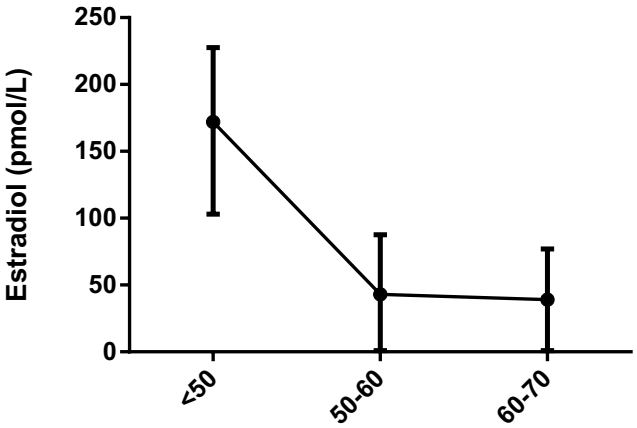

Figure 1. Association between levels of estradiol and age in women before and after menopause.

for macrophages (mean group 1: 0.54 (SEM: 0.16), mean group 4: 1.29 (SEM:0.21), overall P = 0.053, Figure 2C). The association of estradiol with collagen (Figure 2A) was not significant after correction for age in a logistic regression model (OR 1.912 (95% CI: 0.729-5.014)). Lipid core, presence of intraplaque haemorrhage, amount of smooth muscle cells and calcification did not associate with estradiol levels (Supplemental figure 1). Sensitivity analyses revealed no clear difference between the absence and presence of cardiovascular risk factors and the association between microvessels and estradiol levels in women (Supplemental figure 2). Neither did we find evidence for an age-effect (Supplemental figure 3). Interestingly, we observed no association between estradiol levels and intraplaque microvessels in men (mean group 1: 8.85 (SEM: 0.85), mean group 4: 8.74 (SEM: 0.72), overall P = 0.808) and a trend in macrophage content in men (mean group 1: 0.44 (SEM: 0.14), mean group 4: 0.90 (SEM: 0.12), overall P = 0.079, Supplemental figure 4).

Discussion

In a cohort of women with severe atherosclerotic disease, the atherosclerotic plaques overall showed features of stability such as high collagen content and small lipid cores. In contrast, within this plaque type, serum estradiol levels were associated with increased microvessel density and showed a trend towards increased numbers of macrophages, independent of age. The results of this study suggest that women who have experienced cardiovascular disease before 70 years of age have plaques with stable features. In addition, our data suggest that endogenous estrogen levels are implicated in plaque angiogenesis and inflammation in these women. This adds to the controversy of the effects of estrogens in cardiovascular disease in women before, during and after menopause.

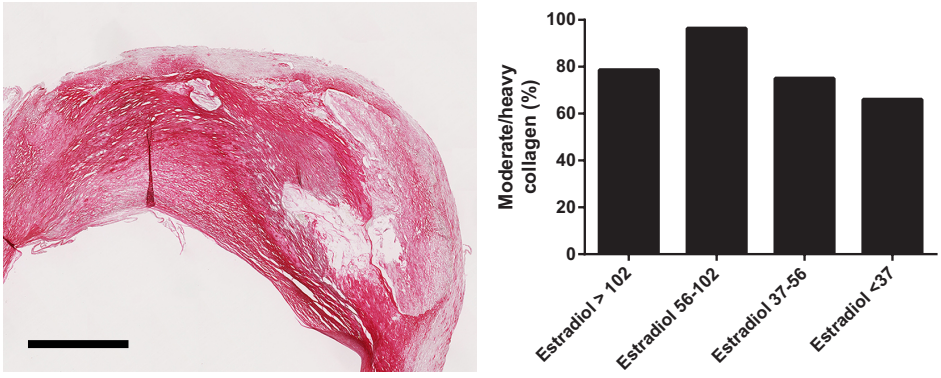

Figure 2A. Typical example of an atherosclerotic plaque with high collagen content

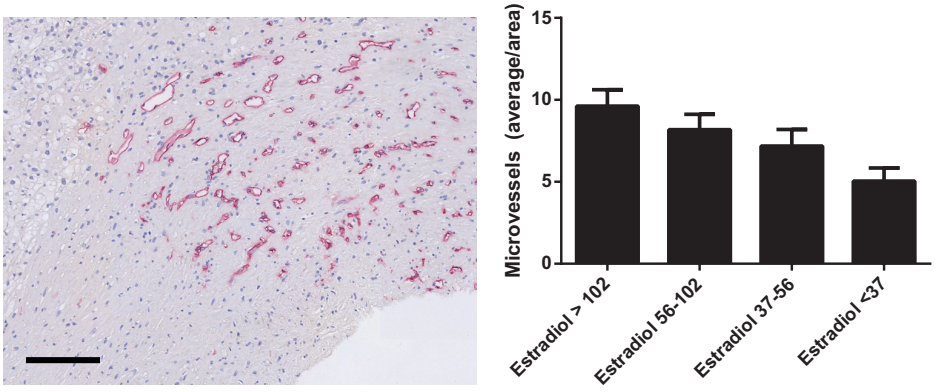

Figure 2B. Typical example of an atherosclerotic plaque with high density of microvessels

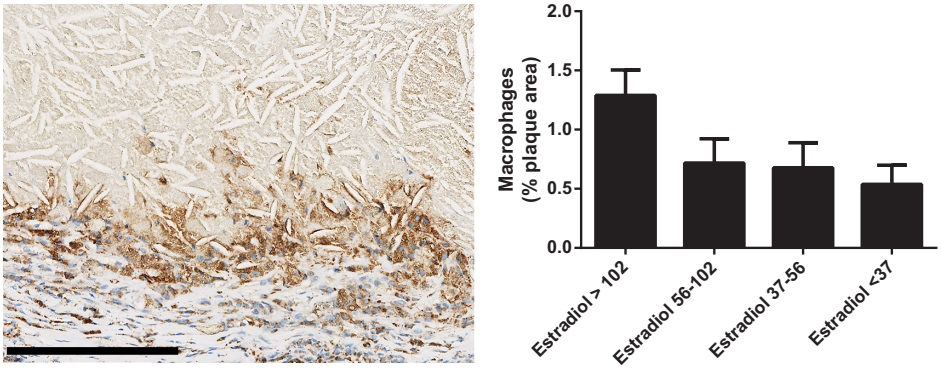

Figure 2C. Typical example of an atherosclerotic plaque with high macrophage content

Our findings are twofold: first, the observed features associated with stable plaque characteristics in women fit into the previously described high prevalence of plaque erosion in young women[5,7,8]. There are multiple substrates for thrombosis overlying an atherosclerotic plaque. Plaque rupture is the most common substrate and the hallmark of the plaque prone to rupture is a thin fibrous cap overlying a lipid rich core[16]. A less common substrate, plaque erosion, is the main cause of coronary thrombosis in women before menopause[6]. The histologic features described by the group of Virmani are abundant surface smooth muscle cells and proteoglycans, and a small or absent lipid-rich core. In our study, around eighty percent of female patients experienced symptoms, either a transient ischemic attack, ocular symptoms or a stroke, suggesting that their atherosclerotic plaque may have given rise to plaque erosion. Second, the increased neovascularization and inflammatory characteristics in these women and the relation with estrogens seems controversial. There is an established link between estrogens and angiogenesis[17,18]. However, angiogenesis is thought to contribute to atherosclerosis and plaque instability, although experimental studies have not yet determined a causal relationship. In human autopsy studies, it was assessed that ~96.5% of intimal neovessels enter the atherosclerotic plaque from the adventitial vasa vasorum and only 3.5% are derived from the arterial lumen. The intimal neovessels that come from the adventitial vasa vasorum are associated with the degree of inflammatory cell infiltration[19]. Indeed, in our study, estrogen levels were associated with both microvessels and macrophages, suggesting that the microvessels in women may indeed be derived from the adventitial vasa vasorum. This may be different in men. In a previous study we observed differences in prevalence of plaque hemorrhage between men and women, with men having more plaque hemorrhage than women. This was despite the observation that the number of microvessels was equal between men and women[15]. One may hypothesize based on these findings that the neovessels in men are therefore more likely to be derived from the lumen, as microvessels from the lumen are indeed associated with plaque hemorrhage, a male predominant feature of the plaque[19].

The clinical implication of our findings is that although estrogens are associated with higher numbers of microvessels that may have paved the way for inflammatory cells, there is no association of estrogens with plaque hemorrhage i.e. "leakiness of vessels" in these women. Estrogens may therefore be involved in neovascularization, and give rise to more stable microvessels that maintain their vascular integrity and thereby limit the development of plaque hemorrhage.

◀ **Figure 2A.** Left panel, typical example of an atherosclerotic plaque with high collagen content semi-quantitatively scored using Picro Sirius red staining. Right panel, % of women with moderate to heavy staining of collagen in the atherosclerotic plaque. Bar: 1mm. **Figure 2B.** Left panel, typical example of an atherosclerotic plaque with high density of microvessels quantified using a CD34+ immuno-stain. Arrow indicates a microvessel. Right panel, averaged number of microvessels per hotspot area in the female plaque in the different estradiol groups, corrected for age. Bar: 150 µm. **Figure 2C.** Left panel, typical example of an atherosclerotic plaque with high macrophage content quantified using a CD68+ immuno-stain. Right panel, mean % of three representive regions of plaque area in the different estradiol groups, corrected for age. Bar: 250 µm.

The earlier clinical observations that CVD was less common in HRT users compared to non-users suggested that hormone replacement therapy (HRT) was a likely candidate for primary CVD prevention[20]. However, the intervention studies in primary and secondary CVD prevention contradicted the findings from observational studies as adverse cardiovascular events were observed with HRT [20–23]. The contradictory results of the different studies have been attributed to the design of the randomized controlled trials, the age of the women, their stage of menopause, preexisting CVD, and the type of estrogen used[24,25]. However, our data adds to the discussion on refinement, as structural changes in the vasculature and the response of atherosclerotic plaque to estrogens may be involved as well. If causal, one may argue that neovascularization may be detrimental, depending on the developmental phase of the atherosclerotic plaque, and the site of neovascularization (adventitial vasa vasorum or lumen), a similar argument holds for the infiltration of inflammatory cells. There are several limitations and strengths to this study. The first limitation is that this is a cross-sectional study in which we cannot prove causality, and can only show associations between estrogens and plaque characteristics. A second limitation is generalizability. We have studied the atherosclerotic plaque in a population of women with severe carotid occlusive disease under the age of 70. Despite the idea that atherosclerosis is a systemic disease, our findings may not be generalizable to atherosclerotic disease in the coronary arteries or other vascular beds. Thirdly this surgical cohort is subject to selection of women. In general, high estradiol levels before and after menopause are believed to prevent women from developing symptomatic CVD. The women we studied who displayed high estradiol levels may somehow not have benefitted from their high levels of estradiol and have other mechanisms that overruled their protection from endogenous estrogen. We can only speculate as to why they developed arterial occlusive disease at a relatively young age. One of the possible explanations is a decreased susceptibility to estradiol. Another explanation could be a high genetic predisposition to developing atherosclerotic disease. Indeed, younger women more often have a positive family history for CVD. However, this is not reflected by a higher prevalence of a history of CVD in the women themselves.

A major strength is that the high numbers of patients within the Athero-Express biobank, 2331 patients thus far, have made it possible to study younger women in a subgroup analysis with sufficient power. This is important as CVD mortality in these women is not declining at the same rate as in men and older women, and the reason for this is unknown. This study observes a role for estradiol in women before and after menopause and reveals an atherosclerotic plaque with features of stability such as high collagen and a small lipid core, suggestive of erosion pathology as a substrate for their event. Yet estrogens in these stable plaques are also associated with angiogenesis and inflammation, a finding that warrants further investigation. This study underscores a growing need for new therapeutic agents that address plaque erosion instead of the classical plaque rupture to combat CVD in women.

Conclusions

In women with atherosclerotic disease all plaques had features of stability, unrelated to serum estradiol levels. However, within these plaques, estradiol levels were associated with increased angiogenesis and a trend towards more inflammation. The implications of these associations warrant further investigation, but add to the controversy surrounding estrogens in women around menopause.

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Supplemental material

Supplemental figure 1. Other histopathological plaque characteristics in women around menopause vs groups of estradiol.

Supplemental figure 2. Sensitivity analyses of the association between estradiol and microvessels.

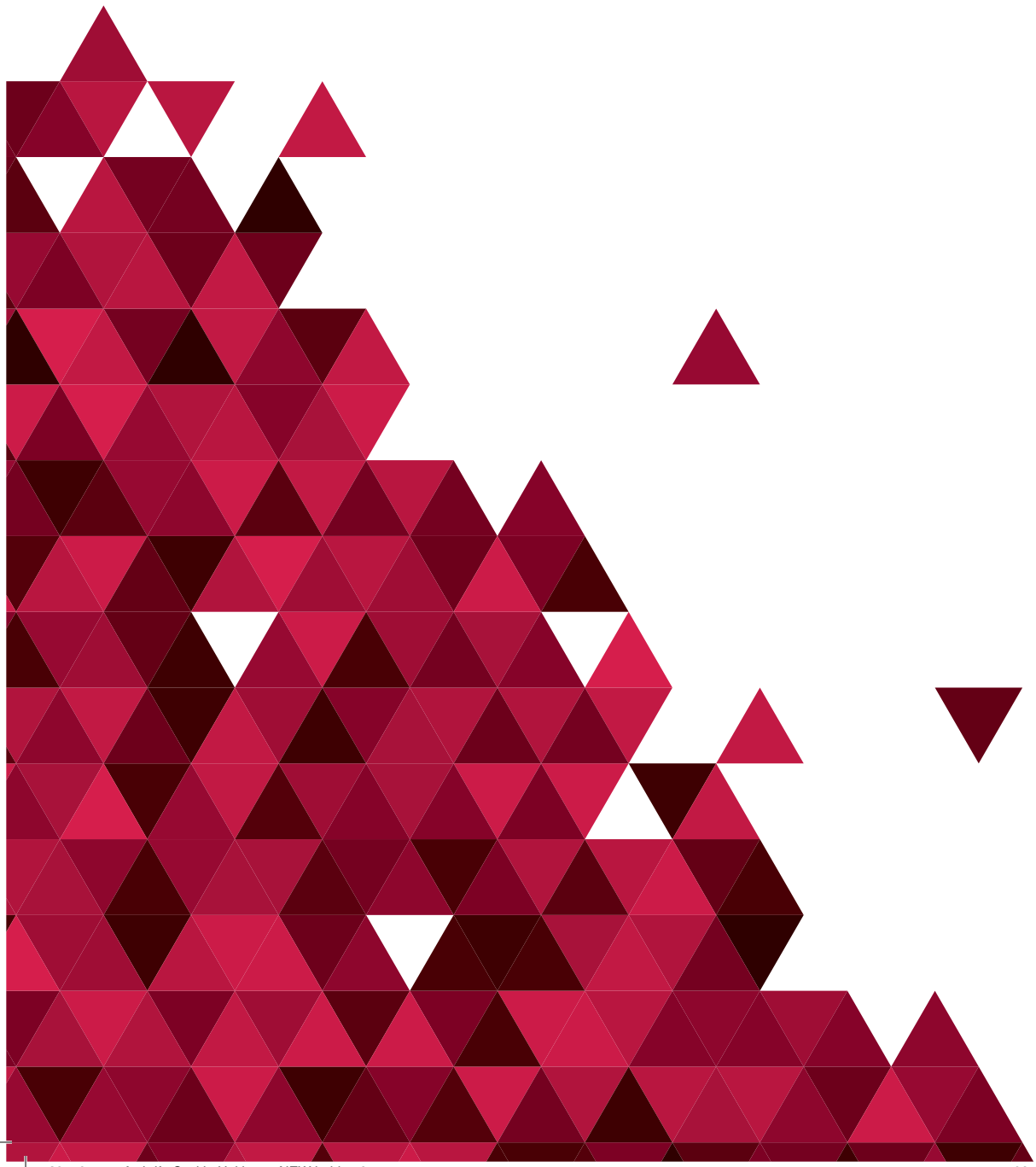
Supplemental figure 3. Sensitivity analysis of the effect of age on plaque characteristics.

Supplemental figure 4. Microvessels and macrophages in men below the age of 70 years vs estrogen groups.

Supplemental table 1. Availability of plaque histological parameters in patients with measured estradiol levels.

Supplemental table 2. Baseline characteristics of the four estradiol groups in women.

Supplemental material is omitted because of space limitations



CHAPTER 5

Patients with diabetes differ in atherosclerotic plaque characteristics and have worse clinical outcome after ilio-femoral endarterectomy compared to patients without diabetes

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Abstract

Background

Diabetes Mellitus (DM) is associated with peripheral arterial disease (PAD) and leads to worse clinical outcome compared with patients without diabetes. Objective of this study was to determine the impact of DM on ilio-femoral artery plaque characteristics and to examine secondary clinical outcomes in patients with diabetes and PAD undergoing surgical revascularization.

Methods

We analyzed 198 patients with and 453 patients without DM from the Athero-Express biobank, a prospective ongoing biobank study, who underwent endarterectomy of the femoral or iliac artery between 2002 and 2013. Seven histological plaque characteristics were compared (calcification, collagen, lipid core, intraplaque haemorrhage, macrophages, microvessels and smooth muscle cells) as well as secondary clinical outcome. Composite outcome consisted of any of the following secondary manifestations of cardiovascular disease: stroke, myocardial infarction, cardiovascular death or peripheral intervention. In addition, target vessel revascularization was examined. The follow-up period was standardized at three years after the procedure.

Results

Patients with diabetes were more likely to have calcified plaques compared with patients without diabetes (OR 2.11 (95% CI 1.43-3.12) P < .01). No other plaque characteristic differed significantly between the two groups. In total, 112 (57.1%) patients with DM and 198 (45.1%) patients without DM reached a composite endpoint during follow-up, of which 21 (10.7%) and 27 (6.2%) died of cardiovascular causes, respectively. DM was an independent predictor of composite cardiovascular events (HR 1.36 (95% CI 1.020-1.801) P = .01) during follow-up. No difference in the incidence of target vessel revascularization was observed between patients with and without DM (31.5% and 30%, respectively) (difference in survival time P = .86) or between longer duration of DM with composite event-free survival (difference in survival time P = .57).

Conclusions

Patients with diabetes who undergo surgical revascularization for PAD with the use of thrombendarterectomy or remote endarterectomy have a more calcified atherosclerotic plaque and an increased incidence in composite cardiovascular events, but no increase in target vessel revascularization.

Introduction

The prevalence of Diabetes Mellitus (DM) is increasing across the world[1]. DM is a known risk factor for atherosclerosis and thus for peripheral arterial disease (PAD)[2]. The ways in which DM influences vascular disease are multifold. DM is associated with increased inflammatory activity and endothelial cell activation causing constrictive remodelling[3–5]. Moreover, DM causes hypercoagulability which plays a role in plaque growth, plaque rupture, and increases the risk of sudden intravascular occlusion or thrombosis[6]. Patients with DM and PAD have a more than two-fold higher risk of cardiovascular morbidity and mortality compared with DM patients without PAD[7,8]. In addition, the duration of DM is associated with higher prevalence of PAD[9]. As the prevalence of DM is expanding dramatically and the disease occurs at an increasingly younger age[10], the number of patients with diabetes affected by PAD in later life will rise, consequently increasing the financial burden on healthcare systems worldwide[3,11].

Plaque characteristics of patients with DM have previously been histologically examined, predominantly in carotid and coronary arteries, but conflicting results have been obtained, presumably due to small patient numbers[3]. A large study performed in carotid patients suffering from diabetes showed no difference in plaque characterization between patients with and without diabetes[12]. Imaging of plaques in PAD patients revealed that diabetes-induced atherosclerosis is mainly located in below-the-knee arteries, instead of the ilio-femoral segments[13,14]. Furthermore, it is associated with calcification of the medial layer instead of the intimal layer of the arteries, known as Mönckeberg sclerosis[15,16]. Histological characteristics of peripheral plaques have to our knowledge never been investigated in a large cohort of PAD patients with diabetes, a prevalent comorbidity. Moreover, it is unclear whether DM is an independent and causative predictor for secondary cardiovascular disease or decreased patency rates in PAD patients undergoing surgical revascularization[5,17–20].

For this study we analyzed a large cohort of patients undergoing plaque removal from the ilio-femoral arteries to determine the impact of DM on plaque characteristics. In addition, the clinical outcome in patients with diabetes and PAD following endarterectomy was assessed. We hypothesized that plaque composition of ilio-femoral arteries in patients with diabetes would be different compared with patients without diabetes, and that patients with diabetes would have worse clinical outcomes and an increased need for target vessel revascularization.

Methods

Patient population

The Athero-Express biobank is a prospective ongoing biobank study that includes blood and plaque specimens of patients undergoing carotid or ilio-femoral endarterectomy surgery in two large tertiary referral hospitals in the Netherlands: the St. Antonius Hospital

in Nieuwegein and the University Medical Center in Utrecht[21]. Plaque removal is conducted according to local and international guidelines, either by direct thrombendarterectomy or remote endarterectomy[22]. The use of a patch was at the clinician's discretion. Clinical data was prospectively collected from patient files and standardized questionnaires.

For this study, the first entry of iliac and femoral endarterectomy for each unique patient was included, without any exclusion criteria. This enabled us to investigate the effect of one of the most prevalent comorbidities in PAD on plaque histology and to compare this with existing literature on other plaque domains such as carotid and coronary plaques. Presence of DM was defined as presence of one of the following: 1) diabetes in medical history extracted from the patient file, 2) the use of either insulin or oral glucose inhibitors extracted from the patient file or 3) self-reported diabetes in the patient questionnaire. Duration of the disease was extracted from the patient questionnaire. A restenotic artery at baseline was defined as surgery on an artery already treated in the past, either by percutaneous intervention or by surgery. Follow-up data during a three-year period was obtained through questionnaires sent to patients. Secondary cardiovascular events were validated using health records kept by general practitioners. Composite secondary cardiovascular events were defined as myocardial infarction (MI), stroke, cardiovascular death or peripheral intervention. Cardiovascular death was defined as one of the following: fatal MI, fatal stroke (either bleeding or ischemic), fatal ruptured abdominal aneurysm, fatal heart failure, sudden death. Stroke was defined as neurological symptoms lasting >24 hours and diagnosed by a neurologist as a stroke. A history of coronary artery disease was defined as either one of the following: MI, percutaneous coronary intervention or coronary artery bypass grafting surgery. Peripheral interventions consisted of either surgery or percutaneous events, including thrombolysis, in any artery other than the coronary arteries or the aorta. Patients could have had more than one event, but only the first occurrence of any of the secondary endpoints was used for the survival analyses on composite endpoints. To determine a measure of patency, target vessel revascularization (TVR) was defined during a three-year follow-up as peripheral reintervention on the same operation side and artery as the entry surgery. If patients suffered from more than one TVR, the first was used as an endpoint for the survival analysis of TVR-free survival. The study was approved by respective medical ethics boards of both hospitals. The study is conducted in accordance with the declaration of Helsinki. All patients provided written informed consent prior to participation in this study.

Sample collection

A detailed description of the sample collection within the Athero-Express biobank can be found elsewhere[21]. In short, blood is collected preoperatively from the radial artery and subsequently stored at -80 degrees. The plaque is processed immediately after surgical removal. The culprit lesion is identified, stored in 4% formaldehyde, decalcified (softening the calcification in the plaque for handling purposes without fully dissolving

it) and embedded in paraffin for histological analysis. The rest of the plaque is cut into pieces of 5 mm and stored at -80 degrees.

Histological assessment

The transverse cross-sections of plaque are used for histological assessment[23]. Plaque specimens were stained for macrophages (CD68), smooth muscle cells (α-actin), collagen (picro-sirius red), extent of calcification (haematoxylin-eosin, HE) and microvessels (CD34). The presence of plaque thrombosis was determined using a combination of luminal thrombi, intraplaque haemorrhage, HE-staining and the presence of fibrin by Mallory's phosphotungstic acid-haematoxylin staining. The presence of either luminal thrombosis, intraplaque haemorrhage or both was considered as positive plaque thrombosis. Computerized analyses were used to quantify macrophages and smooth muscle cells as percentage of plaque area. Microvessels were counted in three hotspots after morphological identification and averaged per slide[24]. Collagen and calcification were scored semiquantitatively into no, minor, moderate or heavy staining at 40x magnification. These categories were grouped into bins (no/minor and moderate/heavy) for the present analyses, where "no/minor" represents absent staining or staining with a few clustered cells and "moderate/heavy" represents larger areas of positive staining. The size of the lipid core was assessed using polarized light and cut off at an area of 10% of the plaque. Digital image microscopy (AnalySiS version 3.2, Soft Imaging GmbH, Munster Germany) was used for quantitative plaque characteristics and for determination of plaque area by tracing the internal elastic lamina as described before[25]. All histological slides were assessed by the same blinded technician[23].

Statistical analysis

Baseline characteristics were compared between patients with and without diabetes using Mann-Whitney U tests for non-normally distributed continuous variables and χ^2 tests for categorical variables. The data were imputed using single imputation. All variables with a p-value <.1 were considered possible confounders for the association between DM and plaque characteristics or outcome. Possible confounders were univariably tested against plaque characteristics or outcome, and if an association was found (cut-off point p<.1), the variable was added to the final model of the association between DM and plaque characteristics or outcome. If in the univariable analysis a possible confounder had been identified with any of the plaque characteristics, it was added to the final models of the association between DM and plaque characteristics. Possible confounders that were identified were BMI, hypercholesterolaemia, current smoking, hypertension, history of coronary artery disease, total cholesterol levels, LDL cholesterol levels and statin use. If in the univariable analysis a possible confounder was significantly associated with the composite endpoint, it was added to the final model of the association between DM and outcome. Possible confounders determined this way were hypercholesterolaemia, history of coronary artery disease, previous leg amputation, Fontaine classification, and statin use.

We used logistic regression models to determine the difference between patients with and without diabetes on different binary plaque characteristics. Linear models were used to determine this difference between log-transformed continuous plaque characteristics. A Cox proportional hazards model was used to determine the difference between patients with and without diabetes on composite cardiovascular events and TVR during 3-year follow-up. Kaplan Meier survival estimates were used for the analyses of the effect of DM in subgroups, in which strata were compared with log-rank tests. Values with a $P < .05$ were considered statistically significant. Single imputation was carried out with the R computing platform, version 3.0.2. All other analyses were carried out in SPSS version 21.0.

Results

Baseline characteristics

A total of 651 patients were included in this study, of which 198 (30.4%) were diagnosed with DM at the time of inclusion. Of these patients with diabetes, 64 (32.3%) were using insulin. Cardiovascular risk factors such as high BMI, high blood pressure and self-reported hypercholesterolemia were more prevalent in DM patients. Patients with diabetes smoked less and were more often treated with statins, probably as a consequence of secondary prevention strategies, resulting in lower cholesterol levels compared with the patients without diabetes. Patients with DM presented with longer duration of PAD symptoms and had a more severe clinical indication for surgery, including tissue loss (34.1% versus 16%), and rest pain (26.3% vs 21.6%), between patients with and without DM respectively. Patients with DM more often had a history of vascular events, including (partial) amputation of the contralateral leg. All patient characteristics are shown in Table 1.

Plaque phenotype

To determine the impact of DM on peripheral plaque phenotypes, seven plaque phenotypes were compared between patients with and without diabetes (Supplemental table 1). After correcting for possible confounders using a multivariable logistic regression model, patients with diabetes had significantly more calcification in their plaques compared with patients without diabetes (OR 2.11 (95% CI 1.43-3.12), $P < .01$, Supplemental figure 1). No other histological plaque characteristic differed significantly between the two groups (Table 2).

Clinical outcomes

To assess whether DM influences clinical outcome in patients with PAD, the incidence of composite secondary cardiovascular outcome rate during three-year follow-up was analyzed. Three-year follow-up information was available on 635/651 (97.5%) of patients (Table 3, Supplemental figure 2).

Table 1. Baseline characteristics

	Patients without diabetes mellitus n = 453 n (%)	Patients with diabetes mellitus n = 198 n (%)	P - value
Male sex	325 (71.7)	153 (77.3)	.14
Age in years (median; IQR)	68; 61-74	69; 63-74	.15
BMI (median; IQR)	25.2; 22.6-27.7	27.0; 24.4-29.8	<.01 [a]
Current smoker	203 (45.5)	66 (33.5)	<.01 [a]
Hypertension	309 (69.1)	154 (80.2)	<.01 [a]
Hypercholesterolaemia	277 (66.3)	132 (77.2)	<.01 [a]
History of CAD (coronary)	170 (37.5)	96 (48.5)	<.01 [a]
History of stroke	21 (4.8)	9 (4.7)	.96
History of (leg) amputation	12 (2.8)	17 (9.1)	<.01 [a]
Fontaine classification			<.01 [a]
- Fontaine IIb	230 (57.6)	74 (44.3)	
- Fontaine III	105 (26.3)	36 (21.6)	
- Fontaine IV	64 (16.0)	57 (34.1)	
Stenosis grade[b]			.84
- 50-70%	32 (9.4)	13 (7.9)	
- 70-99%	98 (28.7)	47 (28.5)	
- Occlusion	211 (61.9)	105 (63.6)	
Contralateral stenosis[b]			.51
- 0-50%	68 (39.3)	24 (34.8)	
- 50-100%	105 (60.7)	45 (65.2)	
Operated artery			.25
- Femoral	398 (89.8)	179 (92.7)	
- Iliac	45 (10.2)	14 (7.3)	
Operation Type			.55
- REA	117 (27.5)	46 (25.1)	
- TEA	309 (72.5)	137 (74.9)	
Plaque area in mm^2 (median; IQR)	18.8 (13.7-27.1)	18.9 (11.3-27.7)	.93
Restenosis[c]	59 (14.6)	24 (13.5)	.72
Ankle-brachial index (median; IQR)	.59; .46-.71	.60; .43-.69	.59
Years since diagnosis PAD	5 (1-10)	6 (3-10)	.04 [a]
eGFR in mL/min/1.73 m^2 (median; IQR)	74.5; 59.4 – 96.0	77.9; 57.1 – 101.0	.87
Triglycerides in mg/dL (median; IQR)	140.7; 100.9-192.0	161.5; 103.5-196.7	.31
Total cholesterol in mg/dL (median; IQR)	184.9; 158.3-216.2	167.2; 138.2-195.8	<.01 [a]
HDL in mg/dL (median; IQR)	44.1; 35.5-52.3	44.3; 36.7-52.5	.95
LDL in mg/dL (median; IQR)	104.2; 84.4-128.6	81.9; 61.8-103.1	<.01 [a]
Glucose in mmol/L (median; IQR)	5.7; 5.2-6.2	7.7; 6.2-9.3	<.01 [a]
Use of statins	327 (72.3)	157 (79.3)	.06 [a]
Use of antiplatelets	379 (84.0)	157 (79.3)	.14

Table 1. *Continued*

	Patients without diabetes mellitus n = 453 n (%)	Patients with diabetes mellitus n = 198 n (%)	P - value
Use of anti-coagulants	72 (15.9)	41 (20.7)	.14
Use of oral antidiabetics	-	136/198 (68.7)	*NA*
Use of insulin	-	64/198 (32.3)	*NA*
Years since diagnosis DM (median; IQR)		9.5 (4.0-15.3)	*NA*

DM: diabetes mellitus; IQR: interquartile range; CAD: coronary artery disease; PAD: peripheral arterial disease; REA: remote endarterectomy; TEA: thrombendarterectomy; GFR: glomerular filtration rate;
[a] p<0.05
[b] stenosis grade of the operated artery and its contralateral counterpart
[c] restenosis was defined as surgery on an artery already treated in the past, either by percutaneous intervention or by surgery

After correcting for possible confounders with the use of a multivariable cox regression model, DM was an independent predictor for secondary manifestations of cardiovascular disease (HR DM 1.36 (95% CI 1.07-1.72), P = .01) (Figure 1). In patients undergoing peripheral reintervention, no difference in TVR between DM patients (31.5%) and non-DM patients (30.0%) was observed, (Kaplan Meier mean survival estimate 2.16 years (1.88-2.43) in DM patients vs 2.16 years (1.97-2.35) in non-DM patients, P-log rank = .86 (Supplemental figure 3)).

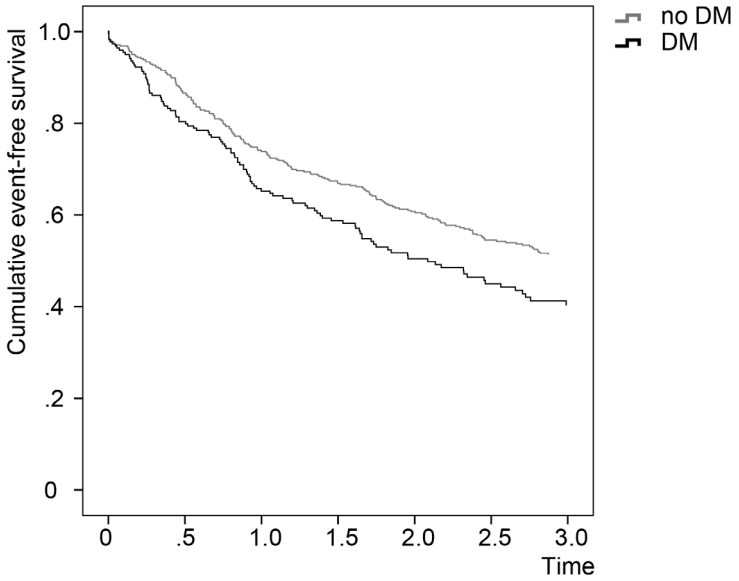

Figure 1. Cox survival plot for event-free survival for composite cardiovascular events of patients with and without diabetes
HR DM = 1.36 (95% CI 1.07-1.72), p=0.01

Table 2. Association of diabetes with plaque phenotype

Plaque characteristic	Beta value of diabetes (95% CI)	OR of diabetes (95% CI)	p-value
Calcified plaque	*NA*	2.11 (1.43-3.12)	<.01*
Collagen rich plaque	*NA*	1.60 (.93-2.74)	.09
Lipid core >10%	*NA*	.98 (.63-1.53)	.92
Presence of IPH	*NA*	.73 (.51-1.04)	.08
Macrophages[a]	-.13 (-.46 – .20)	*NA*	.44
Smooth muscle cells[a]	-.06 (-.35 – .22)	*NA*	.67
Vessel density[b]	.08 (-.17 – .33)	*NA*	.52

OR: odds ratio; CI: confidence interval; IPH: intraplaque haemorrhage. Model corrected for BMI, hypercholesterolaemia, current smoking, hypertension, history of CAD, total cholesterol, LDL cholesterol, glucose and statin use. * p<0.05. [a] log-transformed % of plaque area. [b] log-transformed number of vessels per hotspot

Table 3. Occurrences of clinical outcomes in patients with and without diabetes

	Patients without diabetes n = 453 (%)	Patients with diabetes n = 198 (%)
Composite endpoints[a]	198/439 (45.1)	112/196 (57.1)
Cardiovascular death	27/439 (6.2)	21/196 (10.7)
Coronary artery disease	35/439 (8.0)	15/196 (7.7)
Stroke	11/439 (2.5)	6/196 (3.1)
Peripheral intervention		
PTA or TEA	162/439 (36.9)	90/196 (45.9)
of which TVR[b]	*51/162 (31.5)*	*27/90 (30.0)*
Leg amputation	*51/162 (31.5)*	24/196 (12.2)
of which ipsilateral	x*16/19 (84.2)*	*18/24 (75.0)*

CAD: coronary arterial disease, PTA: percutaneous transluminal angioplasty, TEA: thrombendarterectomy. Model corrected for hypercholesterolaemia, history of CAD, previous leg amputation, Fontaine classification and statin use. [a] *Composite cardiovascular endpoints include cardiovascular death, coronary artery disease, stroke and peripheral intervention.* [b] *TVR: target vessel revascularization, intervention (either surgical or endovascular) on the artery that was operated on during the index surgery.*

Effect of restenosis

As restenotic plaques show significantly different histological features[26,27], a subanalysis on restenotic patients was performed. Data on the incidence of previous intervention (either percutaneous or surgical) on the same artery was available in 582 (89.4%) of the patients. Of these, 83 (14.3%) were reinterventions. We observed the same direction of effect of diabetes on calcification in restenotic plaques (70.8% vs 48.3% moderate/heavy calcification in patients with and without diabetes respectively, P = .06) as in the de novo operated patients (74.5% vs 55.7% moderate/heavy calcification in patients with and without diabetes respectively, P < .01).

Furthermore, analysis showed a similar effect of diabetes on composite event-free survival in patients suffering from restenosis (mean survival time estimate 1.5 (95% CI: 1.0-2.0) vs 1.9 (95% CI: 1.6-2.2) years for patients with and without diabetes respectively, log-rank test P = .17). Patients with diabetes who were operated on de novo lesions had a decreased composite event-free survival (mean survival estimate 1.8 (95% CI: 1.7-2.0) vs 2.2 (95% CI: 2.1-2.3) years for patients with and without diabetes respectively, log-rank test P = <.01, Supplemental figure 4).

Effect of duration of DM

Most detrimental vascular effects of DM arise after years of exposure to hyperglycaemia, and we hypothesized an association might exist between length of suffering from DM before surgery and plaque characteristics. Within the cohort of patients with diabetes (n=198), data of duration of diabetes was available in 130 patients (66%). No association was observed between the severity of calcification of the plaque and duration of diabetes (median of 10 years (interquartile range (IQR) 5.25-15) vs median of 8 years (IQR 3.5-15.5) for no/minor vs moderate/heavy calcification respectively, Supplemental figure 5).
Moreover, when stratified on the median of years of suffering from DM (9.5 years), no association was found of longer duration of DM with composite event-free survival (mean survival time estimate 1.9 (95% CI 1.6-2.1) vs 1.7 (1.4-2.0) for DM diagnosis >9.5 years ago vs =<9.5 years ago respectively, log-rank test P = .57, Supplemental figure 6).

Discussion

In 651 consecutive iliac and femoral endarterectomy patients significantly more calcification in the atherosclerotic plaque was found (OR 2.11 (95% CI 1.43-3.12), P < .01) and a higher occurrence of secondary cardiovascular events during three-year follow-up (HR DM 1.36 (95% CI 1.07-1.72), P = .01) in patients with diabetes as compared to patients without diabetes.
Although one might expect a relation between duration of symptoms on plaque calcification as a measure of plaque maturation, this association was not observed in our data. Patients with DM had longer duration of symptoms than patients without DM, but this was not associated with increased calcification. Neither did we observe an association of duration of DM with plaque calcification. This might implicate that exposure time to hyperglycaemia does not increase calcification burden in plaques of DM patients operated for PAD. These findings resemble imaging studies of patients with diabetes which also showed a more calcified plaque in PAD lesions[14,16]. However, not all imaging studies found this association[28]. Calcification may lead to thrombotic events in patients already suffering from hypercoagulability[3,6,29]. Though previous analyses showed no effect of increased calcification on secondary clinical outcomes in carotid arteries[24], further research into the effect of plaque characteristics on secondary clinical outcome is needed in PAD patients.

When we compare our plaque findings with earlier published histological plaque analyses in different arteries, striking differences can be noted. In a large cohort (n=1455) of our own Athero-Express biobank, no differences were found between carotid endarterectomy plaques of patients with and without diabetes[12]. This difference could very well be due to the late stage intervention in ilio-femoral artery stenosis, where patients first undergo walking exercise and gradual remodelling of an unstable into a stable fibrous lesion is more likely to occur. These late stage interventions are in sharp contrast with carotid surgery, which is increasingly executed within days to weeks following a cerebrovascular event. Likewise, we were not able to replicate the larger necrotic core and increased inflammation previously found in coronary plaques from sudden cardiac death patients, although these plaques are also derived from acute events, which might also reflect a different stage of remodelling[5,30,31].

Clinical outcome was worse in the patients with diabetes, including a total of 10.7% cardiovascular deaths in three years, in accordance with current literature[9]. A 3-fold increase in leg-amputation was observed in patients with diabetes within 3 years of initial inclusion (12.2% vs 4.3%), although literature reported even higher amputation rates of up to 30%[9]. Surprisingly, no difference was seen in MI or stroke. This could be a reflection of the overall poor three-year prognosis of the whole cohort, independent of diabetes. Another possibility is that secondary prevention programs in patients with DM lead to prevention of more acute disease in the macro vasculature. One could speculate that such programs are less able to prevent peripheral microvascular alterations seen in patients with diabetes, which is reflected by high rates of peripheral interventions and high amputation rates observed within this cohort. Another explanation for the higher amputation rates could be decreased sensitivity towards lower limb wound or ulcers as a result of diabetic neuropathy[4,8]. Moreover, diabetes is known to lead to constrictive plaque remodelling[3]. Surprisingly, comparison of TVR between patients with and without diabetes operated on for ilio-femoral disease showed no increased risk of restenotic lesions in patients with DM that require reintervention. This indicates that other secondary events account for the significantly higher composite cardiovascular events in patients with diabetes.

A previous analysis in our cohort showed that patients with a larger plaque area on histological examination are more prone to secondary cardiovascular events[25]. Plaque area as a potential confounding factor in the relationship of diabetes and secondary outcome was examined, but no evidence for any association was found.

This study has some limitations. First, all patients underwent surgical therapy for PAD, which is most often preceded by a period of optimal conservative therapy. We are, therefore, most likely observing the remodelling phase of the peripheral plaque and cannot make any statements about plaque initiation. In fact, this study still observes large differences between DM and non-DM patients, maybe even pointing out that a more calcified plaque in patients with diabetes is a mere reflection of disturbed plaque remodelling due to endothelial dysfunction in this patient group[8,9]. This mechanism could also be the explanation of the absence of DM-specific characteristics in plaques derived

from semi-acutely operated patients with carotid artery disease[3]. Second, TVR was used as a proxy for patency rates, which is normally used to measure durability of vascular reconstruction. Unfortunately the current study protocol and ethical approval allow only for prospective data collection of events during follow-up with the use of questionnaires. Furthermore, as the UMC Utrecht and the St. Antonius Hospital Nieuwegein are tertiary referral centers, many of the patients return to their peripheral hospital after the surgery for postoperative follow-up. As a result, no systematic duplex follow-up for our patients is available, and patency, including patency rates, are hard to define in this cohort. The use of TVR could be an incomplete measure. Therefore, our results might underestimate restenosis. However, this effect is most likely equally divided between both patients with and without diabetes. Similarly, data regarding concomitant endovascular procedures, which could have a positive effect on the number of secondary peripheral procedures, were not available in the Athero-Express biobank study, so we were not able to correct for this effect. Yet, we do not think this effect differs between patients with and without diabetes and therefore cannot confound the observed effect. Furthermore, the Athero-Express Biobank Study unfortunately does not contain information regarding the type of diabetes of which patients suffer from. This prohibits us from making any claims as to what extent patients with type I diabetes differ from patients with type II diabetes. In addition, arteries below the knee were not covered by our biobank as these are either treated conservatively or with percutaneous or endovascular procedures. The large effect of DM on below the knee arteries could explain the increased rate of leg amputations[4,11]. Next, the Athero-Express Biobank Study does not collect information regarding previous radiation therapy for pelvic or inguinal malignancies. Although we think the occurrence of these therapies are evenly distributed between patients with and without diabetes, patients with diabetes have a higher chance of developing malignancies and as radiation therapy gives rise to arterial occlusive disease, this could have confounded the relationship between more diabetes and a more calcified plaque. Last, although our biobank has been collecting plaque material since 2002, a number of 198 patients with diabetes remains modest. Still our analysis holds, as far as we know, the first comparison of plaque characterization in a cohort of patients undergoing ilio-femoral endarterectomy. As the search for modifiable causative predictors of secondary cardiovascular outcome is ongoing, we would encourage other researchers to analyze large patient groups with a variety of cardiovascular risk factors and to collect long-term follow-up.

Overall, patients with diabetes, presenting with PAD requiring surgical revascularization belong to a very vulnerable subgroup of an already threatened group of patients. PAD patients often present with multiple other comorbidities and often do not achieve as adequate risk factor control as other (cardiovascular) patients[32]. We observed more secondary cardiovascular events during follow-up in the patient group with DM and PAD, which leads to increased disease burden. Therefore, these patients need to be under tight surveillance to optimize disease control and (early) treatment of possible comorbidities[3,8].

Conclusion

Patients with diabetes who undergo surgical revascularization for PAD with the use of thrombendarterectomy or remote endarterectomy have a more calcified atherosclerotic plaque and an increased incidence in composite cardiovascular events, but no increase in target vessel revascularization.

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Supplemental material

Supplemental table 1. Prevalence of plaque characteristics

Supplemental figure 1. Example of a femoral atherosclerotic plaque with major calcification from a patient with diabetes mellitus and of a femoral atherosclerotic plaque with no calcification from a patient without diabetes mellitus.

Supplemental figure 2. Kaplan-Meier survival curve for event-free survival for peripheral events (A), cardiovascular death (B) and leg amputation (C) for patients with and without diabetes.

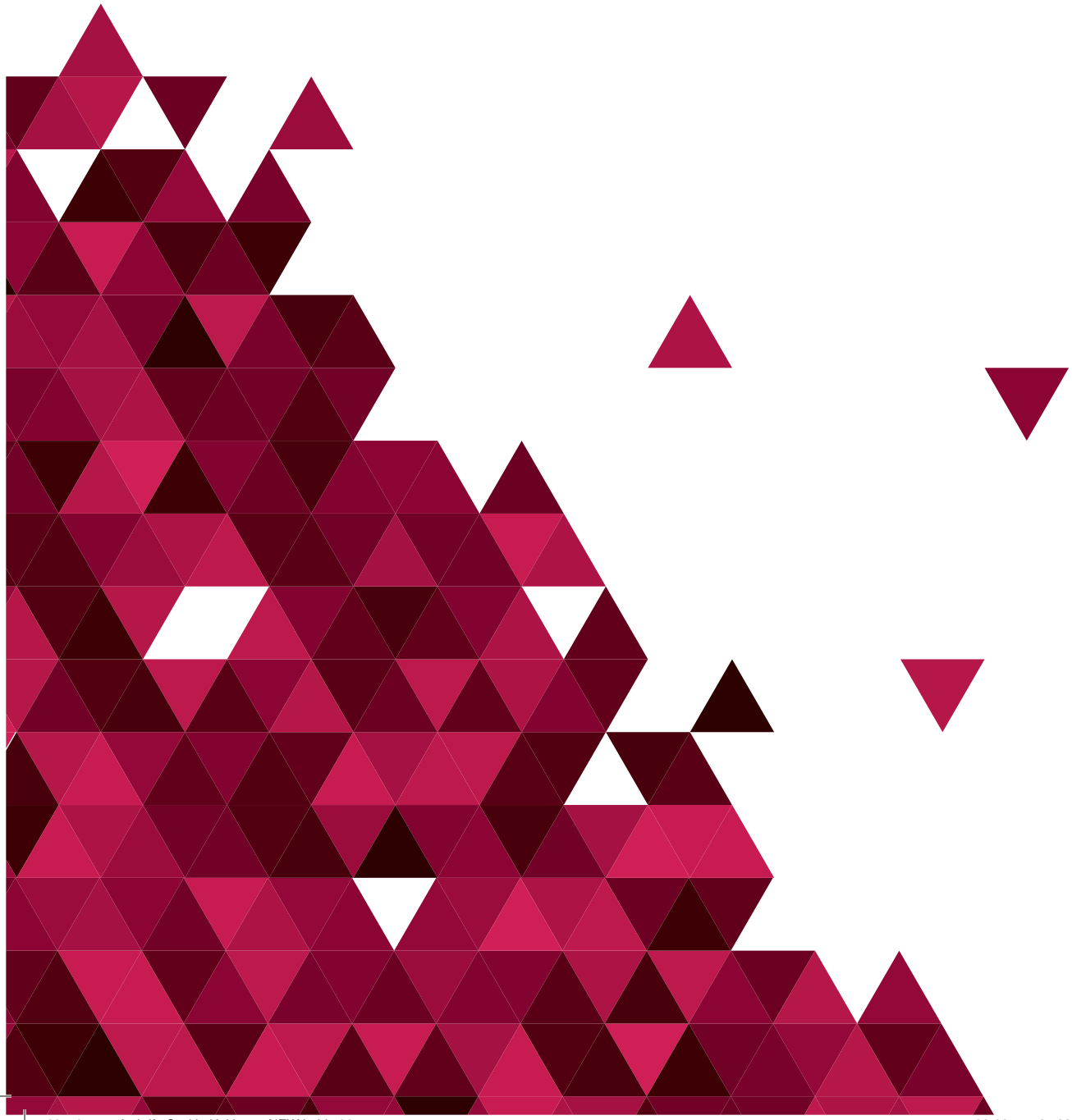
Supplemental figure 3. Kaplan-Meier survival curve for TVR-free survival in patients undergoing peripheral revascularization with and without diabetes

Supplemental figure 4. Kaplan Meier survival curve for composite event-free survival for patients operated for restenosis (A) and de novo (B) for patients with and without diabetes.

Supplemental figure 5. Distribution of no/minor calcification and moderate/heavy calcification for duration of diabetes in years.

Supplemental figure 6. Kaplan Meier survival curve for composite event-free survival for patients with duration =< 9.5 years and >9.5 of diabetes mellitus.

Supplemental material is omitted because of space limitations



CHAPTER 6

Time-dependent differences in femoral artery plaque characteristics of peripheral arterial disease patients

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Abstract

Aim

Peripheral arterial disease (PAD) is a common manifestation of atherosclerosis with an increasing incidence worldwide. The disease is still associated with high morbidity and mortality risks. Previous research in carotid arteries indicates that atherosclerotic plaque characteristics have stabilized over time in patients considered for surgery. It is currently unknown whether this time-dependent stabilization occurs in ilio-femoral arteries as well. Our objective was to analyze whether local ilio-femoral atherosclerotic plaque characteristics have changed over time.

Methods

497 patients within the Athero-Express biobank who underwent primary endarterectomy of the iliac or femoral artery between 2002 and 2013 were analyzed. We investigated six histological plaque characteristics: calcification, collagen, fat content, intraplaque haemorrhage, macrophages and smooth muscle cells.

Results

Over the course of 10 years we observed a lower percentage of all plaque characteristics that are considered indicators of a vulnerable plaque, such as: plaques with a large lipid core from 37.9% to 14.9% and plaques with intraplaque haemorrhage from 69.0% to 34.8% when the two-year cohorts 2003-2004 and 2011-2012 were compared, respectively. Multivariable analyses showed that time-dependent changes occurred independent of changing procedural and patient characteristics.

Conclusions

In this cohort of peripheral arterial disease patients undergoing primary endarterectomy, we observed a time dependent shift of plaque characteristics towards a less lipid rich lesion with less intraplaque haemorrhage. These findings indicate research in cardiovascular disease would benefit from contemporary patient characteristics and plaque specimens to optimize translational potential.

Introduction

The prevalence of peripheral arterial disease (PAD) is increasing worldwide, with a current estimated prevalence of 200 million people in the world. Expanding obesity rates, and a higher incidence of diabetes and smoking, will only increase the number of affected people, especially in high and middle income countries[1]. Although vascular risk factor management and improved (endovascular) treatment for atherosclerotic disease have lowered the disease burden and prolonged the survival of PAD patients, the disease is still associated with high morbidity and mortality risks[2,3]. Nonetheless, recent reports have shown that in patients presenting with claudication complaints, there is a sharp decline in the number of lower leg amputations[4,5]. Several explanations for this decline have been proposed, such as the importance of early screening, preventive (medical) care and the increased use of endovascular revascularization after 2000[5].

In patients undergoing carotid endarterectomy for atherosclerotic lesions, it has been shown that the characteristics of the lesions have shifted significantly over the last decade towards less lipid rich and less inflammatory plaques[6]. In coronary arteries atherosclerotic plaque characteristics have not been studied over time. However, we are facing an evident absolute decrease in the number of ST elevated myocardial infarctions[7,8], with a relative increase of the number of reported non ST elevated myocardial infarctions. It is well established that ST elevated myocardial infarctions are associated with more lipid rich inflammatory lesion characteristics[9]. The temporal changes in plaque characteristics may be a consequence of improved treatment strategies (e.g. cholesterol lowering drugs) or life style changes such as reduction of passive smoking[10]. A simultaneous time-dependent shift in ilio-femoral plaque characteristics towards less destabilizing characteristics could go hand in hand with less progression of disease and the observed improved outcome, such as the lower incidence of leg amputations. It is currently unknown if such a change in plaque characteristics has occurred in symptomatic ilio-femoral artery lesions.

Several atherosclerotic plaque characteristics are considered to represent stable and unstable features that could give rise to a thrombotic occlusive vascular event. Characteristics of unstable plaques include active inflammation, a thin fibrous cap with a large lipid core and intraplaque hemorrhage (IPH)[11,12]. Fibrotic lesions are regarded as hallmarks of more matured and stabilized plaques[13].

To investigate the systemic nature of the differences in plaque characteristics over time, we assessed whether characteristics of ilio-femoral plaques, which were dissected from patients with peripheral arterial disease, have changed over a 10-year period.

Methods

Patient population

All patients undergoing carotid and ilio-femoral endarterectomy surgery in two large tertiary referral hospitals in the Netherlands (the St. Antonius Hospital in Nieuwegein and the University Medical Center in Utrecht) are asked to participate in the Athero-Express biobank study. This prospective ongoing biobank study includes the blood and plaque specimens of these patients as well as three year follow-up data from patient files and through standardized questionnaires[14]. Experienced surgeons removed the plaques in accordance with local and international guidelines[3]. This study is conducted conform the declaration of Helsinki and has been approved by the ethical board in both hospitals. Patients gave informed consent prior to inclusion in the study.

In and exclusion criteria

All patients undergoing iliac or femoral endarterectomy in either one of the hospitals between 2002 and 2013, with an available plaque in the Athero-Express biobank, were included in the current analysis. Standardized questionnaires and patient files were used to collect clinical data. Research indicates that restenotic plaques have different characteristics, therefore we decided to only include primary endarterectomies and to exclude all patients with previous treatment in the target vessel[15,16].

Sample collection

The sample collection protocol of the Athero-Express biobank has been described elsewhere in detail[14]. To summarize: preoperatively, blood is collected and stored at -80 degrees. Immediately after surgery the plaque was processed. A trained technician selected the culprit lesion, i.e. the segment with the smallest lumen. In case of a total occlusion, the segment with the largest plaque diameter was chosen. This segment was stored in 4% formaldehyde, decalcified, and embedded in paraffin for histological analysis; the rest of the plaque was stored at -80 degrees.

Histological assessment

Histological slides were assessed with the use of a previously validated protocol[17]. In brief, transverse cross-sections through the culprit lesions of the plaques were made by an experienced technician and per patient stained for each of the following: CD68 (macrophages), α-actin (smooth muscle cells/myofibroblasts) and picro-sirius red and elastin von Gieson (collagen). Plaque thrombosis was considered to be present if either luminal thrombosis, intra-plaque hemorrhage or both were present in the cross-sections. Hematoxylin-eosin and Mallory's phosphotungstic acid-hematoxylin staining (fibrin) were used for determination of IPH. Collagen and calcification were scored semiquantitatively at 40x magnification and binned into binary groups of no/minor and moderate/heavy staining for current analysis, where "no/minor" represents absent staining or staining with a few clustered cells, and "moderate/heavy" represents larger

areas of positive staining. The size of the lipid core was assessed with the use of polarized light and cut off at an area of 10% of the plaque. Furthermore, computerized analyses were used to quantify macrophages and smooth muscle cells as percentage of plaque area (AnalySiS version 3.2, Soft Imaging GmbH, Munster Germany). The plaques were assessed directly after staining. The same experienced technician assessed all histological slides[17]. Several validity checks have been performed in the Athero-Express biobank over the years, such as inter-segmental plaque differences, and inter- and intra-observer variability, which showed excellent replication[17,18].

Statistical analysis

To evaluate whether plaque characteristics differed over time we split the cohort into groups of two subsequent operation years. The analyses were based on methods previously described[6]. The data were imputed using single imputation. We used chi-square tests to compare categorical baseline characteristics of patients across the different time cohorts and Kruskall-Wallis tests for continuous, non-normally distributed variables. Logistic regression models were used to study the association of operation year with binary plaque characteristics and linear regression models for continuous plaque characteristics. For multivariable analyses, we added the time-dependent baseline characteristics that associated ($p<0.1$) with the plaque characteristic to the multivariable model for each plaque characteristic separately (for calcification: operation type, for collagen: total cholesterol, for lipid core: stenosis grade, for IPH: hypertension and anticoagulant use, for smooth muscle cells: hypertension and operation type, and for macrophages: hypertension, stenosis grade, operation type, total cholesterol and LDL cholesterol). Values with a $p<0.05$ were considered statistically significant. The R computing platform version 3.0.2 was used to carry out single imputation. SPSS version 21.0 was used for all other analyses.

Results

Patient population

A total of 651 unique femoral and iliac endarterectomy patients were included in the Athero-Express biobank study between 2002 and 2013. After removal of all restenotic lesions (n = 154) from the database, 497 patients were included in the present analyses. The majority of included patients were male (72.8%) with a median age of 69 years (61-75 IQR). 44.4% of patients suffered from chronic rest pain or critical limb ischemia (Fontaine III-IV) and 63.2.% was treated for an occluded segment in the target artery (Supplemental table 1).

Table 1. Baseline characteristics

	Cohort (years)					
	2003-2004	**2005-2006**	**2007-2008**	**2009-2010**	**2011-2012**	**P value**
Number of patients	87	127	71	82	118	
Male sex	62 (71.3)	100 (78.7)	49 (69.0)	55 (67.1)	90 (76.3)	0.917
Age in years (median; IQR)	67 (59-74)	68 (59-76)	68 (62-74)	69 (63-73)	70 (63-75)	0.459
BMI (median; IQR)	26.5 (22.9-29.0)	25.5 (23.3-28.3)	25.3 (22.9-29.0)	25.8 (23.4-28.9)	26.1 (23.1-27.8)	0.977
Current smoker	44 (50.6)	55 (44.0)	25 (36.2)	36 (44.4)	51 (43.6)	0.448
Diabetes	25 (28.7)	44 (34.6)	25 (35.2)	26 (31.7)	32 (27.1)	0.531
Hypertension	62 (72.1)	75 (59.1)	50 (72.5)	62 (77.5)	92 (80.7)	0.005 *
Hypercholesterolaemia	59 (68.6)	76 (60.3)	46 (73.0)	43 (69.4)	73 (70.2)	0.337
History of CAD	31 (35.6)	50 (39.4)	31 (43.7)	28 (34.1)	48 (40.7)	0.729
History of stroke	16 (18.6)	22 (17.6)	7 (9.9)	9 (11.3)	13 (11.3)	0.059 *
History of PAD	38 (43.7)	50 (39.4)	27 (38.0)	32 (39.0)	45 (38.1)	0.494
History of Amputation	3 (3.5)	7 (5.5)	1 (1.6)	2 (2.9)	4 (3.4)	0.607
Amputation during 3-year FU	3 (3.5)	6 (4.8)	7 (10.1)	6 (7.6)	5 (4.4)	0.636
Duration of CI, years (median; IQR)	5 (2-8)	5 (1-10)	3 (1-9)	3 (1-8)	4 (2-9)	0.451
Fontaine Classification						0.014 *
Fontaine IIb	52 (61.9)	75 (60.0)	33 (54.1)	30 (51.7)	43 (46.7)	
Fontaine III	18 (21.4)	27 (21.6)	13 (21.3)	20 (34.5)	21 (22.8)	
Fontaine IV	14 (16.7)	23 (18.4)	15 (24.6)	8 (13.8)	28 (30.4)	
Stenosis grade						0.015 *
50-70%	2 (2.7)	8 (8.5)	1 (2.1)	1 (1.3)	24 (22.4)	
70-99%	33 (31.1)	23 (24.5)	17 (36.2)	20 (26.0)	27 (25.2)	
Occlusion	49 (66.2)	63 (67.0)	29 (61.7)	56 (72.7)	56 (52.3)	
Stenose contralateral						0.233
0-50%	23 (43.4)	9 (33.3)	3 (60.0)	8 (20.0)	27 (35.5)	
50-100%	30 (56.6)	18 (66.7)	2 (40.0)	32 (80.0)	49 (64.5)	
Operated Artery						0.002 *
Femoral	75 (86.2)	108 (85.7)	62 (91.2)	77 (95.1)	108 (95.6)	
Iliac	12 (13.8)	18 (14.3)	6 (8.8)	4 (4.9)	5 (4.4)	
Operation Type						<0.001 *
REA	40 (46.0)	46 (36.5)	16 (27.1)	15 (18.8)	20 (17.1)	
TEA	47 (54.0)	80 (63.5)	43 (72.9)	65 (81.3)	97 (82.9)	
Ankle-brachial index (median; IQR)	0.62 (0.48-0.72)	0.60 (0.45-0.71)	0.62 (0.46-0.80)	0.58 (0.43-0.70)	0.56 (0.42-0.72)	0.465
eGFR in mL/min/1.73 m^2 (median; IQR)	72.5 (54.7-91.2)	77.9 (58.6-104.0)	80.1 (57.2-101.7)	73.2 (58.8-95.2)	78.3 (65.0-110.6)	0.329

Table 1. *Continued*

	Cohort (years)					
	2003-2004	**2005-2006**	**2007-2008**	**2009-2010**	**2011-2012**	**P value**
Mean arterial pressure (median; IQR)	105 (97-116)	100 (90-107)	103 (92-112)	100 (92-107)	100 (93-111)	0.119
Triglycerides in mmol/L (median; IQR)	2.1 (1.3-2.9)	1.5 (1.1-2.2)	1.9 (1.1-3.0)	1.5 (1.0-2.2)	1.5 (1.2-2.2)	0.015 *
Total cholesterol in mmol/L (median; IQR)	5.3 (4.5-5.9)	4.6 (4.0-5.2)	4.7 (3.8-5.2)	4.8 (4.0-5.5)	4.8 (4.0-5.5)	<0.001 *
HDL in mmol/L (median; IQR)	1.2 (0.9-1.6)	1.2 (1.0-1.4)	1.1 (1.0-1.3)	1.1 (0.9-1.3)	1.1 (0.9-1.3)	0.138
LDL in mmol/L (median; IQR)	2.9 (2.2-3.5)	2.6 (2.1-3.2)	2.3 (1.7-3.2)	2.7 (2.2-3.5	2.7 (2.1-3.4)	0.016 *
Statin use, yes	56 (64.4)	89 (70.1)	58 (81.7)	59 (72.0)	94 (79.7)	0.018 *
Antiplatelet use, yes	74 (85.1)	100 (78.7)	54 (76.1)	76 (93.8)	99 (83.9)	0.290
Anti-coagulant use, yes	16 (18.4)	30 (23.6)	16 (22.5)	5 (6.1)	13 (11.0)	0.005 *
Antihypertensive drug use, yes	68 (78.2)	103 (81.1)	65 (91.5)	61 (74.4)	97 (82.2)	0.861

Categorical variables are depicted as number of patients (percentage), continuous variables are depicted as medians (interquartile ranges). IQR: interquartile range. CAD: coronary artery disease. CI: claudication intermittens FU: follow-up. PAD: peripheral arterial disease. REA: remote endarterectomy, TEA: thrombendarterectomy, HDL: high density lipoproteins, LDL: low density lipoproteins *p<0.05

Patient characteristics alter over time

Patient characteristics altered over time. We observed more patients presenting with severe symptoms as determined by Fontaine classification over the course of 12 years (p=0.014) although we did not observe an increase in amputation during 3-year follow-up. Hypertension became a more prevalent comorbidity in this patient group (p=0.005), but most other clinical parameters, including duration of complaints, did not shift. Medical treatment advanced to an increased use of statins (p=0.018), with concomitant lower triglycerides and cholesterol concentration in patients (p=0.015 and p<0.001, respectively), and less use of anticoagulants (p=0.005) (Table 1).

Ilio-femoral plaque characteristics alter over time

Over the course of 12 years, the plaque characteristics IPH, fat, collagen, calcification, SMC, and macrophages all altered in ilio-femoral atherosclerotic plaques (Table 2, Figures 1 and 2). Most distinct and consistent was the lower number of plaques with a large lipid core (from 37.9% to 14.9%) and the lower number of plaques with the presence of intraplaque haemorrhage (from 69.0% to 34.8%) when comparing the cohorts 2003-2004 with 2011-2012, respectively (Figure 3).

Table 2. Plaque characteristics for two-year cohorts

Plaque characteristics	Cohort 2003/2004 n = 87	Cohort 2005/2006 n = 127	Cohort 2007/2008 n = 71	Cohort 2009/2010 n = 82	Cohort 2011/2012 n = 118	P value
Binary						
Calcification						
Moderate/heavy	54 (62.1)	89 (70.1)	57 (80.3)	47 (57.3)	51 (44.7)	0.001 *
Collagen						
Moderate/heavy	81 (95.3)	105 (84.0)	61 (85.9)	74 (90.2)	92 (80.7)	0.042 *
Fat content						
Fat > 10%	33 (37.9)	31 (24.4)	13 (18.3)	11 (13.4)	17 (14.9)	<0.001 *
IPH						
Present	60 (69.0)	78 (61.4)	36 (50.7)	35 (42.7)	40 (34.8)	<0.001 *
Continuous (computerized)						
Macrophages						
Median (IQR)	0.05 (0.01-0.20)	0.17 (0.03-0.61)	0.12 (0.04-0.58)	0.07 (0.01-0.18)	0.04 (0.02-0.10)	<0.001 *
SMC						
Median (IQR)	0.82 (0.37-1.54)	1.12 (0.55-1.67)	1.10 (0.73-1.60)	0.93 (0.41-1.32)	0.84 (0.44-1.33)	0.021 *

Categorical variables are depicted as number of patients (percentages) for moderate/heavy staining, as opposed to no/minor staining. Continuous variables are depicted as median (inter-quartile range) of log-transformed plaque area. IPH: Intraplaque hemorrhage. SMC: Smooth muscle cells. *p < 0.05

Multivariable analyses

Multivariable analyses showed a time-dependent decrease in: fat, calcification, collagen, the presence IPH, smooth muscle cells, and macrophages, independent from changing patient characteristics and procedural characteristics, when comparing more recent cohorts with the cohort 2003-2004 (Table 3). Particularly consistent was the decline in plaques with high fat content and IPH. Overall, we observed a tendency towards what are considered to be more stable ilio-femoral plaque characteristics.

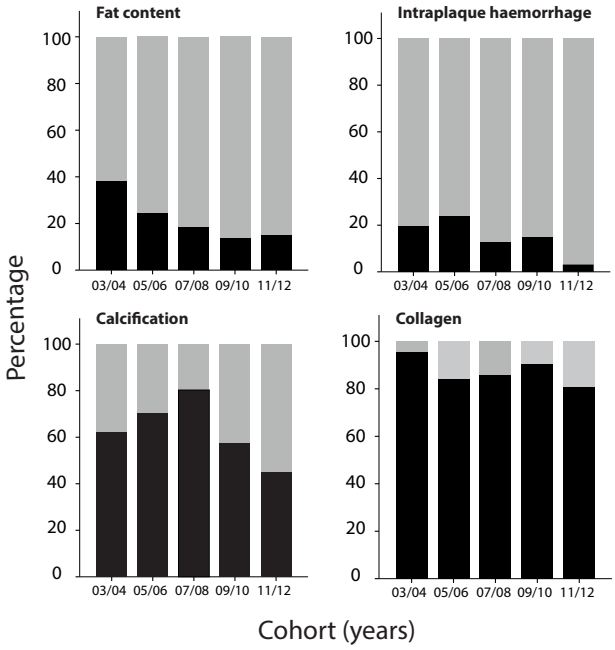

Figure 1. Percentage of plaque characteristics per two-year cohort
Overview of semi-quantitatively scored changing ilio-femoral plaque characteristics over time. Grey: no/minor (no), Black: moderate/major (yes). Fat content: <10% (grey) / >10% (black)

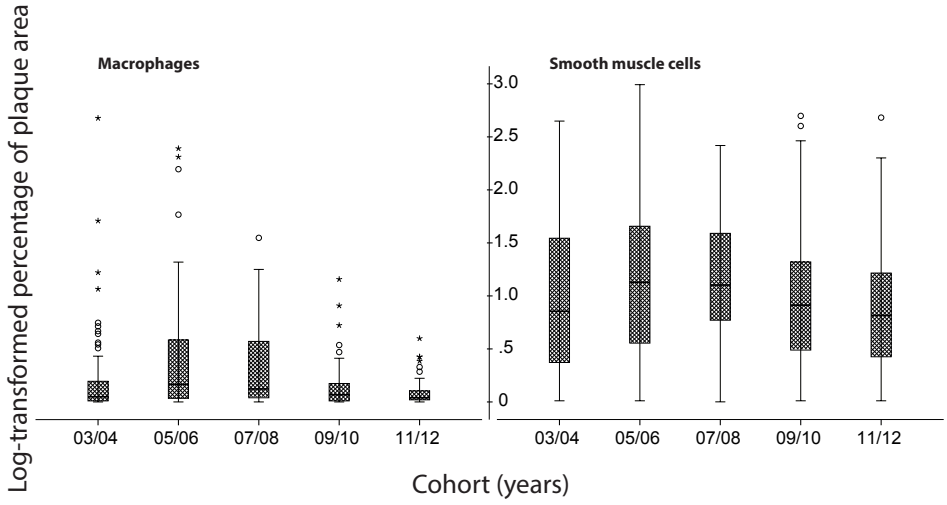

Figure 2. Boxplots of continuous plaque characteristics per two-year cohort
Overview of computerized measurements of ilio-femoral plaquecharacteristics over time. Bullet: 1.5 times IQR outlier, Asterisk: 3 times IQR outlier.

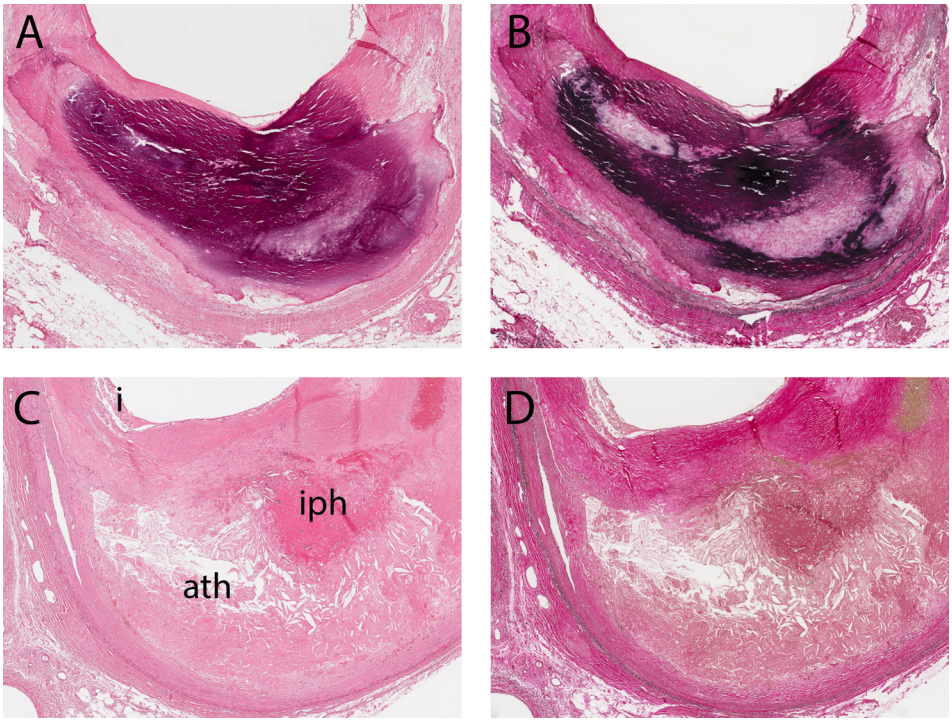

Figure 3. Example of stable and unstable plaque
Representative histological example of stable and unstable atherosclerotic plaque in femoral artery. A and B, stable fibrocalcified plaque with a large calcified area and fibrous connective tissue. C and D, instable fibrous cap atheroma with a large atheroma (ath) with an intraplaque hemorrhage (iph) and inflammation (i) in the shoulder of the plaque. A and C: hematoxylin-eosin stain, B and D: elastin van Gieson stain

Discussion

We analyzed a consecutive cohort of 497 plaques, obtained during femoral and iliac endarterectomy in patients treated for PAD, for differences in plaque characteristics over time. We observed an independent plaque stabilizing trend over the past decade that is in line with observations in other vascular territories.

A number of small studies have investigated post-mortem plaques or plaques in subgroups of patients with PAD, but a large study conducting ilio-femoral plaque histology has been lacking so far[15,19–23]. Current radiological imaging examinations, trying to predict systemic plaque vulnerability, could benefit from increased knowledge concerning risk measures that could be of clinical relevance in femoral and iliac plaques[24,25]. To our knowledge, this is the first large histological study analyzing plaque characteristics, including plaque changes over time, in the ilio-femoral tract. Our results indicate that PAD plaques have independently stabilized in more recent years, even when (changing) patient characteristics are taken into account.

Table 3. Multivariable analysis

Binary plaque characteristics								
	OR of 2005-2006 vs 2003-2004 (95% CI)	**P value**	**OR of 2007-2008 vs 2003-2004 (95% CI)**	**P value**	**OR of 2009-2010 vs 2003-2004 (95% CI)**	**P value**	**OR of 2011-2012 vs 2003-2004 (95% CI)**	**P value**
Calcified plaque								
	1.39 (0.78-2.50)	0.27	1.94 (0.90-4.15)	0.09	0.72 (0.38-1.37)	0.32	0.40 (0.22-0.72)	0.002 *
Collagen rich plaque								
	0.28 (0.09-0.86)	0.03*	0.33 (0.10-1.13)	0.08	0.49 (0.14-1.70)	0.26	0.22 (0.07-0.67)	0.008 *
Fat content >10%								
	0.55 (0.30-1.00)	0.05	0.36 (0.17-0.77)	0.01*	0.25 (0.12-0.55)	<0.001*	0.33 (0.17-0.65)	0.001 *
Presence of IPH								
	0.68 (0.38-1.22)	0.19	0.45 (0.24-0.87)	0.02*	0.35 (0.19-0.67)	0.001*	0.24 (0.13-0.44)	<0.001 *
Continuous computerized plaque characteristics								
	beta of 2005-2006 vs 2003-2004 (95% CI)	**P value**	**beta of 2007-2008 vs 2003-2004 (95% CI)**	**P value**	**beta of 2009-2010 vs 2003-2004 (95% CI)**	**P value**	**beta of 2011-2012 vs 2003-2004 (95% CI)**	**P value**
Macrophages								
	0.16 (0.05-0.27)	0.004*	0.13 (0.00-0.26)	0.05	-0.07 (-0.19-0.06)	0.32	-0.10 (-0.22-0.02)	0.11
Smooth Muscle Cells								
	0.21 (0.03-0.39)	0.02*	0.25 (0.03-0.47)	0.03*	0.07 (-0.13-0.27)	0.49	0.02 (-0.17-0.22)	0.81

Odds ratios (OR) and betas are given for depicted cohorts compared with the cohort 2003-2004. IPH: intra-plaque haemorrhage, CI: confidence interval

The observed changes in fat and IPH over time resembles earlier research from the same biobank in carotid arteries[6]. This is of particular interest, when taking into account the differences between ilio-femoral and carotid plaques. While extracted carotid plaques are generally more unstable and removed in an acute setting within days to weeks after the initial event, ilio-femoral plaques are often the end product of years of remodelling, which is the result of conservative therapy or postponed surgery due to improved endovascular options, and therefore often display a more stable plaque type. Yet, even when these differences are taken into account, plaques in ilio-femoral arteries show time dependent changes comparable with the previously reported observed changes in the carotid artery. This strengthens the idea that the alteration of atherosclerotic plaque characteristics is a more widespread and systemic phenomenon.

Possible explanations for the consistent decrease of lipid-rich plaques and plaques with IPH over time are multifold. For example, the use of statins increased significantly during the course of this biobank from 64.4% to 79.7% of patients, and statin use is known to affect atherosclerotic plaques[26]. Indeed, during the same period, blood lipid and cholesterol levels dropped significantly. Still, the decline in vulnerable plaque characteristics were also seen in the patient group that did not use statins (data not shown). Moreover, we corrected for these changing patient characteristics in the multivariable analyses. Further improvements in preventive and conservative care could have contributed towards more stabilizing plaques, as the ilio-femoral plaque matures and remodels into a more stable plaque[27]. A shift towards longer conservative treatment before surgical intervention may also explain the observed increase in the patients operated with a higher disease severity, as measured by Fontaine classification and higher stenosis grades. Yet, the duration of complaints of intermittent claudication before surgical intervention did not change over time. In addition, one could speculate that the decrease of anticoagulant therapy might contribute towards plaque alterations, predominantly IPH, but we found no evidence for this[26]. Furthermore, the increasing prevalence of hypertension in this patient group could have caused plaque changes, but we found no significant effect in our cohort. Better treatment of hypertension could lead to relative hypoperfusion of the lower extremity, which could cause earlier complaints and therefore less stable plaques upon examination due to earlier resection of the plaque. Although we cannot exclude better compliance, we found no increased subscription of antihypertensive medication in more recent years and no lower mean arterial pressure. Furthermore, in the Dutch governmental smoking policy has changed significantly over the last decade, leading to a significant reduction in passive smoking. It has been established that such policies result in a decline in myocardial infarction in non-smokers[28] Although, whether this also applies to peripheral arterial disease is unknown. Finally, over the course of the years, the biobank protocol for handling and analysis of the plaques and cross-sections has not changed. The histological plaque analysis has been performed by the same dedicated technician and algorithms for computerized measurements were not adjusted over the years. Moreover, our methods have undergone substantive quality control. The phenotyping was validated by an independent observer, and have shown excellent intra and extra-observer variability measures[17,18].

A limitation of this study is that we used semi-quantitative measurements of several plaque characteristics that are binned into two categories for the current analyses. Our results indicate that particularly fat-rich plaques and plaques with IPH are in decline, but we observed no increase in characteristics that indicate a stable plaque. Our analyses could be biased by the fact that local intensity of the picro-sirius red staining could have been taken into account in the current visual assessment of collagen content. It cannot be ruled out that the total collagen content actually increases. A continuous computerized measurement for these stable characteristics, rather than semi-quantitative plaque phenotypes that we studied, could ameliorate the analyses.

Recently is has been shown that PAD patients have a high disease burden when compared

to cardiac and carotid disease[29]. These patients often have chronic complaints or disabling symptoms, leading to a high disease burden for both patient and society[29]. It is important to keep studying this patient group and to find measures upon which secondary prevention can be based. Moreover, the changing plaque characteristics in both carotid and ilio-femoral arteries point towards a changing concept of disease progression. The current observations in the ilio-femoral tract, combined with the earlier reported observations in carotid lesions, may raise doubt about the validity for extrapolation of outcomes obtained in the past to current clinical practice. Especially when these outcomes are based on atherosclerotic plaque biobanks that included patients more than 10 years ago. The differences in plaque characteristics, which have been observed both in the carotid and femoral vascular bed, might have several clinical implications. First, femoral artery revascularization suffers from significant restenosis rates. The outcomes of mechanical and surgical treatment strategies have been described to be influenced by the type of underlying plaque characteristics[30,31]. Therefore, the benefit of revascularization may change over time. Second, if the stabilization of the atherosclerotic lesions is a systemic process, secondary event rates might decline because of stable plaque formation in other vascular beds. Future research should look into the implications of the changing plaque characteristics on secondary cardiovascular outcome.

The current results support the view that the current concept of the vulnerable plaque as the main determinant of plaque progression and arterial occlusive disease may be critically appreciated. Our data suggest that there will be a continuous demand for ongoing collection of tissue and patient information of recently affected patients to study atherosclerotic disease.

Conclusion

In this cohort of peripheral arterial disease patients undergoing primary endarterectomy, we observed a time dependent shift of plaque characteristics towards a less lipid rich lesion with less intraplaque haemorrhage. These findings indicate research in cardiovascular disease would benefit from contemporary patient characteristics and plaque specimens to optimize translational potential.

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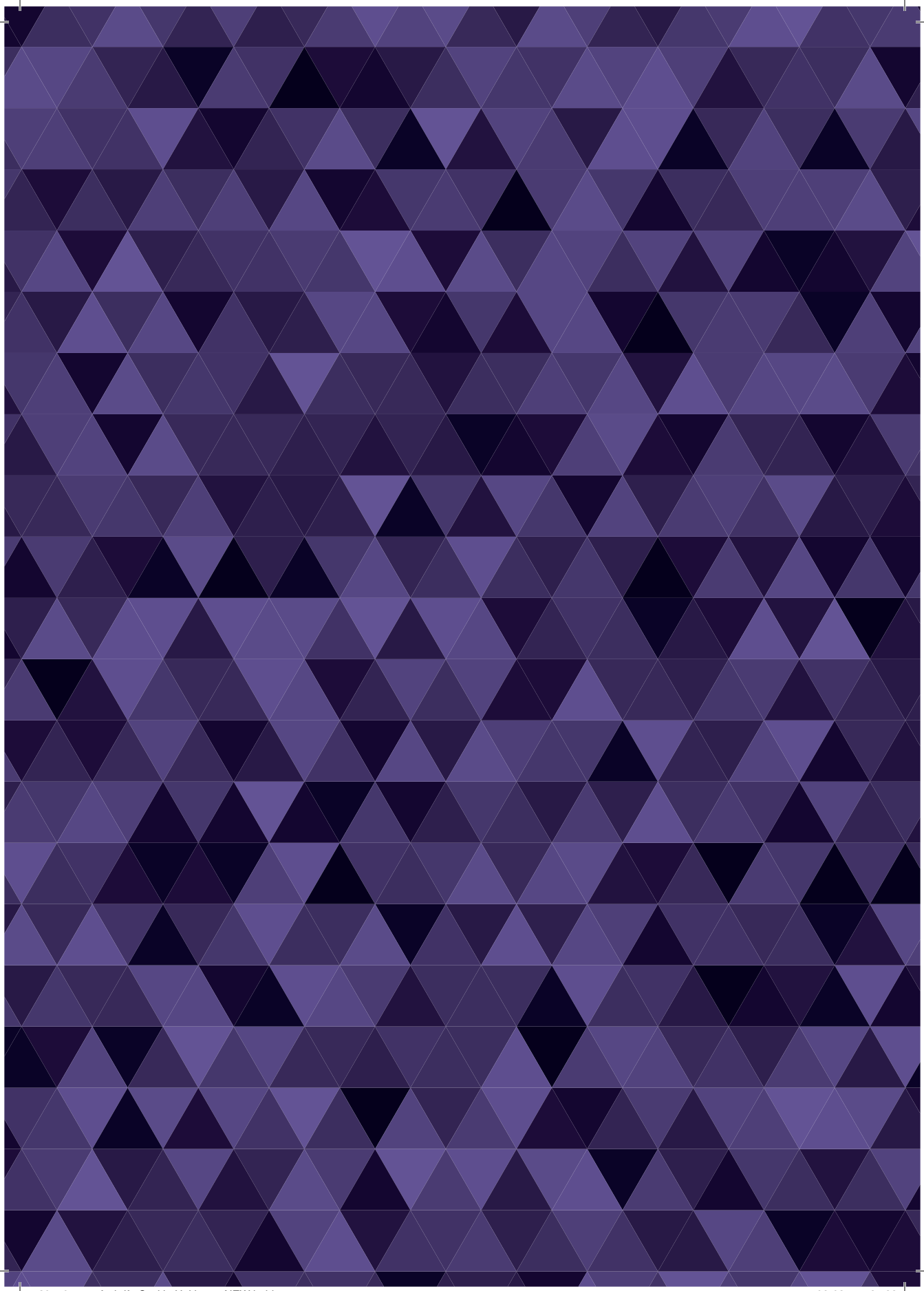
Supplemental material

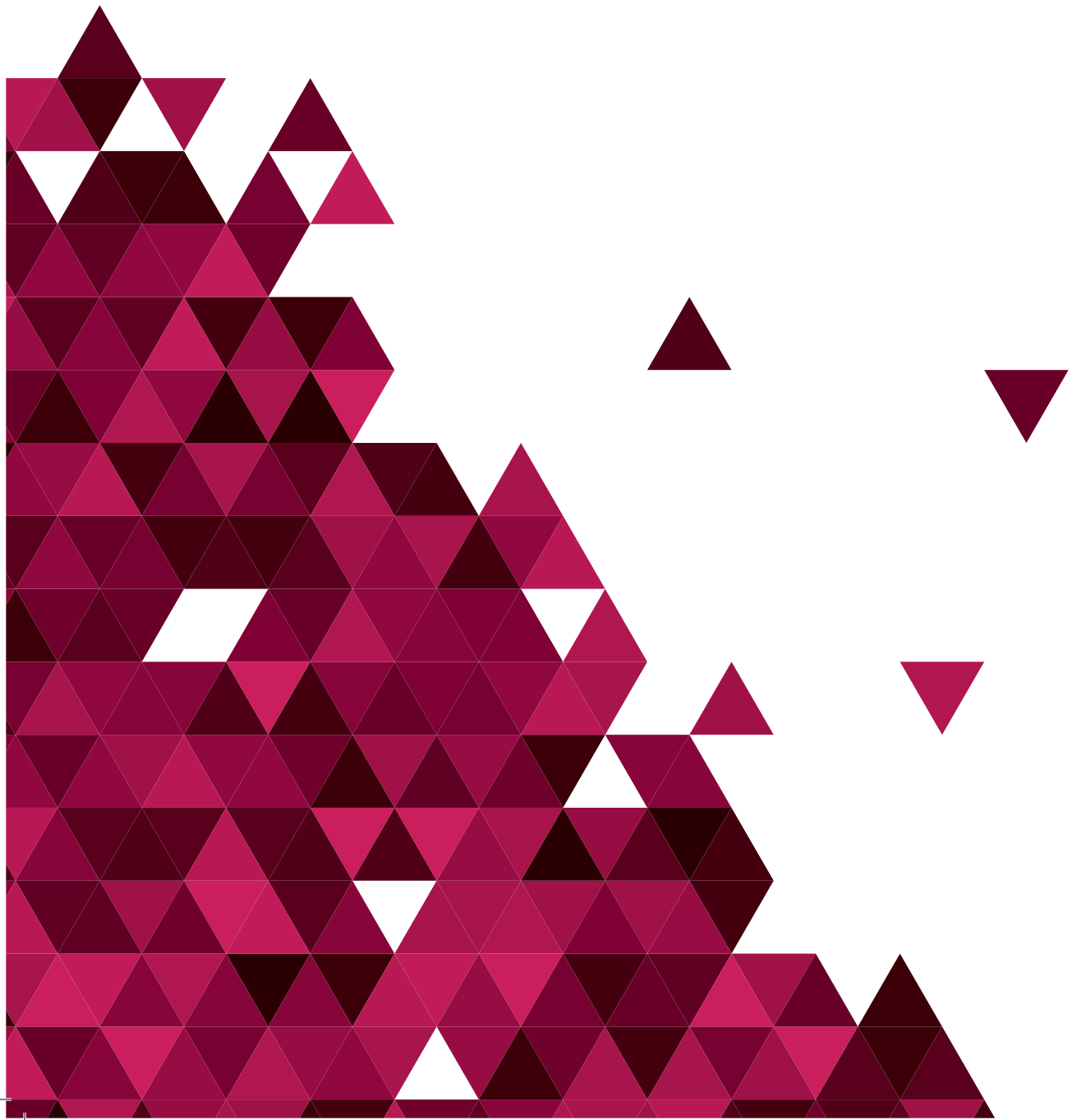
Supplemental table 1 Baseline characteristics of the whole cohort

Supplemental material is omitted because of space limitations



PART III

(Epi)genetic studies in the Athero-Express Biobank



CHAPTER 7

Genetic variation within the Y chromosome is not associated with histological characteristics of the atherosclerotic carotid artery or aneurysmal wall

Submitted

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Abstract

Background

Haplogroup I – a common European paternal lineage of the Y chromosome is associated with increased risk of coronary artery disease in British men. It is unclear whether this haplogroup or any other haplogroups on the Y chromosome is associated with histological characteristics of the diseased vessel wall in other vascular manifestations of cardiovascular diseases showing a male preponderance.

Methods

We examined Dutch men undergoing either carotid endarterectomy from the Athero-Express biobank (AE, n=1,217) or open aneurysm repair from the Aneurysm-Express biobank (AAA, n=393). Upon resolving the Y chromosome phylogeny, each man was assigned to one of the paternal lineages based on combinations of single nucleotide polymorphisms of the male-specific region of the Y chromosome. We examined the associations between the Y chromosome and the histological characteristics of the carotid plaque and aneurysm wall, including lipid content, leukocyte infiltration and intraplaque haemorrhage, in all men.

Results

A majority of men were carriers of either haplogroup I (AE: 28% AAA: 24%) or haplogroup R (AE: 59% AAA: 61%). We found no association between Y chromosomal haplogroups and histological characteristics of plaque collected from carotid arteries or tissue specimens of aneurysms. Moreover, the distribution of frequency for all Y chromosomal haplogroups in both cohorts was similar to that of a general population of Dutch men.

Conclusions

Our data show that genetic variation on the Y chromosome is not associated with histological characteristics of the the plaques from carotid arteries or specimens of aneurysms in men of Dutch origin.

Background

Historically, the human Y chromosome was considered genomic wasteland. It was considered to be implicated only in sex determination and subject to rapid decline[1]. However, more recently the Y chromosome gained interest, as it was found to contain dosage-sensitive regulators of gene expression and its loss was associated with smoking, cancer and death in different studies in men[2–4].

The major part of the Y chromosome (male-specific region, MSY) is inherited essentially unchanged from father to son. Phylogenetic analysis is a powerful tool to examine the ancient paternal ancestry of the Y chromosome[5,6]. The resulting chromosomal haplogroups are characterized by numerous specific genetic mutations and follow a distinct geographical distribution[6]. In Western Europe, haplogroups I and R and their subfamilies are among the most frequently observed Y chromosomal haplogroups although their prevalence differs between countries.

Genetic variation of the MSY was previously implicated in cardiovascular diseases (CVD)[7–11]. Common bi-allelic polymorphisms of MSY were associated with blood pressure, circulating concentrations of LDL-cholesterol, measures of a pro-atherogenic fraction of LDL-cholesterol and a paternal history of myocardial infarction[8,9]. In addition, men with haplogroup I showed a 50% increase in coronary artery disease (CAD) risk in two British populations[12]. Gene expression analysis revealed down-regulation of pathways of adaptive immunity together with up-regulation of pro-inflammatory response in the macrophages of haplogroup I carriers when compared to men from other Y chromosomal haplogroups[12]. However, in a recent Dutch effort, haplogroups did not show a predisposing effect on first or recurrent venous thrombosis[13].

The previous analyses were conducted primarily in relation to CAD or its modifiable risk factors[8,9,12]. Whether haplogroups are also related to the risk of other cardiovascular disorders, or the characteristics of the underlying atherosclerotic plaque is unclear. In addition, there is limited data on the association between Y chromosomal haplogroups and susceptibility to CVD in non-British cohorts.

We have conducted a MSY phylogenetic analysis of 1,610 Dutch men with available histological characteristics of the diseased vascular wall, obtained during carotid endarterectomy or open aneurysm repair. Both disorders show a "male disadvantage", with men much more commonly affected than women[14]. Characteristics of the vessel wall have been shown to be associated with presenting symptoms, in occluded as well as in aneurysmatic vessels[15–17]. The use of histology has advantages over using clinical diagnosis as it may shed light on the biological mechanism behind the observed increased CVD risk associated with haplogroup I.

Methods

Athero-Express population

The Athero-Express biobank (AE) is an prospective ongoing cohort study including all patients undergoing carotid endarterectomy in two large tertiary referral hospitals in the Utrecht area of the Netherlands: the University Medical Center Utrecht in Utrecht and the St. Antonius Hospital in Nieuwegein[18]. Patient characteristics are collected through standardized questionnaires. Blood is collected preoperatively and stored together with the atherosclerotic plaque. Patients are asked to return a short follow-up questionnaire each year for three years. When they indicate a possible cardiovascular event, this is validated through health records kept by their general practitioner. Patients gave written informed consent and the study is approved by the ethics boards of both hospitals.

AAA-Express population

The AAA-Express biobank (AAA) started as a spin-off biobank of the AE, including patients undergoing open aneurysm repair in the same hospitals[19]. Questionnaires and follow-up were collected in the same fashion. Instead of atherosclerotic plaque, aneurysmal tissue was stored. Patients gave written informed consent and the study is approved by the ethics boards of both hospitals.

Processing of patient material in AE and AAA

The processing of patient material from the AE and AAA has been described previously[18,19]. In short, atherosclerotic plaque and aneurysm tissue were immediately processed after removal. One segment, for AE the culprit lesion, was identified, stored in 4% formaldehyde, decalcified and embedded in paraffin for histological slide preparation. The remaining tissue was cut into fragments of 0.5 cm and stored at -80 degrees. Using histology, we studied picro-sirius red staining for collagen, CD68 staining for macrophages and α-actin staining for smooth muscle cells. For plaque histology we additionally studied CD34 staining for the presence of microvessels. For aneurysm wall histology we also conducted CD3 staining for T-lymphocytes, CD20 staining for B-lymphocytes and CD138 staining for plasma cells. Plaque thrombosis was determined combining the presence of luminal thrombi or intraplaque haemorrhage, assessed by hematoxylin-eosin staining and Mallory's phosphotungstic acid-hematoxylin staining for fibrin. Collagen and calcifications were semi-quantitatively assessed at 40x magnification and grouped into no (1), minor (2), moderate (3) or heavy (4) staining. The categories were dichotomized into no/minor and moderate/heavy for the current study. For AAA, leukocyte infiltration was scored at 100x magnification, where <100 positively stained cells was considered minor staining and >100 positively stained cells was considered moderate/heavy staining. Lipid core size was cut off at an area of 40% of plaque size using polarized light. For AE, macrophages and smooth muscle cells were quantitatively assessed using computerized analysis and analyzed

as percentage of plaque area. Microvessels were counted in three hotspots after morphological identification and averaged per slide subsequently. A dedicated technician assessed all histological slides.

Haplogrouping of the Y chromosome

For AE, patients were genotyped using eleven MSY SNPs (Supplemental table 1) tagging 8 Y chromosomal lineages and subsets of the R haplogroup. In AE and AAA, patients were additionally genotyped using the Y chromosomal probes of the Infinium HumanExome BeadChip v1.2 and Illumina HumanCoreExome BeadChip v1.1 respectively, following genotyping of the Ygen consortium[20]. In this case the combination of 68 MSY SNPs was used to discriminate between 65 possible haplogroups. Each individual was assigned to the haplogroup that best fitted his genotypes, allowing for no more than one mismatch and 3% of missing genotypes. In cases where the two genotyping methods did not correspond (n=9), the ExomeChip haplogroup was kept for further analyses. Haplogroup lineages were further grouped into haplogroup E, F, G, H, I, J, N, Q, R and T for the current analyses.

Statistical analyses

Differences in baseline characteristics were tested using ANOVAs and non-parametric Kruskall-Wallis tests, where applicable, for continuous variables. Categorical variables were compared using chi-square tests. Post-hoc testing for ANOVA was performed using Tukey tests, for chi-square test by observing the standardized residuals. Continuous plaque characteristics were log-transformed before analyses. They were analyzed at once for each cohort using MANOVA. Binary categorical plaque characteristics were analyzed using chi-square tests. A multiple-testing corrected *P* value was considered significant. Multiple-testing correction was performed with the use of Bonferroni correction (for binary plaque characteristics in AE: 0.05/4 = 0.0125, for continuous plaque characteristics in AE: 0.05/5 = 0.01, for aneurysm characteristics in AAA: 0.05/11 = 0.0045). All statistical analyses were performed in SPSS version 21.

Results

Prevalence of Y chromosomal haplogroups

We included a total of 1,610 male patients, from Athero-Express (AE, n=1,217) and AAA-Express (AAA, n=393) in this study. Patients displayed a variety of Y chromosomal haplogroups (Supplemental table 2). Most patients were carriers of haplogroup I (AE: 28% AAA: 24%) or haplogroup R (AE: 59% AAA: 61%). Because of low patient numbers in the other haplogroups, for all subsequent analyses, only patients with haplogroups E, G, I, J and R were included (Figure 1).

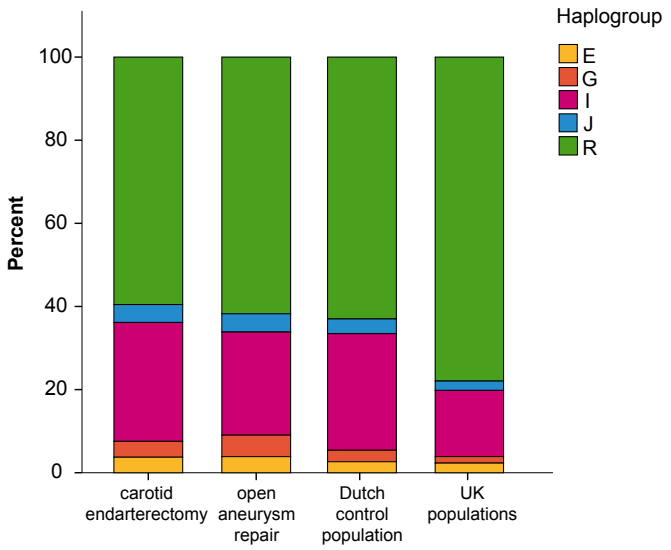

Figure 1. Distribution of the five largest Y chromosomal haplogroups
Distribution of the five largest Y chromosomal haplogroups in two Dutch CVD cohorts: carotid endarterectomy patients from the Athero-Express Biobank Study, aneurysm patients from the Aneurysm-Express Biobank Study, Dutch healthy controls of the Forensic Laboratory for DNA Research and the two UK populations (British Heart Foundation Family Heart Study (BHF-FHS) and West of Scotland Coronary Prevention Study (WOSCOPS) from *Charchar et al.*[12]

Clinical characteristics

The men within the Athero-Express cohort exhibited characteristics of a severely cardiovascular compromised population (Table 1). They were on average 69 years old, 23.7% had a history of diabetes and 71.5% were hypertensive. Of all men, 26.4% presented with a stroke before undergoing CEA. After correction for multiple testing, there were no significant differences in any available clinical characteristics among carriers of the most common haplogroups of the Y chromosome in this population. Patient characteristics of the AE and AAA-Express can be found in Supplemental table 3 and 4.

Atherosclerotic plaque characteristics in Athero-Express

There were no significant differences in macrophage content, mast cell content, neutrophil content, smooth muscle cell content and vessel density in the atherosclerotic plaque, either together at once using MANOVA (Wilks' Lambda: 0.99, $P = 0.33$) or tested independently (Figure 2, Supplemental table 5) between the MSY haplogroups. No significant differences were observed between the MSY haplogroups in calcification, collagen, fat content and intraplaque haemorrhage (Figure 3, Supplemental table 5).

Table 1. Patient characteristics of the Athero-Express Biobank

n = 1206	E n=45	G n=46	I n=345	J n=52	R n=718	p value
Age, mean (SD)	67.6 (10.9)	70.6 (7.6)	69.3 (9.5)	68.8 (9.7)	68.7 (8.7)	0.49
BMI, mean (SD)	26.0 (3.7)	25.5 (2.4)	26.3 (3.4)	26.0 (3.2)	26.4 (3.4)	0.33
GFR (MDRD), mean (SD)	72.2 (22.7)	73.6 (21.4)	73.4 (19.9)	73.6 (17.8)	73.6 (20.2)	1
Diabetes Mellitus	11/45 (24.4)	8/46 (17.4)	96/345 (27.8)	11/52 (21.2)	159/718 (22.1)	0.25
Hypercholesterolemia	26/41 (63.4)	19/42 (45.2)	218/324 (67.3)	36/52 (69.2)	444/658 (67.5)	0.06
Hypertension	33/43 (76.7)	32/44 (72.7)	241/338 (71.3)	33/52 (63.5)	498/694 (71.8)	0.68
Current Smoking	9/44 (20.5)	20/45 (44.4)	103/342 (30.1)	23/52 (44.2)	230/708 (32.5)	0.04
History of coronary artery disease	8/45 (17.8)	15/46 (32.6)	116/345 (33.6)	18/52 (34.6)	266/718 (37.0)	0.11
History of stroke	9/42 (21.4)	7/44 (15.9)	77/328 (23.5)	10/48 (20.8)	151/677 (22.3)	0.85
Peripheral arterial occlusive disease	8/45 (17.8)	11/46 (23.9)	69/345 (20)	12/52 (23.1)	169/718 (23.5)	0.68
Total cholesterol, median (IQR)	4.4 (3.7-5.3)	4.7 (3.8-5.5)	4.5 (3.8-5.2)	4.7 (3.7-5.2)	4.5 (3.8-5.3)	0.94
LDL cholesterol, median (IQR)	2.5 (2.0-3.5)	2.9 (2.2-3.3)	2.6 (2.0-3.2)	2.9 (2.0-3.3)	2.6 (2.0-3.3)	0.97
HDL cholesterol, median (IQR)	1.1 (0.9-1.2)	1.0 (0.8-1.5)	1.1 (0.9-1.3)	1.0 (0.9-1.3)	1.1 (0.9-1.3)	0.94
Triglycerides, median (IQR)	1.3 (1-1.9)	1.3 (1.1-1.8)	1.4 (1-2.1)	1.5 (1-2.3)	1.5 (1.0-2.1)	0.68
Antiplatelet use	41/45 (91.1)	40/45 (88.9)	300/343 (87.5)	46/52 (88.5)	625/718 (87)	0.94
Statin use	32/45 (71.1)	36/46 (78.3)	264/345 (76.5)	39/52 (75)	558/718 (77.7)	0.86
Presenting symptoms:						0.19
Asymptomatic	2/45 (4.4)	5/46 (10.9)	46/339 (13.6)	5/52 (9.6)	118/715 (16.5)	
Ocular	24/45 (53.3)	19/46 (41.3)	135/339 (39.8)	26/52 (50)	323/715 (45.2)	
TIA	13/45 (28.9)	12/46 (26.1)	101/339 (29.8)	11/52 (21.2)	177/715 (24.8)	
Stroke	6/45 (13.3)	10/46 (21.7)	57/339 (16.8)	10/52 (19.2)	97/715 (13.6)	
Contralateral stenosis >50%	17/40 (42.5)	22/45 (48.9)	135/305 (44.3)	23/48 (47.9)	303/641 (47.3)	0.89

AE: Athero-Express, SD: standard deviation, BMI: body mass index, GFR: glomerular filtration rate, PAOD: peripheral arterial occlusive disease, LDL: low density lipoprotein, HDL: high density lipoprotein

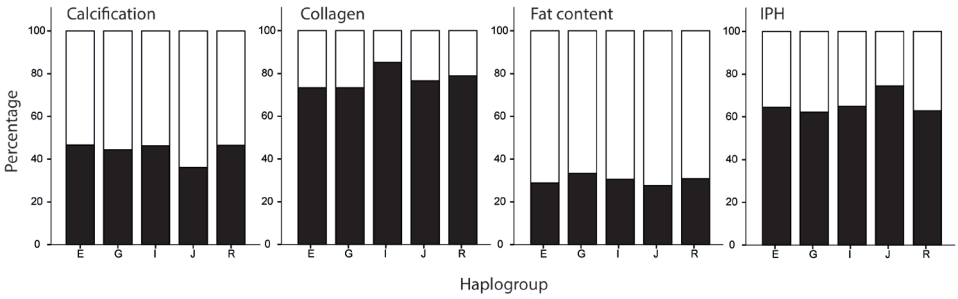

Figure 2. Binary plaque characteristics of the Y chromosomal haplogroups in Athero-Express
Black bars: moderate/heavy staining for calcification and collagen, >40% fat content, presence of IPH; white bars: no/minor staining for calcification and collagen, <40% fat content, absence of IPH.

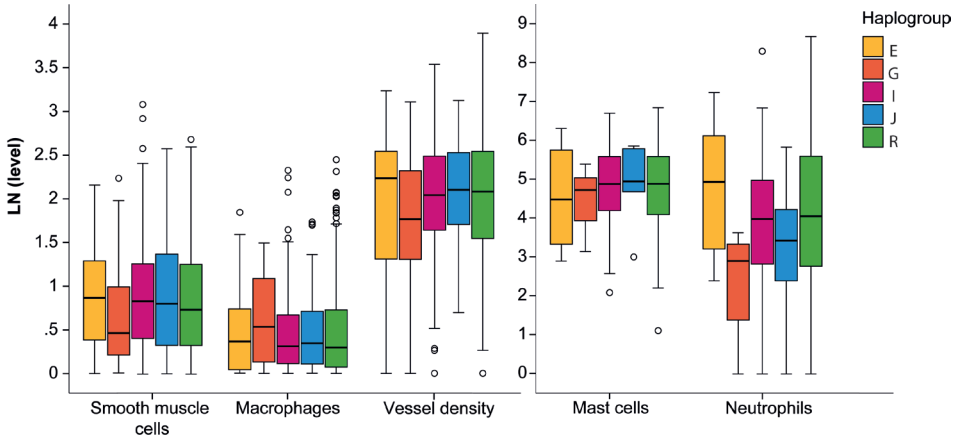

Figure 3. Continuous plaque characteristics of the Y chromosomal haplogroups in Athero-Express

Aneurysm characteristics in Aneurysm-Express

We set out to further explore the association between Y chromosomal haplogroups and vessel wall characteristics in AAA. No significant differences were observed comparing aneurysm wall characteristics of patients undergoing open aneurysm repair between the Y chromosomal haplogroups (Supplemental table 6).

Comparison with the general population

We compared our two cardiovascular disease cohorts with a control cohort of 2,067 healthy Dutch men from the Forensic Laboratory for DNA Research. The haplogroups followed the same distribution in the control cohort as in the cardiovascular disease cohorts with the most men carrying haplogroup I or R (Figure 1). A small difference was observed between the prevalence of haplogroup G in the control population (2.7%) versus

haplogroup G in the diseased populations (AE: 3.8%, AAA: 5.1%, Supplemental table 2). No other differences were found. Based on this finding, an association between one of the haplogroups and risk of carotid occlusive disease or aneurysm development is unlikely. Compared to previously described populations from the United Kingdom, all Dutch populations showed more haplogroup I carriers and less haplogroup R carriers (Figure 1, Supplemental table 2).

Discussion

In our study in 1,610 Dutch men, we found no association of Y chromosomal haplogroups with histological characteristics of the diseased vessel wall. Moreover, we found no difference in distribution of Y chromosomal haplogroups in the general Dutch population versus our patients with severe atherosclerotic cardiovascular disease.

Previous research in two British cohorts found an association between haplogroup I and coronary artery disease[12]. We did not observe an association between MSY haplogroups and characteristics of the diseased vessel wall in the Dutch cohorts. There are several explanations for this apparent discrepancy. First of all, we studied different diseases, namely carotid occlusive disease and aneurysm formation. While atherosclerosis is a shared pathophysiological mechanism in all three cardiovascular diseases, their genetic background and disease mechanism is known to be only partially overlapping[21]. For example, the inflammation in aneurysms is much more driven by infiltration of B and T lymphocytes, whereas in atherosclerotic plaques more macrophages are seen[22–24]. In addition, we investigated cohorts with only diseased patients, in contrast to the British cohorts that included both patients with CAD and apparently healthy controls. If haplogroups would account for the increased risk via the shared pathophysiological background of the three cardiovascular diseases, one would expect a different frequency distribution of haplogroups in our diseased cohorts. Increased risk of CAD that translates into higher rates of cardiovascular mortality in haplogroup I could account for a lower prevalence of patients with haplogroup I in our cohorts that only included patients suitable for surgery. On the contrary, increased risk of CAD due to a higher atherosclerosis risk could also have led to a higher prevalence of patients with haplogroup I in our cohorts, as patients were included based on overt atherosclerotic disease. However, we found the same haplogroup I frequency and distribution of other haplogroups in the Dutch cohorts of patients with vessel disease when compared to a large cohort of healthy Dutch men. Another explanation could be the differences in genetic background of the British and Dutch. Indeed, the distribution of Y haplogroups in Dutch men was different from the one observed in the cohorts from the United Kingdom. In the latter, there were more carriers of haplogroup R and less carriers of haplogroup I. Comparing the Y chromosomal haplogroup distribution of the diseased cohorts to the Dutch control population, we observed a slight enrichment for haplogroup G in the Dutch diseased cohorts (2.7 vs 4.4% for non-diseased vs diseased cohorts, *P* value = 0.004).

This could point towards an increased disease risk for haplogroup G carriers compared to other haplogroups. However, we did not observe differences in patient or disease characteristics for carriers of haplogroup G. An alternative explanation could be that our patients were included mainly in the larger cities in the Netherlands, where people may have a more diverse genetic background whereas the control population participants were included from smaller towns and villages.

There are some limitations to this study. Some Y chromosomal lineages (e.g. F, N, T) were excluded from the analysis because they were only present in few men in the studied populations. Moreover, the subgroups of Y chromosomal haplogroups were binned into larger groups to increase the power of the analysis (e.g. R1, R1a, R1b and R1b1b2 were binned into haplogroup R). We therefore cannot exclude the possibility of an association of any subgroup or smaller haplogroup with cardiovascular disease. In addition, we had low power to detect differences in some lineages that were included in the analysis (e.g. E, J and G) and we cannot exclude a possible association between those haplogroups and cardiovascular disease with certainty. Haplogrouping was performed on several batches of genotyped data. However, for AE we found excellent overlap in haplogroups comparing the ExomeChip haplogroup and the haplogroup determined by genotyping of individual SNPs. We observed some nominally significant associations between haplogroups and baseline characteristics. However, the number of positive associations was low and within the expected range for the number of tests we performed, we therefore considered them false positives.

Since the publication of the association of haplogroup I and coronary artery disease, the cardiovascular research community has become interested in the Y chromosome. Replication of the association has been lacking, and publications in other groups outside of the United Kingdom are scarce[13]. We included two different Dutch cardiovascular cohorts and found no association between haplogroups and histology of the diseased vessel wall. Large efforts might shed more light on the relation between Y chromosomal haplogroups and cardiovascular disease.

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We want to thank Eveline Altena and Peter de Knijff for generously making available the Dutch control population data from the Forensic Laboratory of DNA Research. Furthermore, we want to thank Arjan Schoneveld and Sander van de Weg for the help with the haplogrouping of the AE samples and Evelyn Velema for the histopathological phenotyping of the plaque and aneurysm samples. Lastly, we want to thank all patients included in the two cardiovascular cohorts for their participation.

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CHAPTER 8

Loss of Y chromosome in blood is associated with major cardiovascular events during follow-up in men after carotid endarterectomy

Submitted

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Abstract

Background

Recent studies found an immune-regulatory role for Y, and a relation between loss of Y (LOY) in blood cells and a higher risk of cancer and mortality. Given the involvement of immune cells in atherosclerosis, we hypothesized that LOY is associated with the severity of atherosclerotic plaque characteristics and outcome in men undergoing carotid endarterectomy (CEA).

Methods

LOY was quantified in blood and plaque from raw intensity genotyping data in men within the Athero-Express biobank study. Plaques were dissected, and the culprit lesions were used for histology and the measurement of inflammatory proteins. We tested LOY for association with (inflammatory) atherosclerotic plaque phenotypes and cytokines and assessed the association of LOY with secondary events during 3-year follow-up.

Results

Out of 366 CEA patients, 61 exhibited some degree of LOY in blood. LOY was also present in atherosclerotic plaque lesions (n = 8/242, 3%). LOY in blood was negatively associated with age (beta=-0.03/10yr, r^2=0.07, p=1.6*10^{-7}), but not with cardiovascular disease severity at baseline. LOY in blood was associated with a larger atheroma size (OR 2.15, 95% CI: 1.06-4.76, p= 0.04) however this association was not significant after correction for multiple-testing. LOY was independently associated with secondary major cardiovascular events (HR = 2.28, 95% CI: 1.11-4.67, p=0.02) in blood when corrected for confounders.

Conclusions

In this hypothesis-generating study, LOY in blood is independently associated with secondary major cardiovascular events in a severely atherosclerotic population. Our data could indicate that LOY affects secondary outcome via other mechanisms than inflammation in the atherosclerotic plaque.

Introduction

Loss of the Y chromosome (Loss of Y, LOY) in blood cells was already described in the 60s and affects approximately 15% of the male population of higher age[1]. Only recently LOY was associated with a higher risk of (non-haematological) cancerand overall mortality[2,3]. This relationship was speculated to be due to smoking and a disrupted tumor immunosurveillance[4]. Furthermore, LOY was associated with Alzheimer's disease[5] and the occurrence of auto-immune diseases such as primary biliary cirrhosis[6] and auto-immune thyreoiditis[7]. Indeed, the Y chromosome exhibited an immune-regulatory function by acting as a global trans-expression quantitative trait locus in mice[8]. The Y chromosome directly mediated changes in the transcriptome of CD4+ T-cells and macrophages, contributing to altered gene expression and alternative splicing. A role in global immune response was also found in the monocyte and macrophage transcriptome results of males with haplotype I that exhibited a 50% greater risk of myocardial infarction[9]. Comparison of gene expression data between haplotype I and other haplotypes revealed pathways that are related to inflammation and immunity, revealing down-regulation of adaptive immunity and up-regulation of inflammatory response in haplotype I carriers. Genetic variation on the Y chromosome has been associated with high blood pressure[10] and myocardial infarction[11], independent from traditional cardiovascular risk factors, sex steroids or aggression. Given the global immune-regulatory role of the Y chromosome and the involvement of immune cells in atherosclerosis together with its male predominance, we hypothesized that LOY is associated with more severe atherosclerosis leading to worse outcome in men undergoing carotid endarterectomy (CEA).

Methods

Patient characteristics

The Athero-Express biobank study is an ongoing cohort study that includes atherosclerotic plaques and blood of patients undergoing either carotid endarterectomy (CEA) or femoral endarterectomy in two large tertiary referral hospitals (University Medical Center Utrecht and St Antonius hospital Nieuwegein) in the Netherlands. Clinical data were obtained from medical files and standardized questionnaires. Age was determined as age at surgery. Current smoking was determined as patient-reported smoking in the past year. Hypertension and hypercholesterolaemia were self-reported. Diabetes was considered present in any of the following cases: use of insulin or oral glucose inhibitors, self-reported diabetes mellitus in the patient questionnaire or diabetes mellitus extracted from the medical file. A history of coronary artery was considered present if the patient had suffered a myocardial infarction, or underwent a percutaneous coronary intervention or coronary artery bypass grafting surgery. Peripheral arterial occlusive disease was considered present if the patient either presented with an ankle-brachial index below 0.7, claudication complaints or underwent percutaneous or surgical intervention for

peripheral arterial occlusive disease. Follow-up was obtained by questionnaires sent to the patients by mail 1, 2 and 3 years postoperatively. Major cardiovascular events ((sudden) cardiovascular death, hemorrhagic or ischemic stroke, myocardial infarction, fatal heart failure or fatal aneurysm rupture) were validated using medical records. The medical ethics boards of both hospitals approved of the study, which is conducted in accordance with the declaration of Helsinki.

Sample collection

A detailed description of the sample phenotyping within the Athero-Express study can be found elsewhere[12]. In short, blood was obtained prior to surgery and subsequently stored at -80 degrees. Plaque specimens were immediately processed after removal during surgery. After identification of the area with the largest plaque burden (culprit lesion) the plaque was cut transversely into segments of 5 mm. The culprit lesion was fixed in 4% formaldehyde and subsequently decalcified and embedded in paraffin. Cross-sections were stained for histological examination. Remaining segments were stored at -80 degrees and used for the measurement of inflammatory cytokines and isolation of DNA.

Histological assessment of specimens

Plaque specimens were stained using CD68 (macrophages), α-actin (smooth muscle cells), picro-sirius red (collagen) and CD34 (microvessels). Furthermore the presence of plaque thrombosis was determined, using a combination of luminal thrombi, intraplaque haemorrhage, hematoxylin-eosin staining and Mallory's phosphotungstic acid-hematoxylin staining (fibrin). Either luminal thrombus, intraplaque haemorrhage or both were considered presence of plaque thrombosis. Computerized analyses quantitatively assessed macrophages and smooth muscle cells as percentage of plaque area. Microvessels were identified morphologically and counted in three hotspots and subsequently averaged per slide. Collagen and calcifications were scored semi-quantitatively into no (1), minor (2), moderate (3) or heavy (4) staining at 40x magnification. These categories were grouped into bins (no/minor and moderate/heavy) for the present analyses. The size of the lipid core was assessed using polarized light and cut off at an area of 10% and 40% of the plaque. All histological slides were assessed by the same dedicated technician.

Cytokine measurements of specimens

To determine the effect of LOY on inflammatory phenotypes within the Athero-Express biobank, we analyzed the association between LOY and seven different inflammatory cytokines: IL-6 and TNF-α as pro-inflammatory cytokines, IL-10 as an anti-inflammatory cytokine, RANTES as a marker of T-cell involvement and MCP-1, MCSF and GDF-15 as markers of macrophage involvement. Cytokines were measured by Luminex in plaque lysate (IL-6, TNF-α, IL-10, RANTES, MCP-1, MCSF) or citrate plasma (GDF-15) and normalized to protein content.

Genotyping data and quality control

The methods of the Athero-Express Genomics Study have been described before[13]. Genome-wide SNP genotyping data was collected in 1,858 consecutive CEA patients using DNA from blood or plaque (when no blood was available) and either the Affymetrix Genome-Wide Human SNP Array 5.0 (AEGS1) or the Affymetrix Axiom GW CEU 1 Array (AEGS2). The quality control pipeline consisted of first excluding samples with low average genotype calling and sex discrepancies based on GCOS4 metrics, and thereafter filtering samples with a call rate >97%, variant call rate >97%, minor allele frequencies >3%, average heterozygosity rate $\pm$ 3.0 standard deviations, relatedness (pi-hat >0.20), Hardy-Weinberg Equilibrium ($p < 1.0x10^{-6}$) and based on population stratification (excluding samples >6 standard deviations from the average in 5 iterations during principle component analysis and by visual inspection). After quality control, we kept 1,640 samples for downstream analyses that were imputed using HapMap 2 CEU. For the current study, only the male samples of the AEGS2 (n= 610 total) could be used, as the AEGS1 array does not contain Y chromosomal SNPs.

Determination of Loss of Y

To assess LOY, median log2 ratios (observed intensity/reference intensity) were computed based on the raw intensity data from the male-specific Y chromosomal probes (mLRRY), excluding PAR1 and PAR2. Two blood samples were excluded due to outlying positive mLRRY values (defined as 1.5 interquartile ranges above the third quartile), leaving 366 blood samples and 242 plaque samples for analysis. We first calculated the peak of each mLRRY histogram using the density function in R for kernel density estimation, as previously described[2]. Next, a noise distribution was derived to compute the cut-off value for LOY. To this end, the positive tail of the kernel density was mirrored over the distribution peak of the kernel density estimates (local median), generating a negative tail. The lower bound of the resulting distribution served as the cut-off value for LOY (Supplemental figure 1).

As a validation, LOY was assessed by qPCR of six Y chromosomal genes along the Y chromosome in 9 patients that exhibited dichotomous LOY and 8 patients that did not exhibit dichotomous LOY. Presence one of the genes (TSPY1) was assessed by a commercially available kit (Y-chromosome Detection real-time PCR assay, Primerdesign Ltd). Primer design of the other five primers can be found in Supplemental table 1. Detected DNA content between patients with and without LOY was compared using t-tests and significant for all genes (Figure 1). Primers were first tested on a female control and all yielded no DNA measurement in that sample.

Statistical analyses

Binary LOY in blood was associated with baseline characteristics using χ^2 tests, t-tests and Wilcoxon signed rank tests, where applicable, to determine possible confounders. The data were imputed using single imputation. All variables with a p value <0.1 (age, body mass index (BMI), glomerular filtration rate (GFR), smoking and hypertension) were

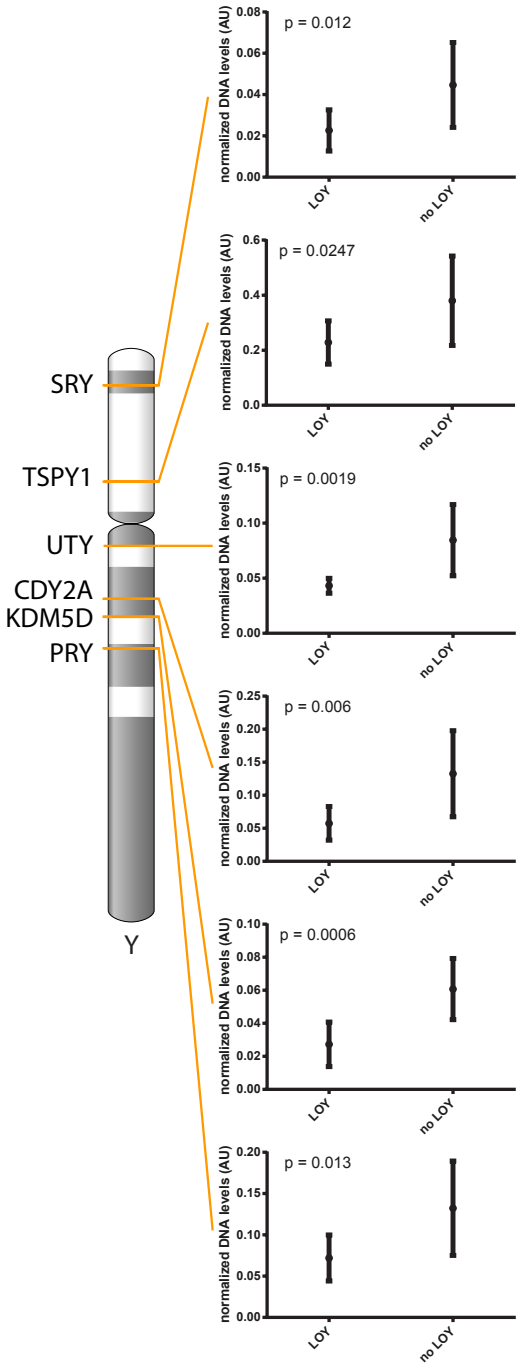

Figure 1. qPCR of Y chromosomal genes
AU: arbitrary units

put into a backstep multivariable model to determine their association with LOY. Remaining significant variables (age and smoking) were put into a multivariable model to assess whether LOY associates with severity of disease characteristics and box-cox transformed plaque phenotypes and inflammatory markers. A Cox proportional hazards model with all covariates that univariably associated with outcome (only age) was used to determine the association between LOY and major cardiovascular events during 3-year follow-up. Values $p < 0.05$ were considered significant. The multiple-testing threshold for plaque characteristics and inflammatory cytokines was set at 0.05/15 tests = 0.003. All statistical analysis were carried out using the R computing platform, version 3.0.2.

Results

Loss of Y in blood

We determined median log2 ratios of Y chromosomal intensity (mLRRY) in 608 patients; in 366 patients we used blood derived DNA. Median log2 ratios of Y chromosomal probes in these patients were negatively associated with age (beta=-0.03/10yr, r^2=0.07, $p=1.6*10^{-7}$, Supplemental figure 1). Of the 366 patients 61 (17%) exhibited dichotomous loss of the Y chromosome (LOY) in blood defined as mLRRY < -0.075 (Table 1, Figure 1, Supplemental figure 2). A trend was seen for more smoking, a lower BMI and less hypertension in the LOY group. No other baseline characteristics were found to differ between patients with and without LOY in blood (Table 1).

Loss of Y in plaque

Within 242 patients we determined mLRRY in atherosclerotic plaque tissue. Median log2 ratios of Y chromosomal probe intensity in plaque were also negatively associated with age (beta = -0.02/10yr, $p=5.02*10^{-8}$, Supplemental figure 1). Of the 242 patients 8 (3%) exhibited dichotomous LOY in plaque defined as median log2 value of Y chromosomal intensity < -0.129 (Supplemental figure 2). Because only eight patients suffered from LOY in plaque, we performed our analyses only on patients of whom we had blood-derived DNA.

No loss of chromosome 21

LOY could be a sign of general intensity loss throughout the genome. We therefore determined whether we could find any evidence for loss of chromosome 21. We found a median log2 ratio of intensity of chromosome 21 probes that was around 0, without any evidence for an association with age (Supplemental figure 3).

Association with smoking

Previous studies point towards a role of smoking in loss of the Y chromosome. We observed a trend towards an association between mLRRY and smoking when corrected for age (beta -0.02 for current smokers compared to non-smokers, p = 0.03). In a backward

step model, age and smoking were found to be most predictive of LOY (AIC for model with only age and smoking = 307. 25 vs AIC for model with age, smoking, BMI, GFR and hypertension = 310.79). Corrected for age, smoking was associated with dichotomous LOY (OR 2.83 (95% CI: 1.50-5.35), p = 0.001).

Association with plaque phenotypes

Because dichotomous LOY showed the largest effect on baseline characteristics, this measure was used to investigate the association between LOY and plaque characteristics and secondary cardiovascular outcome. To investigate whether LOY in blood was associated with a more vulnerable plaque phenotype, we assessed the association between dichotomous LOY in blood and seven classical plaque characteristics: amount of calcification, amount of collagen, atheroma size, presence of intraplaque haemorrhage, macrophage and smooth muscle cell content and vessel density within the plaque. Furthermore, we assessed the association between dichotomous LOY in blood and specific inflammatory or anti-inflammatory cytokines within the atherosclerotic plaque.

Table 1. Baseline characteristics of patients with and without LOY in blood

	Loss of Y (n=61)	No Loss of Y (n=305)	p-value
Age in years (IQR)	75 (69-79)	69 (62-75)	< 0.001
BMI (IQR)	24.9 (23.5-27.0)	25.9 (24.1-28.4)	0.08
Current smoker, yes (%)	25/60 (42)	88/303 (29)	0.08
Diabetes, yes (%)	10/61 (16)	73/305 (24)	0.26
Hypertension, yes (%)	33/59 (56)	203/296 (69)	0.08
Hypercholesterolemia, yes (%)	31/53 (58)	187/281 (67)	0.33
History of coronary artery disease (%)	19/61 (31)	94/305 (31)	1
History of PAOD (%)	12/61 (20)	62/305 (20)	1
Use of antiplatelet therapy (%)	56/60 (93)	271/304 (89)	0.45
Use of lipid lowering drugs (%)	44/61 (72)	244/305 (80)	0.23
Bilateral carotid stenosis (%)	17/48 (35)	129/266 (48)	0.13
GFR (MDRD) mL/min/1.73 m^2 (SD)	68.7 (58.6-82.7)	74.5 (60.4-87.2)	0.12
LDL in mg/dL (IQR)	105 (86-127)	94 (70-124)	0.29
HDL in mg/dL (IQR)	41 (33-43)	39 (32-47)	0.52
Total cholesterol in mg/dL (IQR)	174 (148-186)	162 (135-200)	0.66
Triglyceride levels in mg/dL (IQR)	98 (80-148)	123 (89-177)	0.12
Presenting symptoms (%)			0.27
Asymptomatic	4/60 (7)	42/302 (14)	
TIA	39/60 (65)	172/302 (57)	
Stroke	17/60 (28)	88/302 (29)	

IQR: inter-quartile range, BMI: body-mass index, PAOD: peripheral arterial occlusive disease, GFR: glomerular filtration rate, MDRD: modification of diet in renal disease, SD: standard deviation, LDL: low-density lipoprotein, HDL: high-density lipoprotein, TIA: transient ischaemic attack.

Corrected for age and smoking, dichotomous LOY in blood was nominally associated with a larger than 10% atheroma size (OR 2.15 (1.06-4.76), p= 0.04, table 2, supplemental figure 4).

Association with secondary cardiovascular endpoints

To determine whether dichotomous LOY in blood has an influence on secondary cardiovascular endpoints during follow-up, we used a Cox proportional hazard model correcting for age as this was the only LOY-associated baseline characteristic (p <0.1) that was also associated with major cardiovascular endpoints. During 3 years of follow-up, men with dichotomous LOY in blood had significant more major cardiovascular endpoints (HR = 2.28, 95% CI 1.11-4.67, p = 0.02, figure 2). This effect was present in both smokers and non-smokers (Supplemental figure 5). Atheroma size was not associated with major cardiovascular events during follow-up.

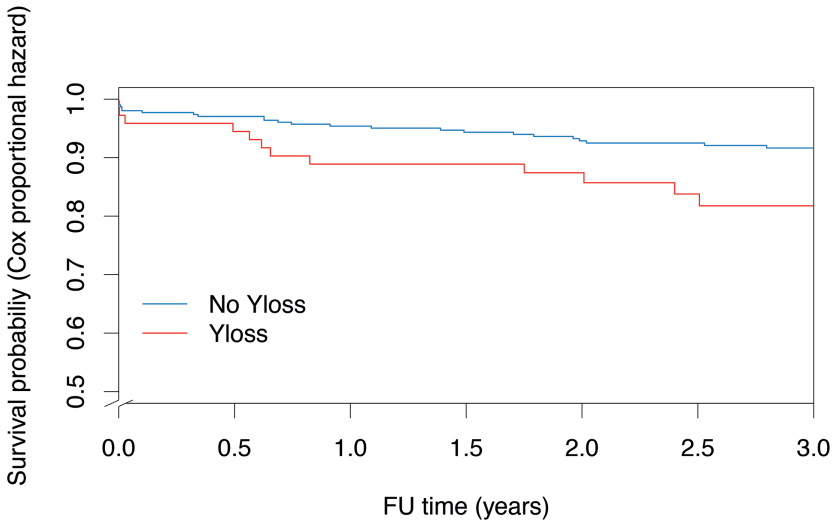

Figure 2. Cox proportional hazards model for major event-free survival. P = 0.02
Model corrected for age and current smoking

Table 2. Associations of LOY with measures of (inflammatory) plaque phenotypes

Plaque phenotype	Beta of LOY (95% CI)	Odds Ratio of LOY (95% CI)	p-value
Atheroma size (>10%)	*NA*	2.15 (1.06-4.76)	0.04
Atheroma size (>40%)	*NA*	1.84 (0.98-3.41)	0.05
Calcification (major)	*NA*	0.86 (0.47-1.58)	0.62
Collagen (major)	*NA*	0.82 (0.39-1.64)	0.59
Intraplaque haemorrhage (present)	*NA*	0.87 (0.48-1.58)	0.65
Macrophage (increase of plaque area)	0.19 (-0.19 – 0.57)	*NA*	0.33
Smooth muscle cells (increase of plaque area)	0.05 (-0.33 – 0.42)	*NA*	0.81
Vessel density (increase per field)	-0.005 (-0.05 – 0.04)	*NA*	0.84
IL-6 in plaque (per pg/mL plaque lysate)	-0.37 (-1.81 – 1.08)	*NA*	0.61
IL-10 in plaque (per pg/mL plaque lysate)	-0.45 (-1.56 – 0.67)	*NA*	0.41
TNF-α in plaque (per pg/mL plaque lysate)	-0.32 (-1.33 – 0.69)	*NA*	0.52
MCSF in plaque (per pg/ug plaque lysate)	0.17 (-0.34 – 0.68)	*NA*	0.51
RANTES in plaque (per pg/ug plaque lysate)	-0.23 (-0.88 – 0.43)	*NA*	0.50
MCP-1 in plaque (per pg/ug plaque lysate)	0.14 (-0.18 – 0.46)	*NA*	0.39
GDF-15 in plasma (per SD pg/mL plasma)	0.11 (-0.11- 0.34)	*NA*	0.33

CI: confidence interval, IL: interleukin, MCP-1: monocyte chemotactic protein, MCSF: macrophage colony-stimulating factor, RANTES: regulated on activation, normal T cell expressed and secreted, TNF: tumor necrosis factor. *Models corrected for age and current smoking. Continuous variables are box-cox transformed.*

Discussion

In this hypothesis-generating study in a population of male carotid endarterectomy patients, loss of the Y chromosome in blood was detectable in both peripheral blood as well as in atherosclerotic lesions. Dichotomous LOY in blood was independently associated with a higher occurrence of major cardiovascular events during a 3-year follow-up period. However, after correction for multiple testing, no associations were found between dichotomous loss of the Y chromosome and systemic and local (plaque) inflammatory status, suggesting that alternate mechanisms may explain the association between LOY and outcome.

We hypothesized that loss of the Y chromosome as an immunomodulating agent in the male genome would lead to a more severe type of cardiovascular disease by increased inflammation in the vascular wall, leading to a more unstable atherosclerotic plaque phenotype, reflected by a macrophage-rich plaque phenotype with a larger lipid pool, more intraplaque haemorrhage and more inflammatory cytokines. While we found an increase in major cardiovascular events and some preliminary evidence pointing towards a larger lipid pool in patients with LOY, we were unable to identify a more inflammatory atherosclerotic plaque in these patients bearing in mind correcting for the testing of 15 different inflammatory phenotypes. One of the reasons could be the different cell-types

in which we identified the LOY (blood) and in which we failed to observe an effect (plaque). However, both blood and plaque take part in the systemic inflammatory response in atherosclerotic disease and macrophages in the plaque derive from circulating monocytes. Furthermore, we also identified LOY in the atherosclerotic plaque itself. Interestingly, the amount of patients with LOY in plaque was lower. Although we cannot be sure as to what cell type is responsible for the detectable LOY in plaque, this lower amount of LOY may possibly be due to the fact that the atherosclerotic plaque does not contain as many rapidly dividing cells as compared to peripheral blood. The difference between LOY in plaque and LOY in blood is also reflected by less variation of LOY between the plaque samples. It could also be due to the fact that the plaque is formed by invasion and division of cells over several decades, during which the Y chromosome is possibly not yet lost. In agreement, from experimental atherosclerosis studies it has been established that plaque macrophages mostly derive from local proliferation rather than continuous infiltration[14].

There are a few other possible explanations for the fact that we did not find any other association with plaque phenotype or inflammation. Firstly, LOY could be so detrimental to the male body that all patients suffering from it die before they develop an operable form of atherosclerosis and thereby simply do not end up in our study. Secondly, LOY could influence atherosclerosis in an earlier phase of the disease, for example affecting disease progression. Patients in the Athero-Express biobank suffer from severe end-stage disease and are, because of the operative guidelines, equally affected. Furthermore, a limitation of the current study is that it is limited in power to detect small but biologically relevant differences because of a relatively small sample size. With an event probability of 12%, to obtain 80% power for observing a hazard ratio of 2.0, one needs 1006 samples and we had only 368 (power of 29%).

A recent study found a relation between LOY in blood and both (non-hematological) cancer and overall mortality in healthy men from the longitudinal ULSAM cohort aged 71-84 years[2]. However, not all increased mortality risk during over 40 years of follow-up could be attributed to malignant diseases. This leaves the question what is causing the other deaths unanswered. In a follow-up study, LOY was also associated with smoking, a risk factor for both cancer and death. Smoking, however, is also a major risk factor for cardiovascular disease. This increased risk is due to several factors, including inflammation but for example also coagulation, endothelial dysfunction and adverse lipid profiles[15]. In our data, smoking was also significantly associated with mLRRY and with dichotomous LOY, when corrected for age. Uncorrected, the absence of a significant association between smoking and dichotomous LOY may be explained by a lack of power (to obtain 80% power for observing a difference between 42% and 29%, one needs 580 samples (of which 20% LOY cases) and we had only 366). In a sensitivity analysis, we observed an effect in both smokers and non-smokers. In summary, we found preliminary evidence to support the hypothesis that the association between LOY and mortality is through a higher risk of major cardiovascular events and that this association cannot be solely explained by smoking as a risk factor.

The mechanism by which the Y chromosome is lost remains elusive. A recent genome-wide approach identified *TCL1A* that is associated to haematological malignancies as a genetic susceptibility locus for LOY at chromosome 14[16]. It might be that loss of the Y chromosome reflects general genomic instability of which the small and last to be replicated Y chromosome is the first victim. Rapidly dividing cells might not take their time to replicate its telomeres and this may lead eventually to loss of the entire chromosome. However, previous experiments blasting the Y chromosome apart have shown that it might be replicated and passed on to daughter cells, even when shattered into pieces even smaller than its original size[17]. Atherosclerosis might also accelerate genomic instability due to the formation of reactive oxygen species. However, we did not find a large proportion of LOY in the atherosclerotic plaque itself.

In our hypothesis-generating study, we found first preliminary evidence that LOY is independently associated with the occurrence of secondary major cardiovascular events in male patients after CEA. More research is needed in a large sample of patients developing cardiovascular disease, preferably a cohort study that recorded cardiovascular disease incidence, to definitively answer the question how LOY is associated with adverse cardiovascular events and specify which events are most likely to be the cause of this association, whether or not smoking is the causative factor and whether or not LOY is also associated with incidence or progression of cardiovascular disease.

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Supplemental material

Supplemental table 1. Primers of the qPCR experiment

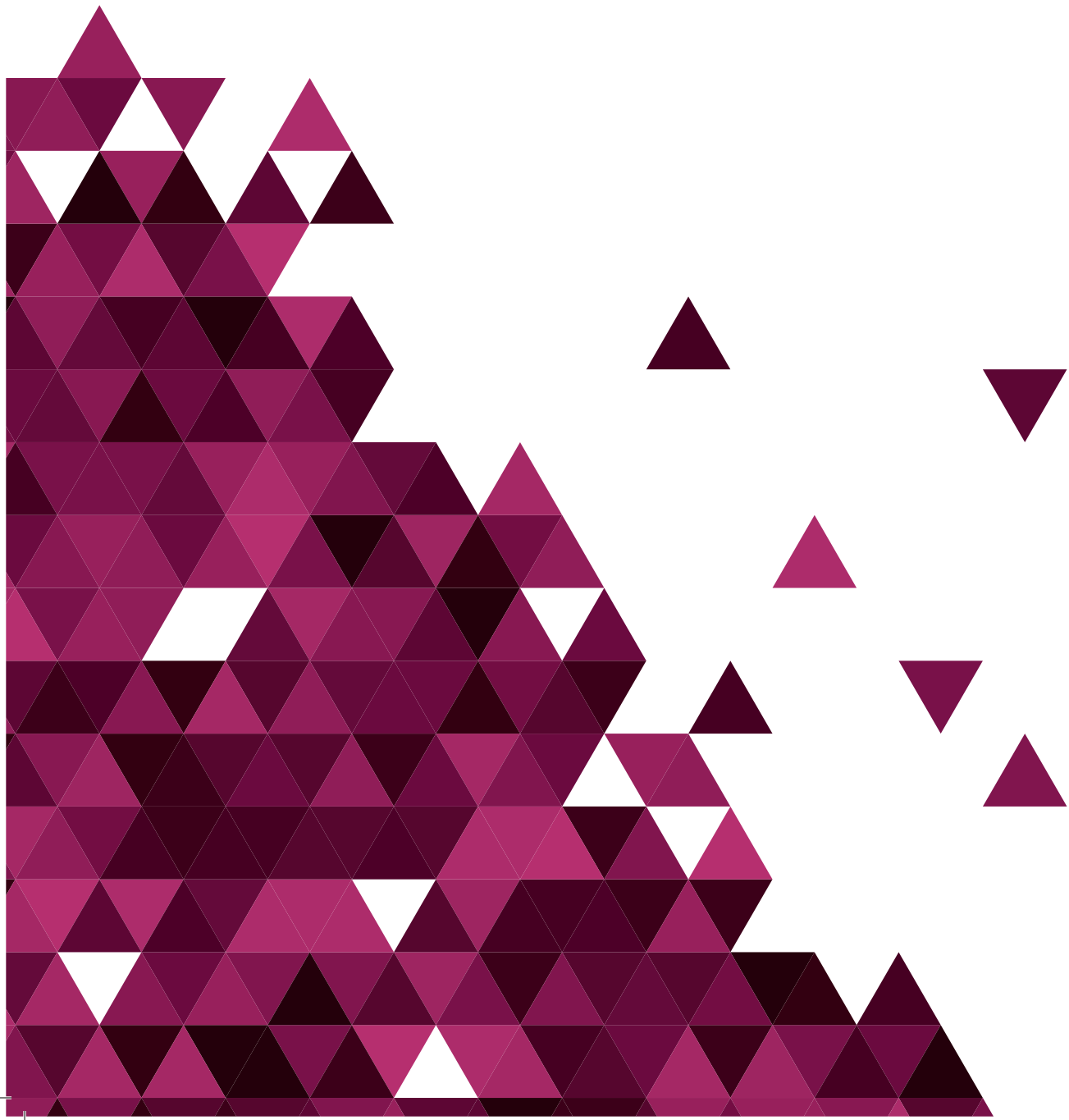
Supplemental figure 1. Determination of LOY cut off for blood and plaque samples

Supplemental figure 2. Association between median log2 ratio of Y chromosomal probe intensity and age in blood and plaque.

Supplemental figure 3. Distribution of median log2 ratio of chromosome 21 intensity and its association with age in blood.

Supplemental figure 4. Percentage of patients with and without LOY with small and large atheroma size

Supplemental material is omitted because of space limitations



CHAPTER 9

Sex-specific differences in DNA methylation in atherosclerotic plaques of 488 carotid endarterectomy patients

In preparation

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Abstract

Background

Sex-differences in the etiology of cardiovascular disease and atherosclerosis have been well defined, yet the extent to which a sex-specific epigenetic signature exists within the atherosclerotic vessel is unknown. We present an epigenome-wide association study (EWAS) into sex-specific differentially methylated regions in carotid atherosclerotic plaques of men and women undergoing carotid endarterectomy.

Methods

We performed an epigenome-wide association study in carotid plaque specimens of 488 patients (148 women, 340 men) who underwent carotid endarterectomy. DNA was isolated from the plaque specimens, bisulfite converted and used to interrogate DNA methylation of 443,872 CpG dinucleotides by means of the Illumina Infinium HM450 Beadchip Array. We validated the results in 92 whole blood samples (31 women, 61 men). Analysis was confined to autosomal chromosomes. DNA methylation differences between the sexes were studied using linear modeling corrected for age. Gene-promotor methylation could be determined based on local CpGs for 14,456 genes.

Results

We identified 3,573 differentially methylated CpGs between women and men in atherosclerotic plaques. We found the majority of top-CpGs to overlap with previously reported sex-differentially methylated CpGs in other tissues and with our DNA methylation data in blood. Analysis of promoter methylation identified 22 gene-promoter regions that were differentially methylated between the sexes. Look ups in expression datasets confirmed a possible relation between differentially methylated promoters and gene expression differences between the sexes.

Conclusions

This epigenome-wide association study shows that DNA methylation profiles are highly sex-specific, even in the severely diseased atherosclerotic vessel wall. Given the sex-specificity of DNA methylation profiles, our results suggest that DNA methylation studies should take sex into account, preferably in a sex-stratified study design.

Background

In many species, large differences have been shown between males and females in the regulation of gene expression[1,2]. Such differences may account for differences in susceptibility to, and pathophysiology of, diseases. Indeed, such sex-differences have been found for DNA methylation marks in leukocytes.[3] Moreover, sex-differences in the etiology of cardiovascular disease and atherosclerosis are well defined. Women tend to form more stable atherosclerotic plaques[4] that are more prone to erosion instead of classic plaque rupture.[5] In line, women are also less likely to develop classical features of cardiovascular disease such as myocardial infarction and stroke early in life[6] and have a favorable prognosis as compared to men.[7]

Differences in DNA methylation between the sexes have been found within numerous tissues and across different races. Most of the studies looking at epigenome-wide sex-differences were carried out in healthy individuals.[8] Recently, genome-wide atherosclerosis-specific profiles of differentially methylated regions were published.[9,10] These studies observed atherosclerosis-specific methylation signatures with genes that play a role in endothelial and smooth muscle functions. Yet, sex-specific analyses were not presented, in spite of the established differences in pathogenesis of atherosclerotic disease between men and women. The latter need is supported by the emerging call for sex-stratified designs in medical research.[11]

Our aim was to investigate sex-differences in DNA methylation in the severely diseased atherosclerotic vascular wall. We present an epigenome-wide association study (EWAS) into sex-specific differentially methylated regions in carotid atherosclerotic plaques of men and women. We compared the results with DNA methylation data in whole blood. To further understand the biological meaning of our findings, sex differences were assessed in publicly available expression data of cardiovascular origin.

Methods

Patient inclusion

The Athero-Express is an ongoing biobank cohort study including patients that undergo carotid endarterectomy at the University Medical Center Utrecht (Utrecht, The Netherlands) or the Sint Antonius Ziekenhuis Nieuwegein (Nieuwegein, The Netherlands). A detailed description of the cohort has previously been published.[12] Clinical data are extracted from patient medical files and standardized questionnaires. The medical ethics committees of both hospitals approved of the study and written informed consent was obtained from patients.

Sample collection

Blood samples were obtained from the radial artery catheter immediately prior to surgery. Carotid plaque specimens were removed during surgery and immediately processed in

the laboratory. Specimens were cut transversely into segments of 5 mm. The culprit lesion was identified, fixed in 4% formaldehyde, embedded in paraffin and processed for histological examination. Remaining segments were stored at -80 degrees.

Methylation Array

DNA was isolated from stored plaque-specimens and patient-matched blood samples. Patient selection was on the basis of plaque, blood and genotype data availability. To exclude selection bias, selected patients were compared with the whole cohort for changes in general characteristics and no significant differences were observed. Isolated DNA was checked for purity and concentration and was equalized to 600ng DNA prior to bisulfite conversion. Bisulfite converted DNA samples were used to measure DNA-methylation by means of the HM450k Methylation Beadchip Array (Illumina, San Diego, USA). Processing of the sample and array was performed according to the manufacturer's protocol.

Quality Control

The raw data from the array was processed using the 'MethylAid' R-package.[13] Samples with low-median signal intensity, high background signal, incomplete bisulfite conversion or a low success rate (<95%) were removed. Probes with low beadcount (<3), with high detection p-value or with low success rate (<0.95%) over samples as well as ambiguously mapping probes were removed. Normalization and batch effects were corrected for using 'Functional Normalisation' with 8 principal components of control probes. Principal components of CpG probes were calculated and used to identify possible mix up of samples by plotting PCs vs sample type (i.e. blood or plaque DNA) and sex. In addition, where available, genotype data from previous studies (using genotyping arrays) was correlated to the raw data of the 65 SNPs included on the HM450k array, and samples with poor correlation ($R < 0.6$) across these 65 SNPs were excluded due to possible mix up. Quality control showed 488 plaque samples (96%) and 92 blood samples (98%) of good quality for further analysis. During quality control, 41,640 probes were excluded (including cross-reactive probes[14]), with 443,872 probes (91.4 %) of good quality remaining. An overview of the sample quality control is depicted in a flow-chart (Supplemental figure 1). For the current analyses, only probes on the autosomal chromosomes were included as X-chromosomal DNA methylation is subject to X-chromosomal inactivation and requires a different methodological approach.

Analysis

Statistical analyses were carried out in R Studio (v0.98.981) using R (v3.2.2). Fast-LMM[15] was used to perform epigenome-wide associations with one methylation principal component in the model, while applying genomic control to correct for residual confounding. Outliers (>3 s.d.) for each probe were removed prior to epigenome-wide analysis. Further association of sex-related CpGs with cardiovascular risk factors and histological parameters was performed by linear regression modelling, using age and sex as covariates.

Athero-Express Genomics Study

Common genetic variation has been examined previously in the Athero-Express, and is described elsewhere.[16] In short, patient DNA samples were genotyped in two batches, and imputation was performed using the 1000 Genomes Project phased haplotypes as the reference panel. Imputed genotypes were available for 454 of the total of 509 patients.

Determining DNA methylation of the gene promoter

For all autosomal DNA-methylation probes, the distance to the transcription start site (TSS) of nearby genes was determined with a custom Perl script using the RefSeq gene annotation (July 2015, GRCh37/hg19). To determine the overall methylation status of the gene promoter, we included all probes within a window of 1000 bp upstream and 500 bp downstream of the gene TSS. Outlier-probes in this windows were discarded (> 0.2 outside of the median methylation-beta of all probes in the window, measured across all samples). We then calculated gene-promotor methylation for each sample as the median of the included probes for each gene.

Gene-centered analysis

We performed an epigenome-wide analysis in plaque on median promoter methylation of 14,456 genes for sex differences, which resulted in 22 differentially methylated gene promoters after genomic control. To further interrogate these genes, we retrieved publicly available expression datasets in the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) that contained human cardiovascular-specific tissues (i.e. plaque or blood) of patients with a form of cardiovascular disease with readily available information regarding sex (Supplemental table 9). We used the GEO2R tool to calculate sex-differentially expressed genes.[17] Datasets with similar cell types were meta-analyzed using weighted Z-statistic meta-analysis. Genes were considered of interest if they showed a consistent effect of promoter methylation and expression (e.g. a combination of higher methylation and lower expression levels or vice versa). Significance was determined by Bonferroni correction ($0.05/22 = 2.2x10^{-3}$). Furthermore, annotation using enrichment and pathway analysis was performed by means of the ToppFun suite.[18]

Results

Population characteristics

DNA-methylation was determined in carotid plaque samples of 488 patients (70% male) and in blood samples of 92 patients (66% male), of which samples of 85 patients were overlapping. Cohort baseline characteristics can be found in Table 1.

Table 1. General cohort characteristics

Characteristics		Men (N = 340)	Women (N = 148)	p value
Age	years	68 (61-74)	69 (62-74)	0.809
SBP	mmHg	153 (135-170)	155 (140-170)	0.239
DBP	mmHg	81 (73-90)	80 (75-90)	0.907
eGFR	ml/min/1.73m^2	74 (61-86)	68 (55-85)	0.051
BMI	kg/m^2	26.1 (24.3-28.4)	26.1 (23.8-28.5)	0.519
hsCRP	mg/l	1.8 (1.1-4.1)	2.8 (1.5-7.9)	0.017
Diabetes	yes	82 (24.1)	28 (18.9)	0.252
Hypertension	yes	247 (75.3)	115 (78.8)	0.483
Statins	yes	257 (75.5)	112 (75.7)	0.999
Smoking	yes	130 (38.5)	64 (44.4)	0.271
Symptoms				0.986
Asymptomatic		56 (16.5)	24 (16.3)	
TIA		152 (44.7)	65 (44.2)	
Stroke		89 (26.2)	36 (24.5)	
Retinal		43 (12.6)	22 (16.0)	

Patient characteristics at time of inclusion stratified by patient sex, excluding patients without data on sex (N = 15). Continuous variables are shown as medians with interquartile ranges. Categorical variables are shown as number with percentage. Symptoms refer to symptoms at presentation, before carotid endarterectomy. Significance shown as p values without FDR adjustment. *Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate by MDRD-formula; BMI, body-mass index; LLDs, use of lipid-lowering drugs; Retinal, retinal infarction and amaurosis fugax.*

Association of CpG methylation with sex

An epigenome-wide association study (EWAS) with covariate age was performed on plaque methylation of 340 men and 148 women to identify CpGs that were differentially methylated between the sexes. After applying genomic control to correct for inflation, 3,573 of 443,872 loci were significantly different between the sexes (Figures 1 and 2, Supplemental table 1, Supplemental figure 3). Loci were more often hypermethylated in women when compared to men (Figure 3). The top 25 differentially methylated CpGs are listed in table 2. Of these 25 CpGs that are now reported to be associated with sex for the first time in atherosclerotic tissue, 24 were previously reported in other tissues and showed overlap with our data in blood from patients undergoing carotid endarterectomy (Supplemental table 2, Supplemental figure 4-5). We validated 351/3,573 CpGs in blood (binomial *P* value $6{,}2 \times 10^{-22}$). Moreover, a similar sex-differential methylation was found across tissues, pointing towards a possible constitutive effect (Supplemental figure 6).

Association of sex-differentially methylated CpGs with cardiovascular parameters

Conceivably, these 3,573 CpGs that are now thought to be constitutively different between the sexes may be confounded by cardiovascular risk factors which may differ

between the sexes. However, none of these CpGs were found to associate with the cardiovascular risk factors LDL levels, HDL levels, current smoking, use of statins, diabetes mellitus, hypertension or BMI (Supplemental table 3). A sex-difference in CpGs may also be due to residual confounding due to cell-type heterogeneity in the plaque. Yet we were unable to show an association of the 3,573 significant CpGs with histological plaque parameters (Supplemental table 4).

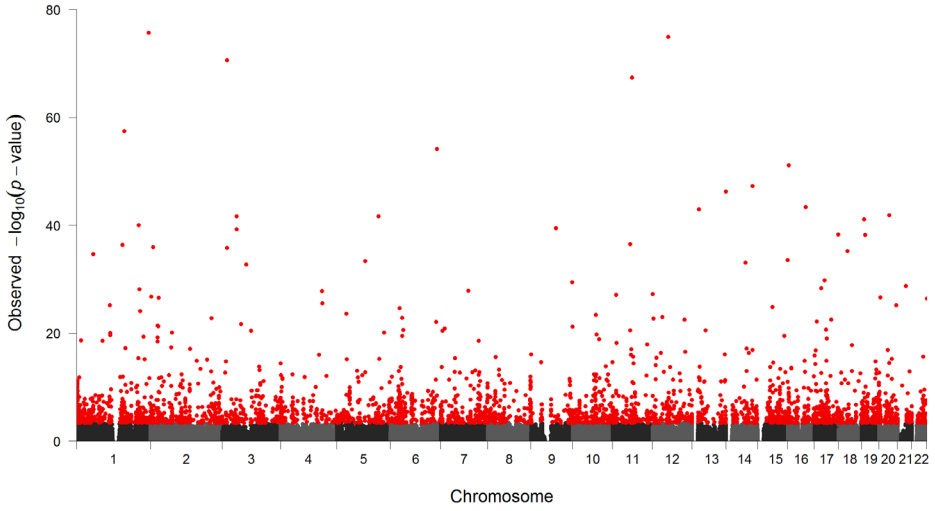

Figure 1. Manhattanplot of sex-specific plaque methylation
Manhattan-plot of differentially methylated CpGs between the sexes in 488 plaque samples, red points denote significant CpGs.

Association of gene promoter methylation with sex

The best evidence for an effect of CpGs on gene expression has been found in CpGs in promoter regions of genes. To grasp the biological meaning of the found associations in CpGs, we therefore calculated a composite measure of promoter methylation. We determined median DNA methylation within the promoter for all genes in each individual patient. Analysis of DNA methylation in gene promoter areas showed relatively minor variability between multiple CpGs in the same promoter (Supplemental figure 2). We studied differences in promoter methylation of each gene between the sexes, which yielded 22 gene promoters that showed sex-specific promoter methylation in atherosclerotic plaque tissue after application of genomic control (Supplemental table 6). We validated 9 of the 22 gene promoters in blood (Supplemental table 6). To investigate whether the differences could be hormone-driven, we annotated all genes with estrogen receptor binding site motifs. The gene promoters with such motifs on average less likely to differ between women and men compared to those without such motifs, arguing against estrogen-driven DNA methylation (Supplemental figure 7).

Table 2. Top 25 of autosomal CpGs that differ significantly between women and men

CpG	Chr	Position	beta	se	Closest Gene	Relation to gene	Relation to Island	FDR p value	Methylation in men	Methylation in women	Lookups in different tissues
cg12691488	1	243053673	0.440	0.008			Island	9.00E-71	0.390	0.190	PFC;LEU;PAN;WB
cg03691818	12	53085038	-0.420	0.008	*KRT77*	Body	Open Sea	2.50E-70	0.030	0.150	PFC;LEU;PAN;WB
cg11643285	3	16411667	-0.420	0.008	*RFTN1*	Body	Open Sea	3.50E-66	0.740	0.870	PFC;PAN;WB
cg25294185	11	65487814	0.420	0.009	*RNASEH2C*	Body	Island	4.30E-63	0.150	0.052	COL;PFC;LEU;PAN;WB
cg03618918	1	160865097	0.410	0.010			Open Sea	3.20E-53	0.810	0.730	PFC;LEU;PAN;WB
cg25568337	6	157098338	-0.400	0.010	*ARID1B*	TSS1500	Open Sea	5.30E-50	0.140	0.230	PFC;LEU;PAN;WB
cg26921482	16	2570283	-0.390	0.011	*AMDHD2*	TSS200	Island	4.80E-47	0.250	0.460	PFC;LEU;PAN;WB
cg02325951	14	89878619	0.390	0.011	*FOXN3*	Body	North Shelf	2.90E-43	0.730	0.620	PAN;WB
cg26355737	13	114292172	0.390	0.012	TFDP1	Body	North Shore	2.60E-42	0.890	0.840	PFC;LEU;PAN;WB
cg04946709	16	59789030	0.370	0.012	*LOC644649*	Body	Island	1.80E-39	0.820	0.700	PFC;LEU;PAN;WB
cg06710937	13	23489940	-0.380	0.012			Island	4.20E-39	0.044	0.120	PFC;LEU;WB
cg08906898	20	34319899	0.370	0.012	*RBM39*	Body	Open Sea	4.70E-38	0.900	0.850	PFC;LEU;WB
cg13323902	5	140090859	-0.380	0.012	*VTRNA1-1*	TSS200	Open Sea	7.10E-38	0.210	0.290	PFC;LEU;WB
cg02758552	3	49395714	-0.370	0.012	*GPX1*	1stExon;5'UTR	Island	7.10E-38	0.240	0.330	PFC;PAN;WB
cg04590718	19	8660758	-0.480	0.015	*ADAMTS10*	Body	North Shore	2.10E-37	0.590	0.680	
cg15817705	1	209406063	0.390	0.013			South Shore	2.50E-36	0.810	0.740	PFC;LEU;PAN;WB
cg07852945	9	84303915	-0.360	0.012	*TLE1*	TSS1500	Island	8.40E-36	0.130	0.200	PFC;LEU;WB
cg23814743	3	49466685	-0.360	0.012	*NICN1*	1stExon;5'UTR	Island	1.40E-35	0.290	0.340	PFC;LEU;WB
cg22345911	17	80231263	-0.360	0.012	*CSNK1D*	5'UTR;1stExon	Island	1.20E-34	0.038	0.074	COL;PFC;LEU;WB
cg03608000	19	11998623	-0.360	0.012	*ZNF69*	TSS200	North Shore	1.20E-34	0.061	0.088	PFC;LEU;WB
cg17232883	11	59318136	-0.360	0.013			Open Sea	5.90E-33	0.110	0.170	PFC;LEU;WB
cg12177922	1	154245232	-0.360	0.013	*HAX1*	1stExon	Island	7.40E-33	0.190	0.280	PFC;LEU;PAN;WB
cg02989351	2	9770584	-0.440	0.016	*YWHAQ*	5'UTR	Island	1.90E-32	0.160	0.200	PFC;LEU;WB
cg17238319	3	16428391	-0.370	0.013	*RFTN1*	Body	Open Sea	2.50E-32	0.710	0.820	PFC;LEU;PAN;WB
cg25304146	18	30092971	0.350	0.013	*WBP11P1*	Body	Open Sea	1.10E-31	0.680	0.610	PFC;LEU;PAN;WB

Table showing the top 25 sex-differentially methylated CpGs in carotid plaque tissue. Lookups in different tissues refers to previously reported significantly sex-differentially methylation in other tissues using the HM450k chip. Abbreviations: PFC, prefrontal cortex; LEU, leukocytes; PAN, pancreatic islet cells; COL, colon; WB, whole blood from the Athero-Express cohort.

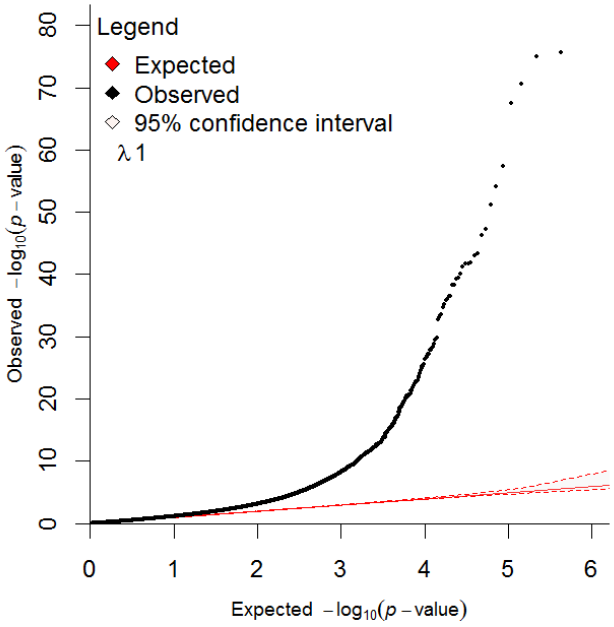

Figure 2. QQplot
Quantile-quantileplot comparing observed and expected p-values.

Association of sex-differentially methylated gene promoters with cardiovascular parameters

To further interrogate these genes, we looked for association of their promoter methylation with cardiovascular risk factors and plaque composition, independently of age or sex, which did not show significant associations (Supplemental table 7-8).

Annotation of sex-differentially methylated gene promoters

To investigate if we could find any more evidence pointing towards a biological mechanism of expression by which the 22 differentially methylated gene promoters possibly exert their effect, we examined publicly available gene expression datasets of human cardiovascular tissue and tested whether our sex-differentially methylated genes corresponded to sex-differentially expressed RNA. We performed association of RNA expression with sex as a phenotype in six different human tissues (plaque- and blood-derived macrophages, stimulated and resting monocytes, PBMCs and whole blood, Supplemental table 9) and examined all sex-differentially methylated genes. In the group of sex-differentially expressed genes with a matching DNA methylation pattern (e.g. higher methylation is lower expression or vice versa), we identified more sex-differentially expressed genes with a nominally significant p-value than expected by chance, especially in the PBMC/whole blood meta-analysis (9/22, binomial P = 4,99x10^{-7}) (Supplemental table 9). This indicates that differential methylation of gene promoters

due to sex may result in expression differences between women and men. Finally, we annotated the genes with sex-differentially methylated promoters using a variety of enrichment and annotation analysis tools united in the ToppFun suite (Supplemental table 10). Gene Ontology analysis showed a nominally significant enrichment for genes involved in sexual reproduction (*NHLH2, ZPBP2, PRDM9, SPESP1, ASZ1* and *LHX8*). Coexpression analysis showed expression in sex-specific cells such as testis and ovary (e.g. *DPPA5, DDX43* and *ASZ1*).

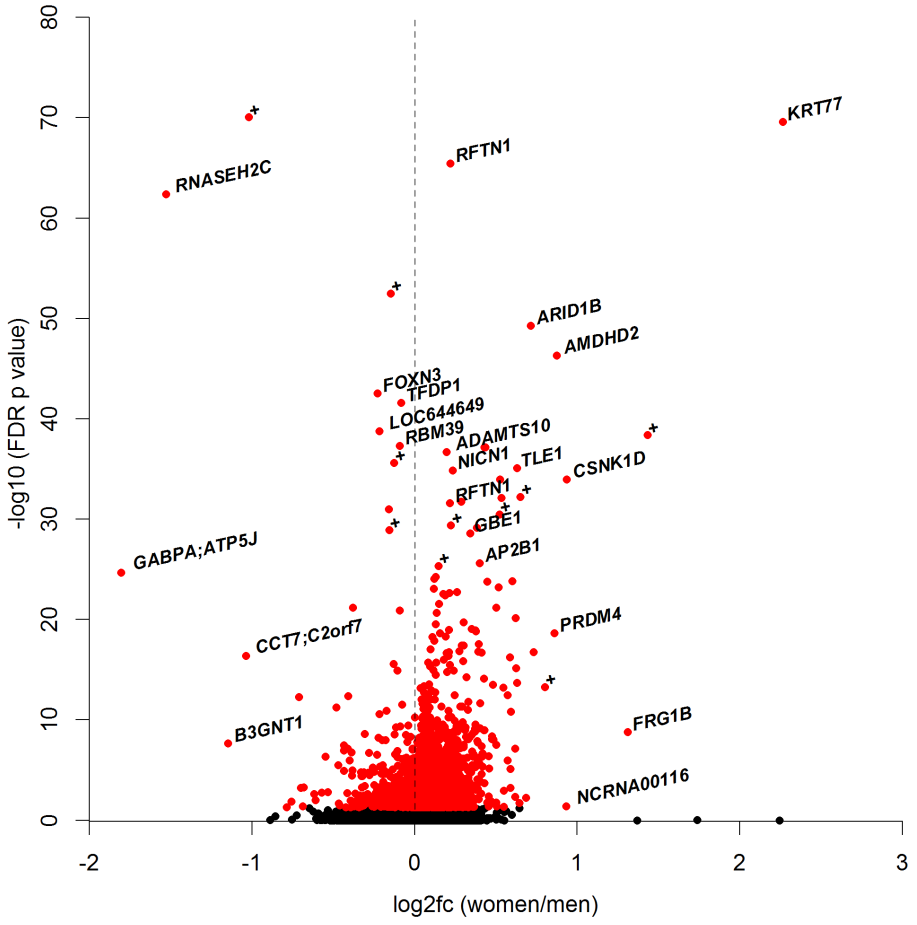

Figure 3. Volcano plot
Volcano plot showing relationship between p-value and (log) fold-change in DNA-methylation between the sexes. X-axis shows beta value for the effect of male sex on DNA-methylation at a CpG in this gene. Y-axis shows the FDR-corrected −log10(p value) of the association with sex.

Discussion

We present an epigenome-wide association study into sex-specific differentially methylated regions on autosomal chromosomes in vascular tissue of a severely atherosclerotic population. We found many differences between the sexes, from an individual CpG as well as from a gene promoter methylation point of view, some of which we were able to validate in blood samples, suggesting that these sex-differences are not tissue-specific. Indeed, it appeared that these sex-differences were also not implicated in cardiovascular disease mechanisms. This suggests that sex by itself may drive DNA methylation profiles of autosomal chromosomes independent of tissue or (cardiovascular) disease.

Sex-differences in DNA methylation have been reported before in healthy individuals in a wide variety of tissues.[8] Interestingly, we replicated many CpGs that have previously been reported to differ between the sexes in prefrontal cortex, peripheral blood cells, intestinal cells and pancreatic islet cells.[19–22] Furthermore, we were able to validate CpGs and promoters in blood of the same patients. This could indicate that sex-differences by itself are much larger than the effect of the disease on modification of DNA methylation. Indeed, we found no association of DNA methylation at CpGs or gene promoters, with any measures of cardiovascular disease severity.

Secondly, our DNA methylation measure is one of a heterogeneous mixture of cells in the atherosclerotic plaque. As DNA methylation is strongly correlated to cell type, this could point towards a consistent sex-effect on DNA methylation although the methylation differs between cell types. This finding is supported by the fact that all previous reports of studies that use the same Illumina HM450k methylation array report the same top hits when comparing men and women methylation. Furthermore, we did not find any association between the differentially methylated CpGs or promoters with plaque characteristics and we found no evidence for a relation between hormone regulation of DNA methylation, as estrogen-specific binding sites were not overrepresented near our significant results. This may be understandable in the light that our female patients are all approximately 20 years post-menopausal, and that the estrogens do not play an important role anymore in this population. One could speculate about the existence of innate sex-differentially methylated regions. A certain methylation profile, irrespective of tissue type or disease status, may thus in itself be informative as a proxy for sex. Whether the novel sex-associated CpGs that we found, are plaque- or disease specific or merely reflect a gain of statistical power remains to be proven. We investigated the possible biological relevance of the differentially methylated CpGs and genes for cardiovascular disease. We found no association of the observed sex-differentially methylated CpGs and promoters between more severely diseased patients (presenting with stroke versus TIA or asymptomatic or absence or presence of contralateral carotid stenosis). Furthermore, enrichment and annotation analysis revealed no specific cardiovascular disease mechanisms of the sex-differentially methylated CpGs and promoters. This is not surprising, given the fact that sex-differences seem to outweigh

both the effects of cell type and disease phenotype with respect to DNA methylation. Still, the innate differences could in itself be the starting point for different biological mechanisms in men and women that contribute to cardiovascular disease, such as lipid accumulation, inflammatory response or coagulation in the vascular wall. As such, they could still account for differences in cardiovascular disease phenotypes as observed in atherosclerotic plaques. However, these subtle differences cannot be found within the large number of sex-differentially methylated CpGs and promoters. DNA methylation of CpGs in a gene promoter region is relatively well linked to gene silencing and we found evidence pointing towards expression differences with co-expression in sex-specific tissues, yet recent research in the field of epigenetics revealed more complex interplay between different regulatory mechanisms for other CpGs sites.[23] Unequivocally, differences in regulation of gene expression add to the phenotypic differences between the sexes in mammals.[1,2]

This study has several limitations. First, due to stringent correction for possible residual confounding using genomic control, this study may result in a fair amount of false-negative discoveries. Thus, there may be many CpGs affected by sex which go undetected in our study. Future studies may solve this through increased statistical power in larger cohorts. Second, due to the heterogeneous cell types in the plaque we cannot ascertain in which cell types the sex-associated differences arise. Neither can we infer the biological meaning of the related genes in the context of atherosclerotic disease. One of the remaining questions is whether the found methylation differences that are only found in the atherosclerotic plaque are the cause or the effect of the disease phenotype or the influence of sex. However, we replicated many loci that have been reported previously in different tissues and we found no association with cardiovascular disease risk factors or disease severity measures strengthening the current interpretation of the results. Third, we were unable to confirm the role of methylation in gene expression due to the absence of expression data in the Athero-Express biobank. It could very well be the case that many of the CpGs we found are affecting the differences between women and men through regulatory mechanisms. Furthermore, we determined promoter methylation of genes based on a non-empirically determined window. This may lead to inaccuracy and as a result, pathway analysis might suffer from a measure of bias. This may have led to an underestimate of the number of gene promoters associated with sex-differences. However, we do not that believe the absence of the mechanism of action of the CpGs or promoters makes the differences in itself less true or interesting. Furthermore, we found evidence for an association between gene promoter methylation and gene expression within publicly available datasets. Fourth, we chose to exclude the X-chromosome in our study as the imprinted X-chromosome leads to complicated analysis that is not comparable to the autosomal data as it reflects another sex-specific biological mechanism. However, we recognize the fact that differential methylation of the (imprinted) X-chromosome can contribute to sex-differences in the atherosclerotic vessel wall.

Conclusions

We present the first epigenome-wide association study into sex-differences on autosomal methylation in the atherosclerotic plaque. We found 3,573 differentially methylated CpGs and 22 differentially methylated gene promoters. Our data confirm that DNA methylation profiles are highly sex-specific, in the severely diseased atherosclerotic vessel wall as well as circulating blood cells. Our underlines the need for sex-stratified designs in medical research. Further research is needed to investigate the sex-differentially methylated CpGs and genes to determine their role in the molecular mechanisms of sex-differences in (cardiovascular) disease.

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Supplemental material

Supplemental table 1. Epigenome-wide analysis in plaque samples

Supplemental table 2. The effect of sex on DNA methylation in different tissues

Supplemental table 3. Association of CpGs with cardiovascular risk factors

Supplemental table 4. Association of CpGs with carotid plaque histology

Supplemental table 5. Methylation of gene promoters in plaque and blood samples

Supplemental table 6. Association of gene promoters with cardiovascular risk factors

Supplemental table 7. Association of gene promoters with carotid plaque histology

Supplemental table 8. Lookup of gene promoters within sex-differential expression data

Supplemental table 9. ToppFun suite analysis

Supplemental figure 1. Flow-chart of quality control

Supplemental figure 2. Variability of DNA methylation in relation to the transcription start site.

Supplemental figure 3. Scatterplots comparing DNA methylation in men and women

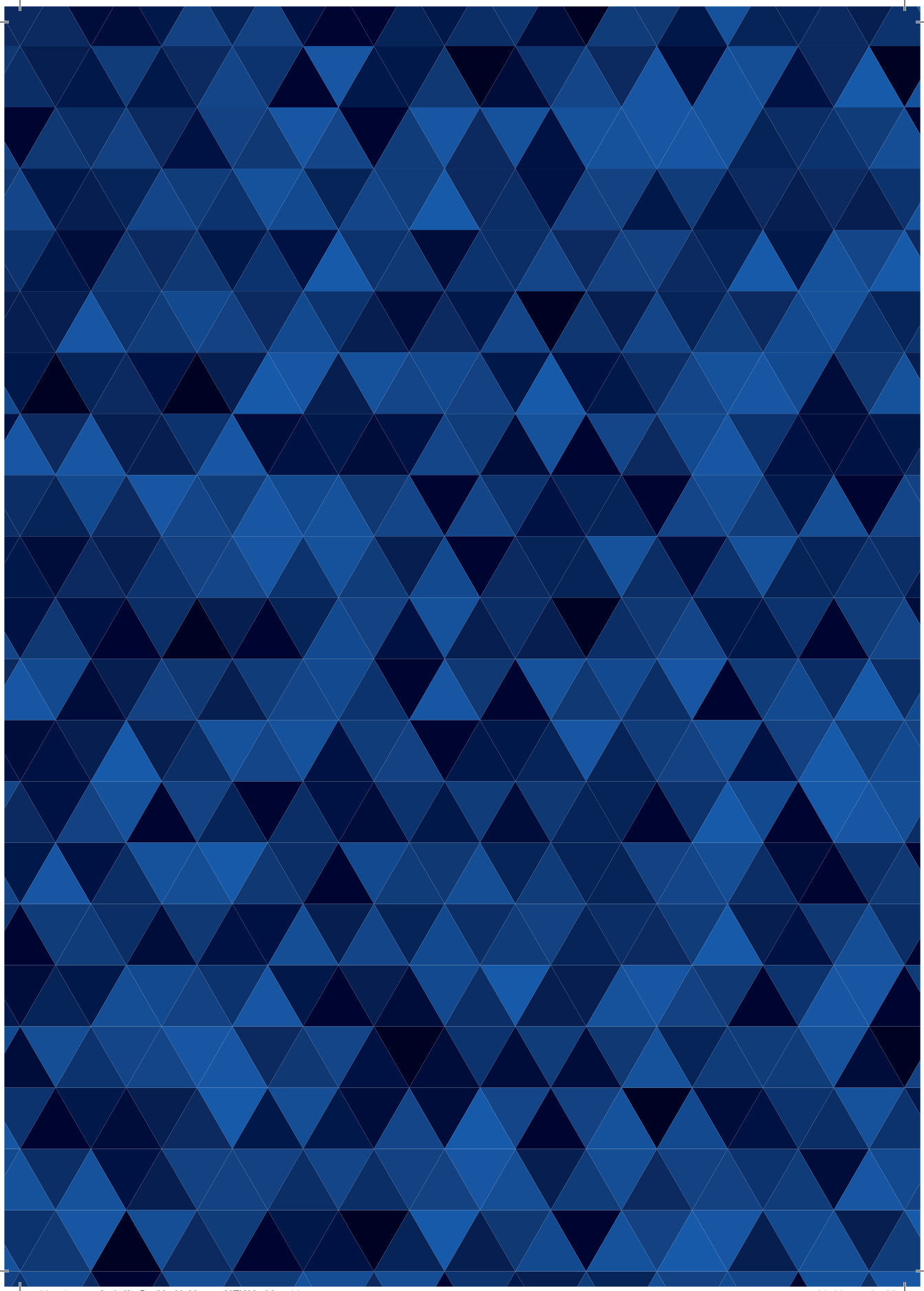
Supplemental figure 4. Manhattanplot of EWAS on sex differences in blood samples

Supplemental figure 5. Qqplot of EWAS on sex differences in blood samples

Supplemental figure 6. Scatterplots comparing DNA methylation across tissues

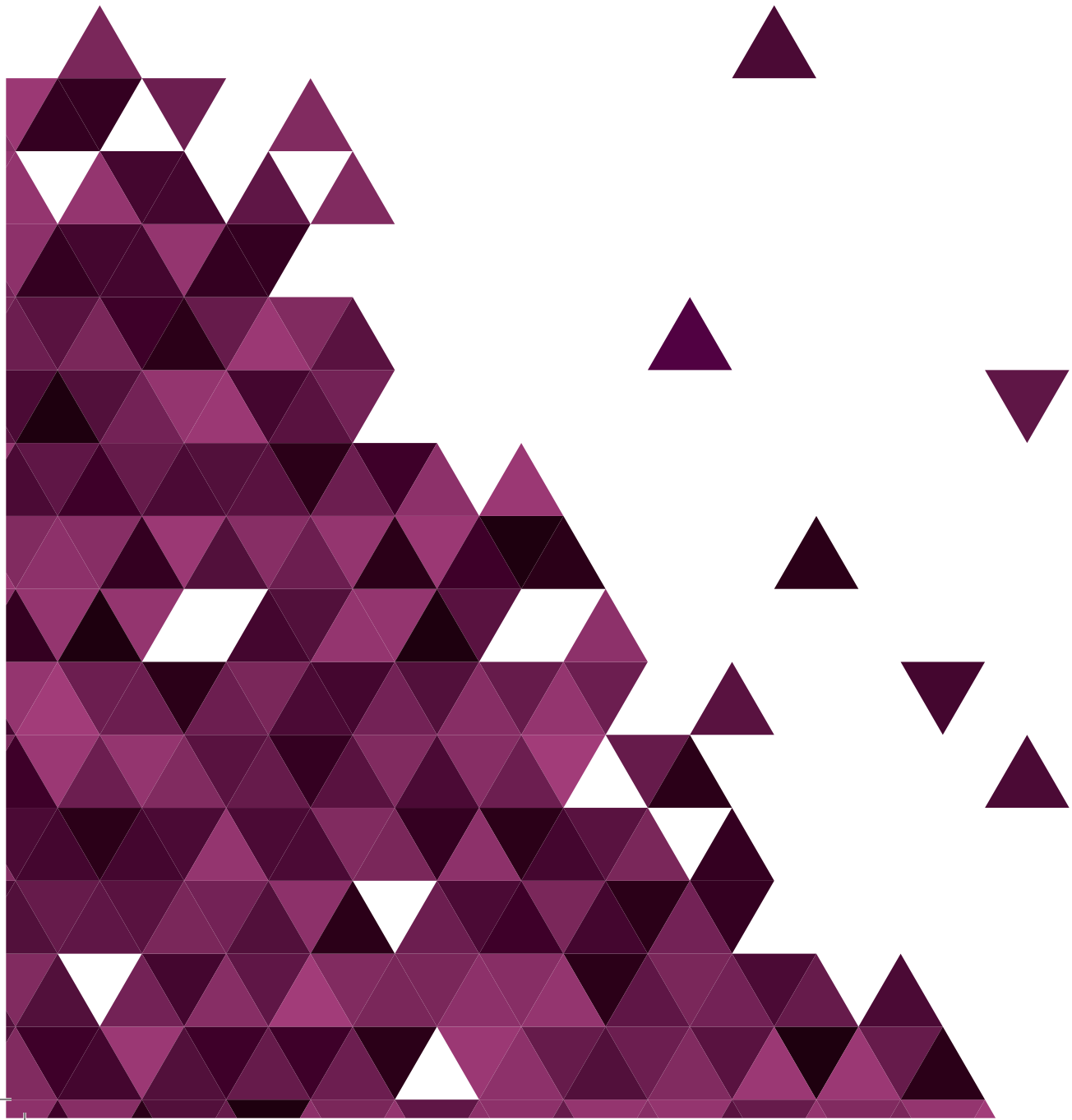
Supplemental figure 7. Scatterplot comparing number of estrogen binding motifs and sex-association of gene promoters

Supplemental material is omitted because of space limitations



PART IV

Studies on clinical outcome



CHAPTER 10

Long-term outcome in men and women after CABG; results from the IMAGINE trial

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Abstract

Background

The aim of this study is to determine sex differences in long-term outcome after coronary artery bypass grafting (CABG).

Methods

The international randomized controlled IMAGINE study included 2553 consecutive patients with a left ventricular ejection fraction of > 40% who underwent isolated CABG. Median follow-up was 32 months (IQR 17 to 42 months). The composite endpoint comprised of death, myocardial infarction (MI), cerebrovascular event, angina, revascularization and congestive heart failure. Cox regression analysis was used to examine sex differences in outcome post-CABG.

Results

Of the 2553 patients, 2229 were men and 324 (13%) were women. Women were older and more often reported diabetes and hypertension. Smoking and impaired renal function were more prevalent in men. Women experienced a higher event rate during follow-up (composite endpoint 18% vs. 12%; P=0.007). Cox regression showed an increased risk of the composite endpoint in women after adjustment for age (HR 1.48 (95% CI: 1.11-1.97)) which was non-significant after additional adjustment for other confounders (HR 1.26 (95% CI: 0.92-1.72)).

Conclusion

Women have a worse long-term outcome after CABG than men in univariate analysis. However, after adjusting for potential confounders female sex became a non-significant predictor for prognosis, possibly due to the small sample size of women. Definite answers regarding sex-differences in long-term outcome after CABG should come from future pooling of studies comprising a larger number of women.

Introduction

Coronary artery disease (CAD) is the main cause of death in women older than 65 years.[1] In 2008 the prevalence of cardiovascular disease in the United States was 35.0% in women compared to 37.4% in men. However age-adjusted mortality rates were higher in women, namely 51.7% versus 48.3%.[1] Previous studies suggest sex differences in treatment and prognosis of CAD, but many discrepancies exist between different studies.[2-16] It remains uncertain whether these differences in outcome are due to a different risk burden between men and women or whether female sex is an independent risk factor of worse outcome and prognosis. Age is a major confounder, as younger, but not older, women have a higher mortality rate than men after myocardial infarction with or without intervention.[17-19] Furthermore, women undergo coronary angiography or percutaneous coronary intervention less often as compared to men.[19-21] The influence of female sex on the outcome after coronary artery bypass grafting (CABG) remains unclear, as previous studies are contradictory.[2, 3, 8, 13] In order to determine possible sex differences in long-term outcome after CABG, data from the Ischemia Management with Accupril post-bypass Graft via inhibition of the coNverting Enzyme (IMAGINE) were analysed. IMAGINE is a multicentre, international randomized controlled trial with extensive data concerning baseline characteristics and operational techniques.

Methods

Patient characteristics

The design and the main results of the Ischemia Management with Accupril post-bypass Graft via Inhibition of the coNverting Enzyme (IMAGINE) trial have been previously described in detail.[22, 23] In brief, the IMAGINE trial is an international, randomized, double-blind, placebo-controlled, multicentre study that investigated whether early administration of an angiotensin-converting enzyme (ACE) inhibitor after CABG reduced cardiovascular events compared to placebo in stable patients. Patients older than 18 years with a left ventricular ejection fraction (LVEF) of $\geq$ 40% who were stable after CABG were included. Exclusion criteria consisted of intolerance or contraindication to ACE inhibitors, insulin-dependent diabetes, concomitant cardiac surgery, serious concomitant disease including severe renal impairment, significant perioperative myocardial infarction, pregnancy and investigational drug use < 30 days. The 2553 patients included in this study between 1999 and 2004 were randomly assigned to quinapril 10-20 mg (n=1280) or to placebo (n=1273). On average patients were randomized 4 $\pm$2 days after CABG, with a maximum of 7 days (10 days in France). The primary endpoint was a composite of time to first occurrence of cardiovascular death or resuscitated cardiac arrest, nonfatal myocardial infarction, coronary revascularization, unstable angina, stroke and congestive heart failure that required hospitalization. Five patients were lost to follow-up (0.2%). For the current analyses all available follow up time was used. The ethics committees of all participating institutions approved the research protocol and all patients gave written informed consent.

Statistical analysis

Patients were stratified by sex. Baseline categorical variables are presented as percentages (numbers). Differences between sexes were calculated by Chi-Square test. Continuous variables are described as the mean value ± standard deviation (SD) if normally distributed or the median value if the distribution was skewed. Possible differences were tested by *t*-test. All statistical tests were two-sided using p <0.05 as level of significance. The primary endpoint was evaluated using a Cox proportional hazard model where men served as the reference category. Results are expressed as hazard ratios (HR) with 95% confidence intervals (95% CI). To identify possible confounders all baseline characteristics and surgical characteristics were related to the composite endpoint separately, adjusted for age. Correlation with the determinant sex was evaluated by a Pearson's correlation chi-square in variables that were significantly associated with the composite endpoint. Those with a p-value <0.1 at Pearson's correlation chi-square, as well as age and sex, were added in the multivariate model. Since previous studies demonstrated that body surface area (BSA) is associated with a worse outcome post-CABG in female sex we used BSA instead of body mass index.[13, 14] Because of the well-documented surgical characteristics, a subanalysis was made regarding the type of grafts used during CABG. All statistical analyses were performed using SPSS Version 21.0.

Results

Patient characteristics

Out of the 2553 included patients 324 (13%) were women. Median follow-up was 32 months in both men and women (IQR 17-42 in men, IQR 15-42 in women). Baseline characteristics are shown in Table 1. Women were on average 5 years older than men and more often reported hypertension and a family history of CAD. Men more often smoked and revealed decreased renal function (all P<0.01).

Characteristics of CABG

On average men received more grafts (3.3 versus 3.0 in women; P<0.01). The percentage of off-pump CABG compared to CABG on cardiopulmonary bypass did not differ between men and women (18% versus 21%, P=0.19). Furthermore, there was no difference in complete revascularization, defined as all vessels > 1 mm with a stenosis > 70% having been bypassed, between women and men (P=0.21).

Endpoint

Women were more likely to experience the composite endpoint, 18% versus 12% in men (P<0.01), as shown in Table 3. This difference is mainly driven by the distribution of unstable angina (5% in women vs 1.9% in men), coronary revascularization (1.2% in women vs 0.4% in men) and congestive heart failure (2.5% in women vs 0.9% in men). Cox regression analysis demonstrated an increased risk of the composite endpoint in

Table 1. Baseline characteristics

	Men (n=2229)	Women (n=324)	P-value
Age, years (SD)	60 ± 10	65 ± 10	<0.01
Median follow-up in months (IQR)	32 (17-42)	32 (15-42)	0.21
Medical history			
Myocardial infarction	40 (887)	35 (114)	0.11
CABG	3 (58)	2 (6)	0.42
Percutaneous coronary intervention	17 (388)	21 (67)	0.15
Peripheral vascular disease	7 (151)	9 (30)	0.10
Stroke/ TIA	1 (33)	1 (4)	0.73
Cardiovascular risk factors			
LDL cholesterol (mmol/L) (SD)	2.9 ± 1	2.9 ± 1	0.95
Diabetes	10 (212)	13 (41)	0.08
HbA1c (mmol/mol) (SD)	39 ± 8	41 ± 32	<0.01
Systolic blood pressure (mmHg) (SD)	121 ± 14	124 ± 15	0.11
Current or former smoker	74 (1658)	52 (167)	<0.01
Family history of coronary artery disease	67 (1480)	73 (235)	0.03
Body surface area (m^2) (SD)	2.0 ± 0.2	1.8 ± 0.2	<0.01
Heart rate (bpm) (SD)	82 ± 13	81 ± 12	0.23
Left ventricular ejection fraction (%) (SD)	60 ± 7	61 ± 10	0.43
MDRD (estimated GFR based on creatinine) (SD)	63 ± 15	108 ± 32	<0.01
Medication			
Acetylsalicyclic acid (ASA)	74 (1567)	72 (205)	0.44
Betablockers	78 (1657)	79 (224)	0.92
Calcium-channel blockers	767 (36)	107 (38)	0.67
Diuretics	9 (184)	9 (25)	0.97
ACE inhibitors	21 (433)	19 (54)	0.55
Statins	65 (1384)	60 (172)	0.09

Continuous variables are presented as mean ± SD; categorical variables are presented as percentages (*n*)
SD, standard deviation; IQR, inter quartile range; LDL, low-density lipoprotein; MDRD, modification of diet in renal disease; GFR, glomerular filtration rate.

women compared to men after adjustment for age with a HR of 1.48 (95%CI 1.11-1.97). Seven other variables were after adjustment for age associated with the composite endpoint, family history of CAD (HR 1.36 (95%CI: 1.06-1.74)), a medical history of PCI (HR 1.65 (95%CI: 1.28-2.11)), CABG (HR 2.28 (95%CI: 1.39-3.72)) or peripheral vascular disease (HR 1.80 (95%CI: 1.30-2.51)), BSA (HR 0.61 (95%CI: 0.38-0.98)), complete revascularization (HR 0.63 (95%CI: 0.48-0.85)) and number of grafts used (HR 0.78 (95%CI 0.71-0.87)). Of these seven variables only a family history of CAD (P= 0.03), number of grafts (P<0.01) and BSA (P<0.01) correlated with sex and were added to the multivariate model (Figure 1). Female sex was not associated with the composite endpoint in the

multivariate analysis (HR 1.26 (95%CI: 0.92-1.72), Figure 1) nor was BSA (HR 0.74 (95%CI: 0.45-1.23). A family history of CAD remained associated with the composite endpoint (HR 1.35 (95%CI: 1.05-1.73)) as well as number of grafts used (HR 0.79 (95%CI: 0.72-0.88)). In the original IMAGINE trial, there were no differences in the incidence of the primary endpoint between the quinapril and placebo group after subdividing by sex.

Table 2. Surgical characteristics

Patient characteristics % (n)	Men (n=2229)	Women (n=324)	P-value
Off-pump CABG	18 (407)	21 (69)	0.19
Number of grafts	3.3 ± 1.1	3.0 ± 1.1	<0.01
Use of LIMA	95 (2120)	92 (297)	0.01
Use of RIMA	19 (415)	9 (28)	<0.01
Use of free IMA	3 (61)	6 (18)	<0.01
Use of other arterial grafts	20 (445)	10 (31)	<0.01
Use of saphenous vein	79 (1757)	79 (257)	0.84
Endarterectomy	6 (111)	8 (22)	0.21
Complete revascularization	88 (1962)	90 (293)	0.21

Continuous variables are presented as mean ± SD; categorical variables are presented as percentages (*n*) CABG, coronary artery bypass grafting; Free artery bypass, composite of radial artery, all other arteries than LIMA or RIMA; IMA, internal mammary artery; LIMA, left internal mammary artery; mixed grafts, arterial or venous grafts; RIMA, right internal mammary artery.

Table 3. Composite endpoint

Patient characteristics	Men (n=2229)	Women (n=324)	P-value
Composite endpoint	12 (273)	18 (57)	<0.01
Cardiovascular death	0.8 (17)	1.2 (4)	
Myocardial infarction (non-fatal)	1 (22)	1.2 (4)	
Documented angina (not req. hosp.)	6.1 (137)	6.2 (20)	
Unstable angina (req. hosp.)	1.9 (43)	5 (15)	
Coronary revascularization	0.4 (9)	1.2 (4)	
Stroke	1.0 (23)	0.3 (1)	
Resuscitation or cardiac arrest	0.1 (2)	0.3 (1)	
Congestive heart failure (req. hosp.)	0.9 (20)	2.5 (8)	

Categorical variables are presented as percentages (*n*). MDRD, estimated GFR based on creatinine; Req. hosp., requiring hospitalization; TIA, transient ischemic attack

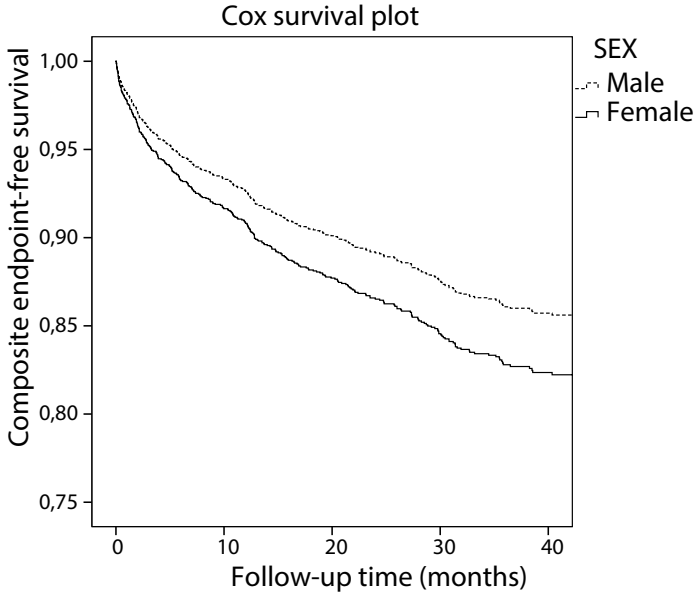

Figure 1. Cox survival plot
Cox survival plot for composite endpoints in women and men

Discussion

The current study demonstrates that women have an increased risk of an adverse outcome after CABG compared to men during 2.5 years of follow-up. However, in the multivariate analysis female sex is not an independent predictor for developing the composite endpoint in this cohort potentially due to lack of power. At baseline women were older and more often had hypertension, a family history of CAD and a smaller BSA. On the other hand men smoked more often and had more frequently renal dysfunction. In regard to other studies both men and women included in the IMAGINE trial reported a relatively low burden of cardiovascular risk factors. Interestingly, our results showed no differences in percentage of off-pump CABG between men and women and no benefit of off-pump CABG for the composite endpoint in both men and women. Previous studies showed an increased risk of adverse outcome in women for CABG on cardiopulmonary bypass, compared to off-pump CABG.[4-8, 10, 14] The majority of prior studies included emergency CABGs whereas we excluded these unstable patients, which makes it difficult to directly compare results.[4-8, 10-15] Furthermore, we used a composite endpoint where others used death as primary outcome. Some studies showed an increased risk in women for early mortality,[2, 8, 10] but the majority found no sex differences.[3, 4, 12-15] Others only found an increased risk for mortality in women after CABG on cardiopulmonary bypass and not after off-pump CABG.[5-7, 11, 16] The higher risk in women we found in the univariate analysis is caused primarily by a higher rate of unstable angina

and coronary revascularization as the number of deaths was equal in both sexes. This is consistent with the finding in this study that the number of grafts used is significant between women and men in the multivariate analysis. The difference in univariate analysis between women and men could therefore point towards a difference in coronary artery diameter: as women are smaller, they have smaller coronary arteries that are technically more demanding in CABG. Indeed, BSA was a confounding factor in this study.

Limitations

Main limitation of this study is the small sample size of women. Women comprised only 13% of our study population compared to 24% on average in other studies.[2-8, 10-16] Unfortunately no screenings log, with numbers screened patients and the reason of exclusion, is available so the low inclusion rate in women remains elusive. One of the possible explanations is the exclusion of patients with severe comorbidities, as women are known to be more severely impaired. The sample size of women introduces an unexpected power problem in the multivariate model, where sex does not seem to associate with the composite endpoint whereas the cox survival plot shows a difference between women and men.

Our results are only applicable to stable patients undergoing CABG since unstable patients were excluded from the study, just as patients with a clinical need for ACE-inhibitors (e.g. severe renal insufficiency and insulin dependent diabetes). We are to our knowledge the first study to include only stable patients and since a large part of the CABG population is stable before surgery, it is relevant to investigate sex differences in outcome in this subpopulation. It could be that sex differences are still present in the unstable group.

Echocardiography testing for diastolic dysfunction which may eventually evolve in to heart failure with preserved ejection fraction (HFpEF) was not performed. As diastolic dysfunction is common in the general population[24] more prevalent among women undergoing cardiac surgery[25] and associated with worse outcome in CAD patients[26], this could be a confounding factor. Also, no data were available on relief of angina symptoms, one of the indications for CABG surgery. However, we do not think this affected the results, since persisting angina was well-documented.

The difference between women and men found in this study was mainly due to differences in 'soft' endpoints such as unstable angina and cardiac revascularization, rather than more robust endpoints such as death. As these 'soft' endpoints are more prone to misclassification, this could potentially have induced non-differential (more in women) misclassification of the outcome. Unfortunately, this type of bias is difficult to overcome and may have overestimated the sex difference.

The duration of follow-up was limited to 2.5 years. Although the majority of the present studies had a limited follow-up of 30 days after CABG,[2, 4, 6, 7, 10-15] some have shown a decrease in the sex gap after long-term follow-up,[3,27] as described earlier by M Claassen *et al*[28]. Future studies should examine a larger number of women during long-term follow-up. For example, an individual participant data analysis of current studies could

improve the power to detect sex-specific differences and their determinants in outcome between women and men after CABG.

Conclusion

Women have a worse long-term outcome after CABG than men in univariate analysis. However, after adjusting for potential confounders female sex became a non-significant predictor for prognosis, possibly due to the small sample size of women. Definite answers regarding sex-differences in long-term outcome after CABG should come from future pooling of studies comprising a larger number of women.

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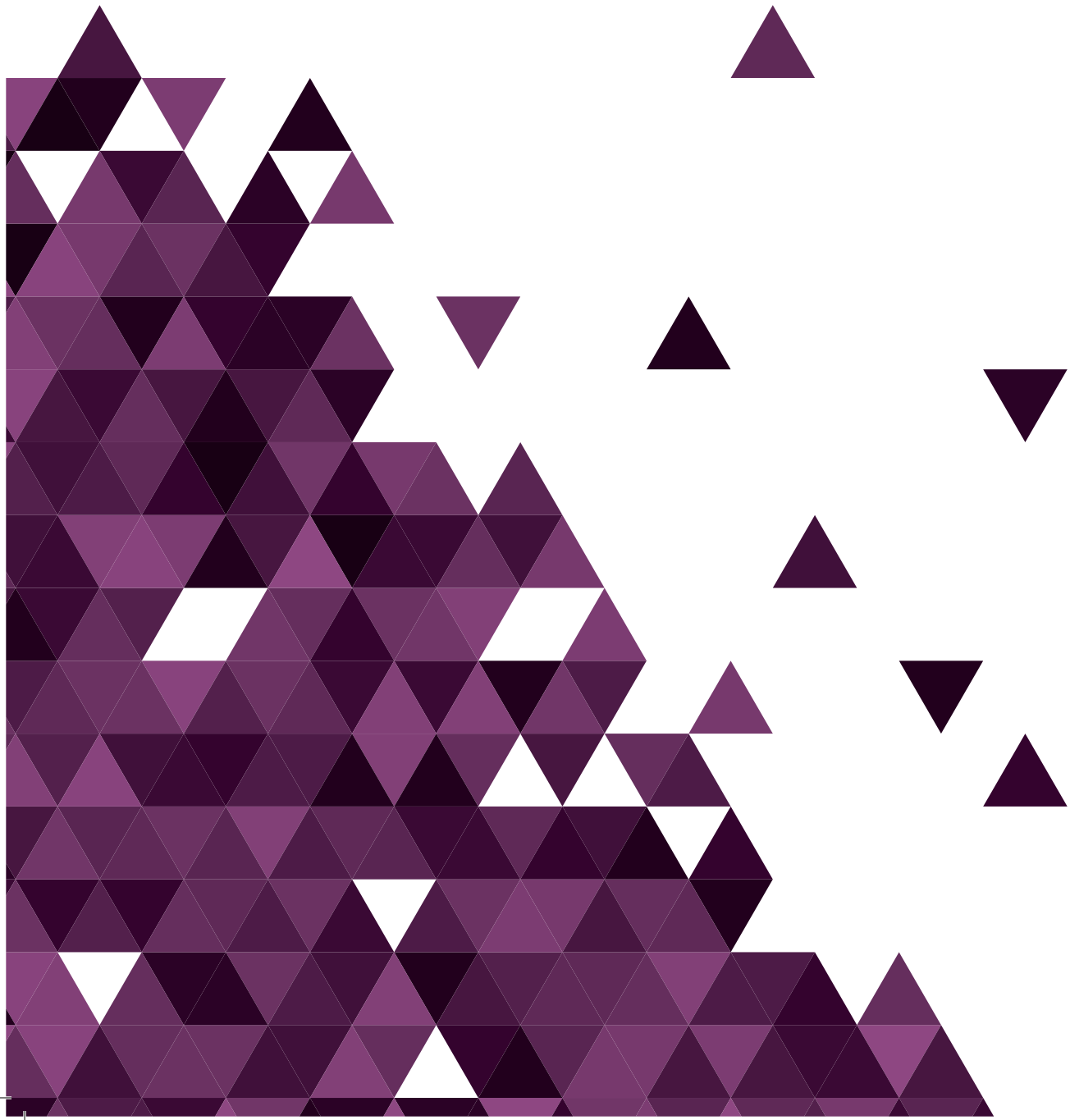

CHAPTER 11

The impact of female sex on long-term survival of patients with severe atherosclerosis undergoing endarterectomy

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Abstract

Background

Long-term age- and sex-specific mortality data in patients undergoing carotid endarterectomy (CEA) and iliac/femoral endarterectomy (FEA) are scarce. We examined long-term mortality in these patient groups, stratified by age and sex.

Methods

Between 2002 and 2012, 1771 patients (1200 men, 571 women) treated by CEA, and 685 patients (495 men, 190 women) who underwent FEA, were included and linked to the national mortality registry of the Netherlands. Absolute mortality risks during follow-up were analyzed by life-table and Kaplan Meier survival analyses in two age groups and stratified by sex, and compared to a matched sample from the general population. In addition, multivariable Cox regression analyses were performed.

Results

After CEA, with a median follow-up duration of 4.3 years (interquartile range 2.0-7.1), 298 all-cause deaths had occurred in men (25%) and 105 (18%) in women. As in the general population, cumulative survival after CEA was significantly better in women compared to men (P = 0.002) and absolute CEA-associated mortality risk in women was similar to that of the general population. For FEA patients, mortality risk was worse than for CEA patients and the general population in both sexes and surprisingly, female sex did not have a favorable effect on survival. Following FEA, 130 men (26%) and 51 women (27%) died after a median follow-up time of 3.0 years (interquartile range 1.5-5.9). Stratifying by age, and adjusting for cardiovascular risk factors did not change these trends.

Conclusions

Long-term mortality after CEA is higher in men than in women, and in women mortality risk is similar to the general population. After FEA, the benefit of women as seen after CEA is lost.

Introduction

Any vascular procedure for obstructive atherosclerosis could have an effect on survival. Carotid and iliac or femoral endarterectomy (CEA/FEA) are procedures that are widely performed by vascular surgeons throughout the world to improve prognosis and/or relieve (ischemic) symptoms in patients with severe atherosclerosis. CEA has shown a benefit in patients with symptomatic carotid artery stenosis and subgroups of asymptomatic patients compared to medical treatment alone[1,2]. FEA is performed in patients with iliac and/or femoral arterial occlusive disease and intermittent claudication or critical limb ischemia. Yet, there is limited information on the short- and mid-term survival of patients undergoing CEA or FEA and noticeably, long-term prognostic data are even rarer (Supplemental material, Tables 1 and 2). In addition, the available data ignore age and sex as important determinants of mortality. Therefore the aim of the present study is to describe absolute mortality risks after CEA or FEA procedures up to 10 years of follow-up, stratified by age and sex.

Methods

Patient population and selection

All studied patients were included in the Athero-Express, a prospective observational longitudinal study in patients undergoing CEA or FEA at the University Medical Center Utrecht, Utrecht, and the St. Antonius Hospital Nieuwegein, the Netherlands, as described in detail previously[3]. Indications for CEA were reviewed in a multidisciplinary meeting and based on recommended criteria by NASCET/ECST/ACST[2,4,5] Patients were treated by FEA if they had at least Fontaine IIb peripheral arterial disease (PAD) and unsuccessful supervised exercise therapy with an indication for iliac or femoral endarterectomy according to guidelines and multidisciplinary consensus[6,7]. All patients scheduled for these procedures were asked to participate, without applying any exclusion criteria. The Institutional Review boards of the hospitals approved the study and all patients provided written informed consent. Patients completed a questionnaire at baseline regarding medication use, cardiovascular risk factors, and medical history. This information was added to clinical data from the electronic hospital databases. For the current analysis, we used data of patients included in the Athero-Express study from 2002 until December 31st, 2012. Duplicate patients were excluded after splitting the total group into CEA and FEA groups (if a patient underwent the same surgical procedure more than once, either bilateral or redo CEA/FEA, only the first operation was included).

Control group from the general population

To compare mortality risk of CEA and FEA patients to a representative control group, we generated an age- and sex-matched random sample from the general Dutch population. For this purpose, the Dutch population registry (PR) was used, which is a longitudinal

dynamic registry containing all Dutch citizens. Statistics Netherlands assigned a personal identifier to everyone in the PR with a unique combination of the variables 'sex', 'date of birth', and '4 digits of postal code'. In the Netherlands, more than 85% of the citizens has a unique combination of these variables and therefore, this personal identifier can be used to identify PR unique individuals in different registries[8]. For each patient in the Athero-Express study, we randomly selected 4 age- and sex-matched unique controls from the PR, as valid on January 1st, 2002 (the start of the Athero-Express cohort). Because mean age was different in CEA and FEA patients, two control cohorts were made, one for each group.

Follow-up: mortality data

Both for our patient and control cohort in this observational study, we linked all individuals to the mortality registry of Statistics Netherlands[9]. Linkage was done for those who were PR-unique using the personal identifier. Quality of linkage of patients from the PR to the mortality registry has been assessed previously. It was shown that the number of false positive linkages was around 1%. The number of missed linkages due to administrative errors in postal codes and missing birth data was approximately 4%[10]. Hence, quality of linkage has been shown to be good. As all-cause death was the outcome to be studied, data on occurrence and date of death were subsequently collected for all individuals, with an ultimate follow-up date on December 31st, 2013. There was no loss to follow-up for this outcome measure.

Statistical analysis

Continuous baseline characteristics were compared by Students t-test, or Mann-Whitney U test, where applicable. Categorical variables were compared by chi-square test. Age stratification was done in 2 groups. The study was limited by power (mainly the female FEA group) to create more (sub)groups. A cut-off value of 70 years was applied, which was based on the mean age of both sexes in the CEA and FEA groups. Survival was analyzed by Life table analysis to get absolute risks, and Kaplan-Meier survival analysis (comparing groups by Log rank test). Because we observed interesting sex-specific trends in this study, Cox proportional hazards models for both CEA and FEA groups were made additionally to evaluate whether these trends were independent of cardiovascular risk factors. These potentially confounding risk factors were previously determined based on clinical relevance. These variables were age, body mass index, smoking, alcohol use, HDL-cholesterol, diabetes mellitus, hypertension, history of peripheral intervention, history of coronary artery disease, acetylsalicylic acid and statin use, osteoporosis therapy (calcium, vitamin D, bisphosphonates, or a combination of these), symptom status (Fontaine stadium in the FEA group), glomerular filtration rate (binned at 60 ml/min), and year of operation[11] (binned at 2006). For CEA, contralateral carotid stenosis was additionally included. These variables were tested in univariable models first. If the associated P-value was <0.25 (to ascertain that important variables were not missed), the variable was also included in the multivariable model (in which covariates were added

simultaneously). A two-sided P-value of <0.05 was considered statistically significant. All statistical analyses were performed with SPSS statistics version 20. Analyses with both population- and mortality registries were performed in agreement with privacy legislation in the Netherlands and performed in a secure environment at Statistics Netherlands. This ensures that results do not reveal information on the individual patient level.

Literature review

In order to provide an overview of the contemporary literature in line with our study aim, a Medline and Embase search was performed on 30 January 2014 combining synonyms for either CEA or FEA and mortality. Fulltext available original articles in English, German, French, Dutch, and Spanish that reported either sex- and/or age-stratified mortality risks after CEA and FEA were included. If only combined endpoints were shown, articles were excluded (Supplemental material for detailed methodology of this review).

Results

Patient selection

A patient cohort of 2740 patients was sent to Statistics Netherlands. After linkage, 2618 records remained (96% complete linkage). These comprised of 1839 CEA and 779 FEA procedures. After removing duplicates (bilateral or redo-CEA/FEA), 1771 unique patients treated by CEA (1200 men, 571 women) for symptomatic or asymptomatic high-grade carotid stenosis were included (Table 1). In addition, 685 unique patients underwent FEA in this period (495 men, 190 women) (Figure 1).

Patient characteristics of CEA patients

Mean age was similar in both sexes (69 years). Most important baseline differences between men and women were prevalence of hypertension (70% vs. 79%, P < 0.001), history of coronary artery disease (36% vs. 24%, P < 0.001), and cholesterol levels (both total cholesterol and HDL cholesterol were higher in women). Kidney function was worse in women, who had a glomerular filtration rate of 68 ml/min compared to 76 ml/min in men (P < 0.001). Symptom status and time from event to CEA were similar (Table 1; complete case analysis was done on all patient characteristics).

Patient characteristics of FEA patients

In this group, mean age was 67 years in both men and women. Male patients had a higher body mass index (26.8 vs 25.1, P = 0.027) compared to women. As in CEA patients, history of coronary artery disease was more frequent in men (45% vs 29%, P < 0.001) and men had a better kidney function (glomerular filtration rate 86 ml/min vs. 70.5 ml/min, P = 0.001). In women there was a trend towards more severe symptoms than in men, with 55% Fontaine III or higher, compared to 44% in men (P = 0.067) (Table 1; complete case analysis was done on all patient characteristics).

Table 1. Patient characteristics

	CEA			FEA		
	Male patients (n=1200)	**Female patients (n=571)**	**P-value***	**Male patients (n=495)**	**Female patients (n=190)**	**P-value***
Age (mean) (SD)	68.8 (8.9)	69.2 (9.9)	0.46	67.4 (8.7)	67.6 (10.7)	0.83
Current smoking	388/1176 (33)	208/548 (38)	0.044	188/482 (39)	87/187 (47)	0.076
Diabetic mellitus	278/1200 (23)	115/570 (20)	0.16	149/495 (30)	52/190 (27)	0.48
Hypertension	815/1162 (70)	441/557 (79)	<0.001	345/479 (72)	135/186 (73)	0.89
Body Mass Index (BMI) (mean) (SD)	26.3 (3.6)	26.3 (4.7)	0.91	26.8 (10.2)	25.1 (4.4)	0.027
Alcohol use						
No	379/1111 (34)	334/520 (64)		74/163 (45)	108/458 (24)	
1 to 10 units/week	417/1111 (38)	131/520 (25)		57/163 (35)	162/458 (35)	
≥ 10 units/week	315/1111 (28)	55/520 (11)	<0.001	32/163 (20)	188/458 (41)	0.003
Hypercholesterolemia	736/1108 (66)	353/516 (68)	0.43	302/445 (68)	117/166 (71)	0.54
History of coronary artery disease	428/1199 (36)	137/569 (24)	<0.001	221/495 (45)	55/190 (29)	<0.001
History of peripheral intervention	204/1195 (17)	112/568 (20)	0.18	231/493 (47)	91/190 (48)	0.81
Bilateral carotid stenosis	510/1074 (48)	217/508 (43)	0.076	NA	NA	
Preoperative acetylsalicyl acid use	998/1197 (83)	500/569 (88)	0.014	389/494 (79)	145/190 (76)	0.49
Preoperative statin use	909/1199 (76)	446/569 (78)	0.23	362/495 (73)	138/190 (73)	0.90
Osteoporosis therapy	28/1199 (2.3)	36/568 (6.3)	<0.001	15/494 (3.0)	22/190 (11)	<0.001
Total cholesterol (mean) (SD)	4.6 (1.2)	5.0 (1.3)	<0.001	4.7 (1.2)	4.9 (1.3)	0.31
HDL (mean) (SD)	1.1 (0.45)	1.3 (0.70)	<0.001	1.1 (0.35)	1.4 (0.47)	<0.001
LDL (mean) (SD)	2.9 (2.4)	3.0 (1.1)	0.43	2.7 (0.95)	2.6 (1.1)	0.76
Glomerular filtration rate, CG (mean) (SD)	76.4 (26.5)	67.9 (24.4)	<0.001	86.4 (56.4)	70.5 (33.4)	0.001

Table 1. *Continued*

Clinical presentation							
Asymptomatic	179/1196 (15)	64/567 (11)		242/428 (57)	70/153 (46)		*Fontaine IIb*
Ocular symptoms	174/1196 (15)	100/567 (18)		101/428 (24)	47/153 (31)		*Fontaine III*
Transient ischemic attack	521/1196 (44)	244/567 (43)		85/428 (20)	36/153 (24)	0.067	*Fontaine IV*
Stroke	322/1196 (27)	159/567 (28)	0.096				
Event to operation time, (median) (IQR)	35 (15-85)	35 (14-79)	0.47	NA			
							Operated artery
				53/177 (30)	116/461 (25)		*SFA/DFA*
				96/177 (54)	260/461 (56)		*CFA*
				14/177 (7.9)	40/461 (8.7)		*Iliac*
				14/177 (7.9)	45/461 (9.8)	0.62	*Combination femoral and/or iliac segments*

CEA: carotid endarterectomy; FEA: femoral endarterectomy; SD: standard devation; HDL: High Density Lipoprotein; CG: Cockroft-Gauld; IQR: interquartile range; SFA: superficial femoral artery; CFA: common femoral artery; DFA: deep femoral artery; NA: not applicable. * comparing men and women

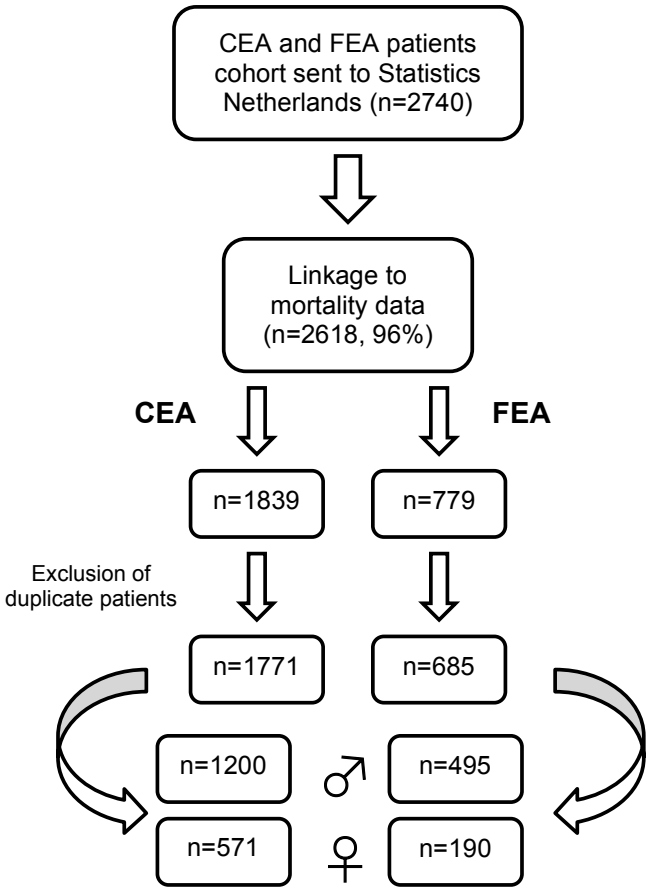


Figure 1. Flowchart of included patients

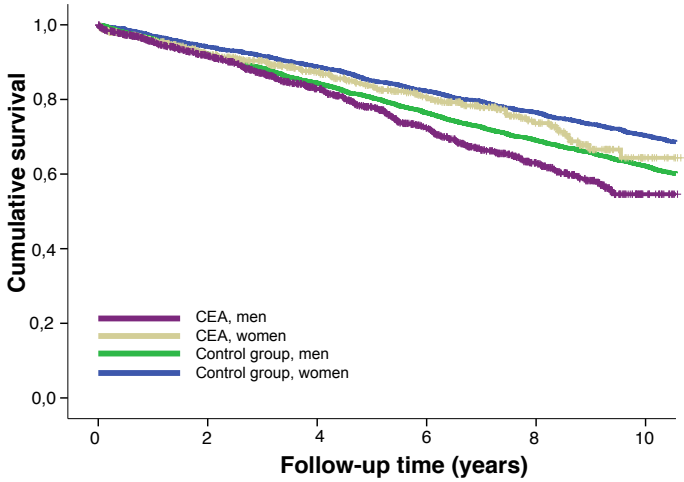
Absolute mortality risks during years of follow-up after CEA by sex and age

In total, 298 men (25%) and 105 women (18%) died during follow-up of the study. Median follow-up duration of all patients was 4.3 years (interquartile range (IQR) 2.0-7.1), in men 4.3 (IQR2.0-7.0) and in women 4.5 (2.1-7.3). Mortality risks per follow-up year were higher in men than in women (P = 0.002), and for women, risks were similar as in the general population. Mortality percentages at 3 and 9 years follow-up in men were 13% and 42%, and 10% and 32% in women, respectively. Interestingly, women had similar mortality percentages after CEA as age-matched controls (Table 2 and Figure 2). The observed differences between men and women in the CEA group were independent of cardiovascular risk factors (hazard ratio for all-cause mortality in men vs. women: hazard ratio (HR) 1.7, 95% confidence interval (CI) 1.2-2.4) (Table 4).After stratifying on age (70 years as cut-off), 656 men in the CEA group were 70 years or younger, and 544 were older.

Tabel 2. Cumulative 10-year mortality risks for men and women after carotid endarterectomy

Follow-up years	1	2	3	4	5	6	7	8	9	10
Men	4 (0.6)	8 (0.8)	13 (1.1)	17 (1.2)	22 (1.4)	28 (1.6)	34 (1.8)	37 (1.9)	42 (2.2)	46 (2.8)
Control group men	4 (0.3)	8 (0.4)	12 (0.5)	16 (0.5)	19 (0.6)	24 (0.6)	27 (0.6)	31 (0.7)	34 (0.7)	38 (0.7)
Women	4 (0.9)	8 (1.2)	10 (1.3)	13 (1.5)	17 (1.8)	19 (2.0)	22 (2.2)	26 (2.6)	33 (3.3)	35 (3.7)
Control group women	3 (0.4)	6 (0.5)	8 (0.6)	11 (0.7)	15 (0.7)	18 (0.8)	21 (0.8)	23 (0.9)	27 (0.9)	30 (1.0)
Age stratified										
Men <= 70	2 (0.6)	4 (0.8)	7 (1.1)	10 (1.3)	13 (1.5)	18 (1.8)	21 (2.1)	24 (2.2)	28 (2.7)	32 (3.5)
Control group men <= 70	2 (0.2)	3 (0.3)	5 (0.4)	6 (0.5)	8 (0.5)	10 (0.6)	12 (0.6)	14 (0.7)	17 (0.7)	19 (0.8)
Men >70	7 (1.1)	13 (1.5)	20 (1.9)	26 (2.2)	34 (2.5)	41 (2.7)	50 (3.0)	56 (3.2)	62 (3.6)	66 (4.2)
Control group men >70	6 (0.5)	13 (0.7)	20 (0.9)	27 (1.0)	34 (1.0)	40 (1.1)	45 (1.1)	51 (1.1)	56 (1.1)	60 (1.1)
Women <= 70	3 (1.0)	4 (1.1)	4 (1.3)	6 (1.6)	8 (1.8)	9 (2.0)	9 (2.0)	12 (2.5)	18 (3.5)	20 (4.3)
Control group women <= 70	1 (0.2)	2 (0.4)	2 (0.4)	3 (0.5)	5 (0.6)	6 (0.7)	7 (0.7)	9 (0.8)	10 (0.9)	12 (0.9)
Women >70	6 (1.5)	13 (2.1)	15 (2.3)	20 (2.7)	26 (3.1)	31 (3.5)	38 (4.0)	43 (4.5)	55 (6.3)	NA
Control group women >70	6 (0.7)	11 (0.9)	15 (1.1)	20 (1.2)	26 (1.3)	31 (1.4)	36 (1.5)	40 (1.5)	45 (1.5)	50 (1.5)

Numbers are percentages representing absolute mortality rates at different years of follow-up. NA: not available due to low numbers at risk during interval. Numbers in parentheses indicate standard error of survival risks at each timepoint.



♂ CEA	1200	1034	895	760	629	516	401	294	197	102	27
♀ CEA	571	494	433	380	315	265	218	158	109	52	15

Figure 2. Survival after carotid endarterectomy and in control population, stratified by gender.

Mortality percentages at 3 and 9 years follow-up were 7% and 28% in young men, and 20% and 62% for older men. For women, total numbers of patients in both age categories were 301 and 270, respectively. Mortality percentages at 3 and 9 years follow-up were 4% and 18% for young women and 15% and 55% for the older female group (Table 2).

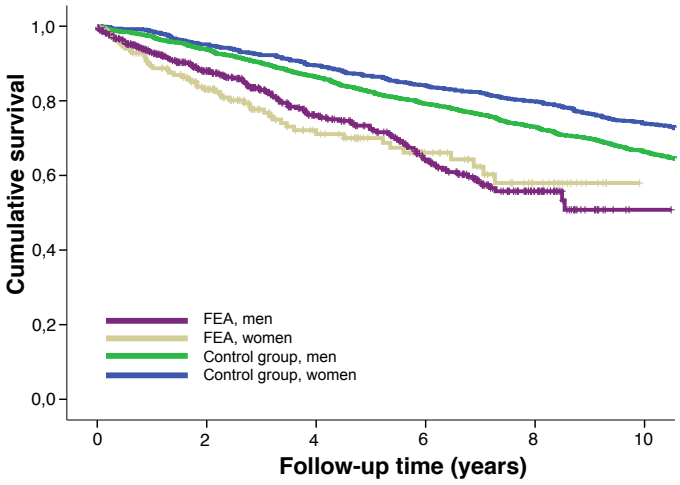
Absolute mortality risks during years of follow-up after FEA by sex and age

During follow-up, 130 men (26%) and 51 women (27%) died. Median follow-up duration of all patients was 3.0 years (IQR 1.5-5.9), in men 3.1 (IQR 1.4-5.9) and in women 2.8 years (IQR 1.5-5.8). Mortality percentages after FEA were similar for both sexes (P = 0.71), and were higher than in the general population. Compared to CEA, survival after FEA was significantly worse in both men and women (P < 0.001 and P < 0.009), indicating a relatively larger disadvantage for women. Mortality percentages at 3 and 9 years follow-up in men were 17% and 49%, and 22% and 43% in women, respectively (Table 3 and Figure 3). After adjusting for cardiovascular risk factors, men did also not have a statistically significant risk of all-cause mortality compared to women in this FEA group (HR 1.1, 95% CI: 0.72-1.7) (Table 4). After stratifying on age, 310 men in the FEA group were 70 years or younger, and 185 were older. Mortality percentages at 3 and 9 years follow-up were 12% and 42% in young men, and 27% and 60% for older men. For women, total numbers of patients in both age categories were 107 and 83. Mortality percentages at 3 and 9 years follow-up were 15 and 26% for young women and 32% and 68% for the older female group (Table 3).

Tabel 3. Cumulative 10-year mortality risks for men and women after iliac/femoral endarterectomy

Follow-up years	1	2	3	4	5	6	7	8	9	10
Men	7 (1.2)	12 (1.6)	17 (1.9)	24 (2.3)	28 (2.5)	36 (2.9)	42 (3.2)	45 (3.4)	49 (4.2)	49 (4.2)
Control group men	3 (0.4)	6 (0.5)	10 (0.7)	14 (0.8)	18 (0.9)	21 (0.9)	24 (1.0)	27 (1.0)	30 (1.0)	34 (1.1)
Women	11 (2.3)	17 (2.9)	22 (3.3)	28 (3.8)	30 (4.0)	34 (4.4)	38 (4.9)	43 (5.6)	43 (5.6)	NA
Control group women	1 (0.4)	5 (0.8)	8 (1.0)	11 (1.1)	13 (1.2)	16 (1.3)	18 (1.4)	20 (1.5)	23 (1.5)	26 (1.6)
Age stratified										
Men <= 70	4 (1.1)	8 (1.7)	12 *(2.0)*	17 *(2.5)*	18 *(2.7)*	28 (3.4)	34 (3.8)	36 (4.2)	42 (5.3)	NA
Control group men <= 70	1 (0.3)	3 (0.5)	5 (0.6)	7 (0.7)	9 (0.8)	10 (0.9)	12 (0.9)	14 (1.0)	16 (1.0)	19 (1.1)
Men >70	13 (2.5)	19 (3.1)	27 (3.7)	37 (4.4)	44 (4.7)	50 (5.0)	57 (5.5)	60 (5.9)	60 (5.9)	60 (5.9)
Control group men >70	5 (0.8)	11 (1.2)	18 (1.4)	25 (1.6)	32 (1.7)	38 (1.8)	43 (1.8)	49 (1.8)	54 (1.8)	59 (1.8)
Women <= 70	9 (2.8)	14 (3.5)	15 (3.7)	20 (4.3)	22 (4.6)	26 (5.3)	26 (5.3)	26 (5.3)	26 (5.3)	NA
Control group women <= 70	1 (0.4)	2 (0.6)	2 (0.7)	4 (0.9)	4 (1.0)	5 (1.1)	7 (1.3)	9 (1.4)	10 (1.5)	11 (1.5)
Women >70	23 (3.8)	20 (4.7)	32 (5.9)	39 (6.7)	43 (7.1)	46 (7.6)	55 (8.6)	68 (9.8)	68 (9.8)	NA
Control group women >70	2 (0.8)	9 (1.6)	16 (1.9)	19 (2.2)	25 (2.4)	30 (2.5)	31 (2.5)	35 (2.6)	40 (2.7)	45 (2.7)

Numbers are percentages representing absolute mortality rates at different years of follow-up. NA: not available due to low numbers at risk during interval. Numbers in parentheses indicate standard error of survival risks at each timepoint.



♂ CEA	495	100	324	253	197	164	121	78	43	11	<10
♀ CEA	190	152	124	88	70	54	42	30	18	<10	<10

Figure 3. Survival after femoral endarterectomy and in control population, stratified by gender.

Literature review

Our Medline and Embase search yielded a total of 3806 unique articles on CEA, of which 69 were eligible for this review. Only 5 studies reported both sex- and age-stratified data, with the majority showing only perioperative mortality data. One study[12] also presented long-term mortality data in 56% of their cohort with a mean follow-up of 61 months. However, age- and sex-specific data were not combined and no other time-points were shown (Supplemental material, Table 1). In general, long-term follow-up data was scarcely reported. For FEA, of 1537 unique articles that were found, 2 were eligible. Both studies reported sex-as well as age-stratified data, but no combined data. One study[13] presented Kaplan-Meier estimated mortality up to ten years stratified by age with limited patient numbers, and only for the younger group (<65 years), which was 28% (Supplemental material, Table 2).

Table 4. Cox proportional hazards models for all-cause mortality

	Univariable, CEA	Multivariable, CEA	Univariable, FEA	Multivariable, FEA
Male sex	1.4 (1.1-1.8)*	1.7 (1.2-2.4)	0.94 (0.68-1.3)*	1.1 (0.72-1.7)
Age	1.07 (1.06-1.08)*	1.08 (1.06-1.1)	1.06 (1.04-1.08)*	1.05 (1.02-1.07)
Body Mass Index	0.96 (0.93-0.99)*	0.96 (0.92-1.0)	0.98 (0.94-1.02)	
Current smoking	1.2 (0.94-1.4)*	1.9 (1.4-2.5)	1.1 (0.82-1.5)	
Diabetic mellitus	1.4 (1.1-1.8)*	1.7 (1.2-2.3)	1.6 (1.2-2.2)*	1.4 (0.95-2.0)
Hypertension	1.2 (0.94-1.5)*	1.5 (1.1-2.1)	0.90 (0.65-1.2)	
HDL-cholesterol	0.52 (0.37-0.74)*	0.61 (0.41-0.91)	1.2 (0.70-2.0)	
Alcohol use (no alcohol as reference)				
1 to 10 units	1.0 (0.80-1.3)		0.86 (0.59-1.3)*	1.00 (0.65-1.5)
More than 10 units	0.95 (0.72-1.3)		0.70 (0.48-1.04)*	0.73 (0.46-1.1)
History of coronary artery disease	1.8 (1.4-2.1)*	1.4 (1.1-1.8)	1.4 (1.03-1.8)*	1.03 (0.71-1.5)
History of peripheral intervention	1.4 (1.1-1.8)*	1.4 (1.0-2.0)	1.1 (0.84-1.5)	
Bilateral carotid stenosis	71.3-1.9)*	1.9 (1.4-2.5)	NA	NA
Preoperative acetylsalicyl acid use	0.46 (0.37-0.59)*	0.60 (0.42-0.83)	0.76 (0.54-1.1)*	0.94 (0.62-1.4)
Preoperative statin use	0.88 (0.71-1.1)		0.74 (0.54-1.007)*	0.76 (0.51-1.1)
Glomerular filtration rate (>60 ml/min)	0.38 (0.31-0.47)*	0.74 (0.54-1.009)	0.36 (0.27-0.50)*	0.59 (0.39-0.90)
Clinical presentation (asymptomatic as reference)				
Ocular symptoms	0.80 (0.55-1.2)*	0.75 (0.45-1.3)	NA	NA
Transient ischemic attack	0.95 (0.71-1.3)*	0.99 (0.67-1.5)	NA	NA
Stroke	1.3 (0.93-1.7*)	1.4 (0.90-2.0)	NA	NA
Fontaine stage (stage 2 as reference)				
3	NA	NA	1.7 (1.2-2.5)*	1.9 (1.2-2.9)
4	NA	NA	2.4 (1.7-3.6)*	2.3 (1.5-3.6)
Operation year (> 2006)	1.2 (0.96-1.5)*	0.98 (0.68-1.4)	0.82 (0.59-1.1)	
Osteoporosis therapy	2.0 (1.3-3.2)*	1.7 (0.94-3.2)	2.1 (1.2-3.7)*	1.0 (0.47-2.2)

Hazard ratios with 95% confidence intervals are shown. CEA: carotid endarterectomy; FEA: femoral/iliac endarterecomy; NA: not applicable. * Indicates hazard ratios with a P-value <0.25 that were included in the multivariable models. Numbers are percentages representing absolute mortality rates at different years of follow-up. NA: not available due to low numbers at risk during the interval.

Discussion

Carotid and iliac/femoral endarterectomy are widely performed procedures within vascular surgery throughout the world. Long-term survival is a very important outcome measure when assessing prognosis of patients undergoing a certain surgical procedure, and is strongly influenced by age and sex. Strikingly, studies that show age- and sex-specific long-term mortality data are very scarce. This study provides absolute age- and sex-specific mortality risks in more than 1700 CEA patients and almost 700 FEA patients, during 10 years of follow-up.

Women operated on carotid stenosis have similar survival compared to the general population

We show that women have a higher life expectancy than men after CEA, just as in the general population. This finding was independent of cardiovascular risk factors, as men had an (adjusted) 1.6 higher chance of mortality during long-term follow-up compared to women in this study. Interestingly, our results even indicate that women with stroke, transient ischemic attack (TIA), amaurosis fugax or asymptomatic women with high-grade carotid stenosis undergoing CEA do not have a worse long-term mortality than the (age-matched) general population. This is an unexpected finding, since it is clear that these women suffer from (presumably systemic) severe atherosclerotic disease, and implies that the surgery is highly effective in preventing long-term mortality in women. However, for asymptomatic women benefit of surgery is still up for debate. Unfortunately, limited group size did not allow for separate analysis of long-term mortality in asymptomatic women.

Long-term survival after FEA is similar in men and women

After FEA, however, the benefit that CEA female patients displayed as compared to men is absent, and women have as worse a prognosis as men. This did not change after adjusting for cardiovascular risk factors. The reason for this relative disadvantage is yet unclear. Intuitively, it may be due to the higher prevalence of comorbidities in women undergoing FEA. Indeed, these women had significantly worse kidney function, with a larger difference as compared to men, than in CEA patients. It is known that kidney function is a very strong predictor of mortality in this population[14]. In addition, women had more severe PAD compared to men, which supports previous observations that women have more extensive disease at presentation[15] and yet are less likely to undergo revascularization procedures than men[16]. On the other hand, several other cardiovascular risk factors were less prevalent in women, opposing the idea that women had a general higher risk than men. One of the factors that may relatively worsen outcome in women undergoing FEA compared to CEA, is their perception of cardiovascular risk and the decision to seek medical care when experiencing complaints[17,18]. Unfortunately we do not have information on the duration and severity of complaints before these patients sought medical care. Still, because PAD is a more chronic occlusive process, we may

speculate that delay in the diagnosis in women with PAD might have played a role. Indeed, asymptomatic PAD or PAD with atypical symptoms has been shown to be more common in women than in men[19,20]. On the other hand, a stroke or TIA which mostly have a thromboembolic origin may be harder to neglect. Fortunately, the awareness that cardiovascular diseases are a leading cause of death among women has nearly doubled in the last 10 years[21], and campaigns to increase awareness of women's cardiovascular risk are currently ongoing[22,23]. Since the Athero-Express study also comprises plaque specimens of included patients, we could determine that women had more stable plaques (containing less fat, plaque hemorrhage and macrophages) than men, as reported previously (data not shown)[24,25]. This finding was present in both CEA and FEA groups, but interestingly this was more pronounced in CEA patients.

Comparison with contemporary literature

Little is known about the prognosis after CEA and FEA in men and women separately, combined with age stratification. In general, it is known that PAD, and ankle-brachial index, are very strong predictors of mortality, independent of the presence of cerebrovascular or coronary artery disease[26–28], and higher mortality in patients suffering from PAD compared to carotid artery disease has also been described in a previous study[29]. However in the current study, differences between patients and an age- and sex-matched control group were smaller, mainly because of higher mortality risks in our control group. Differences in cardiovascular risk profile, geographical region and ethnicity may also play a role, since it is known that these factors influence survival[30,31]. In our study, the majority (>95%) of patients is estimated to be Caucasian, but ethnicity of patients in the previous study was unknown[29]. Other studies in patients with severe PAD undergoing infra-inguinal bypass surgery also indicated that survival in women was similar[32] or even worse[33] than in men. Also regarding other sex-specific outcomes after lower extremity revascularization, such as patency, limb salvage, and perioperative events, a recent literature review on different types of revascularization procedures (including endovascular procedures) did not support a difference between men and women[15]. Long-term mortality data contributing to this review were again very limited.

Clinical relevance, strengths and limitations

The presented results are very relevant for clinical practice, because as already mentioned, a patients' long-term prognosis is an important measure for both patient and doctor when planning or considering a certain treatment. So far, no studies have reported such data, while it may have an important influence on treatment decisions. Other strengths of this study are the validity of registries and linkage methods. Linkage was almost complete: for our patient cohort this was 95%. For the control group, linkage was previously shown to be of good quality, and correct in around 97% of cases (by using a random sample of the PR)[10]. Hence, even if the non-linked group is different, the differences are probably limited[10] and because the size of this group is small, the effect of selection bias due to non-linkage is expected to be minimal. Furthermore, all-cause

mortality has a low chance of being misclassified. The high number of patients with long-term data availability allowed for sex-stratified, absolute mortality risk calculation up to 10 years of follow-up in two age groups. This study is limited by its generalizability to the overall population undergoing CEA or FEA, and subgroups with different symptoms and/or stages of disease were present in our patient groups. However, we have shown many patient characteristics, making this cohort transparent and allowing for comparison with other cohorts. Yet, this study was limited by missing data on risk factors in the control groups, which reduces the possibility to make more precise comparisons between patients and controls. Also, there are other variables that were not measured which can be important determinants of survival. For instance, the effect of postoperative medication use, levels of cardiovascular biomarkers that may have prognostic significance (such as serum uric acid), but also geographical, and hospital/physician factors, were not available. Finally, data of patients with carotid or iliac/femoral stenosis who were treated conservatively, and not operated, were not available, so comparisons between operative and medical treatment could not be made.

Summary and conclusions

Long-term mortality after CEA is higher in men than in women, and in women mortality risk is similar to the general population, which was not to be expected. After FEA, however, mortality is high for both sexes as compared to the general population, and surprisingly, the benefit of women as seen after CEA is lost. These sex-specific trends are not influenced by age.

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Supplemental material

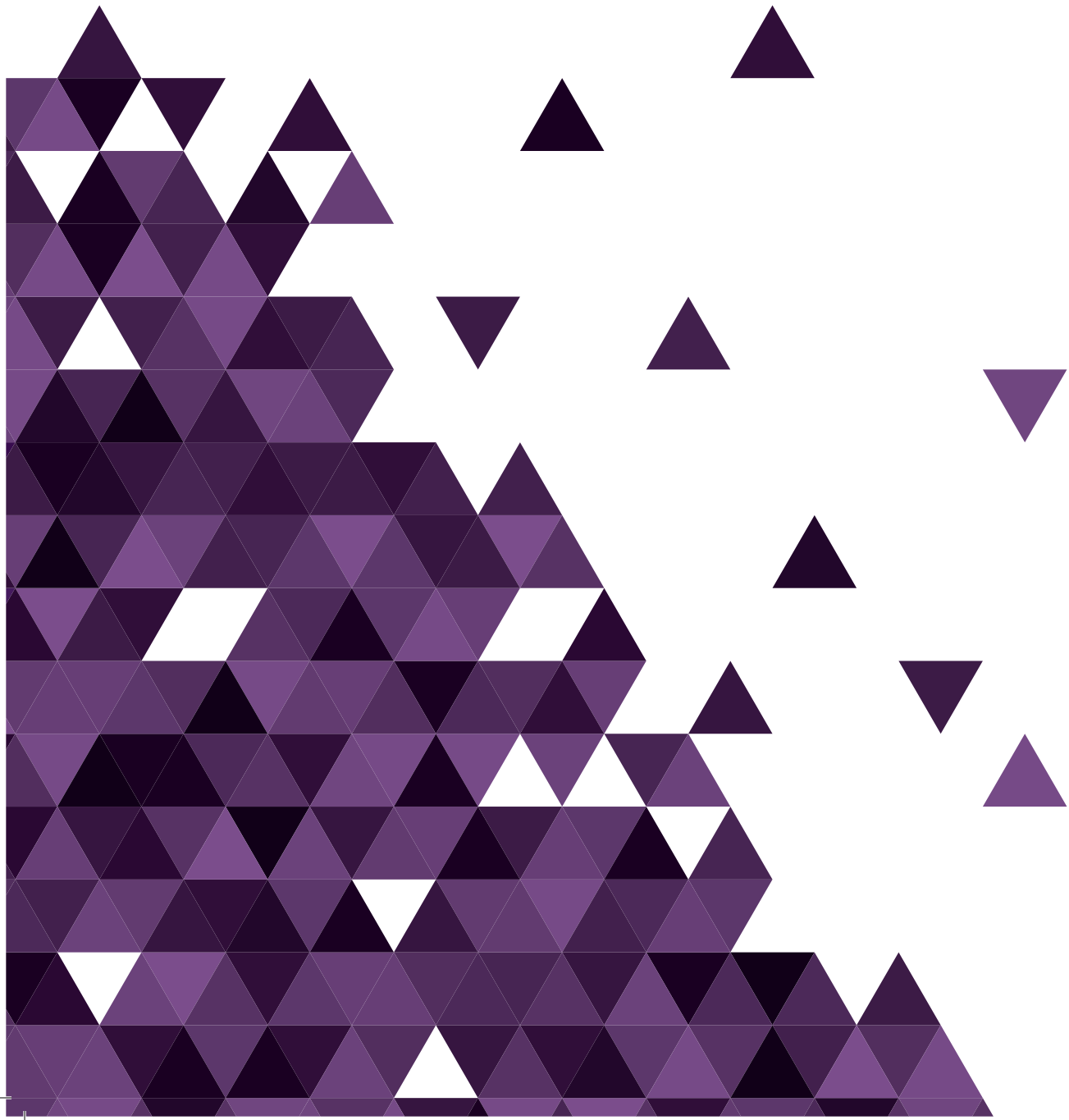
Supplemental methods

Search syntax

Supplemental results

The search on CEA articles yielded 2547 records in Pubmed, and 1529 in Embase. After removing duplicates, 3806 records remained, of which 69 were eligible after review of title and abstract. For FEA, 811 and 1040 records were found in the respective database; 1537 after removing duplicates of which 2 were eligible. A summary of absolute age- and sexspecific mortality data from these eligible articles are shown below (CEA: Table 1; FEA: Table 2).

Supplemental material is omitted because of space limitations



CHAPTER 12

Health-Related Quality of Life is poor but does not vary with cardiovascular disease burden among patients operated for severe atherosclerotic disease

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Abstract

Background

Patients with cardiovascular disease (CVD) are reported to have a poorer Health-Related Quality of Life (HRQoL) compared to healthy age- and gender-matched individuals. Moreover, HRQoL seems to predict survival in CVD populations. We studied HRQoL and the association with outcome during follow-up in a population undergoing surgery for peripheral artery disease or cerebrovascular large artery disease.

Methods

In the Athero-Express biobank cohort study patients filled in a questionnaire containing RAND-36. We stratified the cohort to compare HRQoL scores (range 0-100, higher scores representing better HRQoL) and assessed three-year event-free survival for composite cardiovascular endpoints of patients with good (above median) versus poor (equal to and below median) HRQoL at baseline. Additionally we compared the cohort to a healthy age-matched population.

Results

2,012 and 865 patients undergoing carotid endarterectomy (CEA) or endarterectomy of femoral/iliac arteries (FEA) were included respectively. The median HRQoL was 75 (IQR 0-100 (both patient groups)) for physical role limitations versus 0 (IQR 0-100 (CEA) and 0-66.7 (FEA)) for emotional role limitations. No differences in HRQoL subscores were found, CVD burden did not associate with HRQoL and three-year composite event rates did not associate with the reported HRQoL in both CEA and FEA. Both groups had poor HRQoL scores compared to an age-matched general population, especially regarding emotional role limitations and social functioning.

Conclusions

HRQoL is poor and does not associate with CVD burden within patients suffering from severe atherosclerotic disease. Reported HRQoL was not associated with incident cardiovascular events during follow-up.

Background

Cardiovascular diseases (CVD) are the world's leading cause of death[1]. The majority of CVD are caused by atherosclerosis, a systemic inflammatory disease that manifests in different organs including brain, heart, kidneys and legs. Disease burden of CVD is high as symptomatic atherosclerosis in these organs can be physically as well as mentally disabling. Physical functioning is for example affected by paralysis due to cerebrovascular ischemia, while depression is known to co-occur with cardiovascular diseases[2].

Scoring systems of health-related quality of life (HRQoL), such as RAND-36 (a questionnaire equivalent to SF-36) are developed to measure the subjectively perceived influence of disease on physical, social and emotional functioning[3,4]. Investigating HRQoL in various patient groups is clinically relevant as patients with poor HRQoL might benefit from referral to both occupational therapy for simple practical interventions and psychological support to learn to cope with their emotional and physical problems.

Patients with symptomatic atherosclerosis, such as peripheral arterial occlusive disease[5], carotid artery occlusive disease[6], coronary artery disease[7], abdominal aortic aneurysms[8] and stroke[9], as well as the known risk factors for atherosclerosis, e.g. hypertension[10], diabetes[11] and obesity[12], are reported to have a poorer HRQoL as measured by SF/RAND-36 when compared to healthy age and gender matched individuals even when they are free of symptoms[13]. Patients with risk factors but without overt CVD report declined physical HRQoL scores with almost no effect on mental subdomains of HRQoL. Individuals with diagnosed CVD report even lower scores on the physical subdomains with notably declined mental HRQoL scores as well, although social functioning remains largely unaffected. Moreover, compromised quality of life seems to be an independent predictor for survival in CVD populations[14–16].

The purpose of this study is to investigate whether HRQoL differs between people with different cardiovascular disease burden in a cohort of patients suffering from severe atherosclerotic disease undergoing surgery for peripheral artery disease or cerebrovascular large artery disease. Furthermore cardiovascular event rates during follow-up are compared between groups with differentially reported HRQoL. Additionally we compared the cohort to a healthy age matched population.

We hypothesize that among patients with severe atherosclerotic disease, HRQoL varies depending on cardiovascular disease burden. A negative effect of poor HRQoL on cardiovascular event rates during follow-up is expected. We expect a HRQoL in the patients that is significantly worse than the general population.

Methods

Population

Since 2002 the Athero-Express biobank study is an ongoing cohort study that includes carotid and femoral endarterectomy patients from two large tertiary vascular referral hospitals in the Netherlands without any exclusion criteria. Upon surgery, atherosclerotic plaques are obtained for histological assessment. To date, over 3,000 carotid and femoral endarterectomy patients have been included. In the three years of follow-up time, in which patients are asked to fill in a follow-up questionnaire after 1, 2, and 3 years, approximately 30% of patients reach an endpoint of cardiovascular origin. Study outline and protocol have been described in detail before[17]. Upon inclusion in the Athero-Express biobank study patients are asked to fill in a detailed questionnaire that contains the 36 questions from RAND-36. All patients that were included up to September 2013 and completed the questionnaire were included in the present analysis.

Measurement of HRQoL

HRQoL was measured according to the validated Dutch version of RAND-36. Scores on all nine subdomains (physical functioning (PF), social functioning (SF), role limitations due to physical functioning (RP), role limitations due to emotional functioning (RE), mental health (MH), vitality (VT), bodily pain (BP), general health perception (GH) and health change (HC)) were computed to a scale from 0-100 with higher scores representing better HRQoL. HRQoL measures in healthy controls were obtained from the Dutch RAND-36 manual[3]. The manual describes a validation study in a random sample of 1063 citizens of the Dutch municipality of Emmen in 1992. Among others they were stratified by age.

Measurement of cardiovascular disease burden

Cardiovascular disease burden was determined in several ways. Firstly, symptomatology of the operated artery, e.g. indication for surgery, was determined. Symptomatology of the treated femoral/iliac endarterectomy patients was assessed using Fontaine classification. For carotid endarterectomy patients were stratified according to inclusion diagnosis (stroke, transient ischemic attack patients, patients with ocular symptoms and asymptomatic patients). Secondly, presence of different risk factors (age, male sex, obesity, smoking, hypertension, diabetes, hypercholesterolemia) was assessed. Thirdly, history of a second atherosclerotic organ concomitant to the operated artery (defined by having a history of myocardial infarction, stroke, narrowing carotid artery or peripheral arterial disease) was evaluated. All data were collected from patient files. Presence of risk factors and history of atherosclerotic disease was binned into binary scores (present/absent) as much as possible.

Definition of endpoints

A cardiovascular composite endpoint was defined as having at least one of the following events during follow-up: (fatal) myocardial infarction, (fatal) cerebral infarction or

bleeding, coronary angioplasty or coronary artery bypass grafting, peripheral arterial intervention, fatal heart failure, fatal aneurysm rupture, other cardiovascular death, leg amputation or sudden death. Endpoints were collected from validated follow-up of the Athero-Express biobank cohort. For cardiovascular endpoint analyses, HRQoL scores were binned into 'poor' (equal to and below median) and 'good' (above median).

Ethics

All patients provided written informed consent. The study protocol of the Athero-Express biobank study has been approved by the local medical ethics committees.

Statistical analysis

Descriptive statistics were used to analyze the demographics of the population. To explore response bias as a potential confounder, univariate analysis for associations between baseline characteristics and response rates (chi-square tests for categorical variables and t-tests for continuous variables). P-values <0.05 were considered statistically significant. Because of non-normal distribution of HRQoL scores, nonparametric tests were used to compare HRQoL scores between groups with absence and presence of risk factors, history of previous (symptomatic) atherosclerotic disease and symptomatology. Dichotomous variables were compared using the Mann-Whitney U test, categorical variables were compared using the Kruskall-Wallis test. After correcting for multiple testing (18 tests in each of the 9 HRQoL subdomains) using Bonferroni correction, p-values <0.003 were considered statistically significant. Potential confounders in the relation between symptomatology and any of the nine HRQoL subdomains were identified by ANOVA for continuous variables and linear-by-linear association chi-square tests for categorical variables. Variables with p<0.20 were added to a multivariate regression model. After correcting for multiple testing (nine subdomains in two separate populations) using Bonferroni correction, p-values <0.003 were considered statistically significant. Reference sets for the general population were obtained from the RAND-36 manual. To compare survival of good and poor HRQoL, we plotted Kaplan Meier curves for the composite cardiovascular endpoint stratified to poor and good HRQoL. Differences between the strata were tested with log rank tests. SPSS version 20 was used for all analyses. The reporting of this study conforms to the STROBE statement[18].

Results

The current study included 2012 carotid endarterectomy (CEA) patients and 865 patients with endarterectomy of iliac or femoral arteries (FEA). Of CEA and FEA patients 68% and 72%, was male respectively. Age was 69 (range 35-93) (CEA) and 68 (range 30-100) (FEA). Baseline characteristics are shown in table 1. Loss to follow-up rates were 10.3% in FEA and 9.3% in CEA with median follow-up time of 2.9 years and 3.0 years respectively. In total, 834 patients reached an endpoint of cardiovascular origin, 469 (23%) after CEA and 379 (44%) after FEA.

Table 1. Baseline characteristics

Operating site	**Carotid artery n = 2012**		**Femoral/Iliac artery n = 865**
Age (mean) (SD)	68.96 (9.29)		67.64 (9.14)
Males (n) (%)	1376/2012 (68.4%)		620/865 (71.7%)
BMI (mean) (SD)	26.40 (3.93)		26.28 (8.19)
GFR (mean) (SD)	72.17 (20.70)		79.45 (48.51)
Smoking (n) (%)	672/1953 (34.4%)		340/844 (40.3%)
Hypertension (n) (%)	1438/1948 (73.8%)		623/839 (74.3%)
Diabetes (n) (%)	454/2003 (22.7%)		257/862 (29.8%)
Hypercholesterolemia (n) (%)	1240/1832 (67.7%)		526/765 (68.8%)
History of MI (n) (%)	408/1987 (20.5%)		238/853 (27.9%)
History of stroke/TIA (n) (%)	1272/1889 (67.3%)		113/835 (13.5%)
Restenosis (n) (%)	85/1972 (4.3%)		139/770 (18.1%)
Contralateral stenosis >50% (n) (%)	809/1786 (45.3%)		209/340 (61.5%)
History of intermittent claudication (n) (%)	368/622 (59.2%)	History of narrowing carotid artery (n) (%)	148/844 (17.5%)
Presenting symptom CEA (n) (%)		Ankle-Brachial index operated leg (mean, SD)	0.59 (0.21)
* asymptomatic	272/1998 (13.6%)	Fontaine classification for femorals (n) (%)	
* ocular	310/1998 (15.5%)	* Fontaine IIb	401/736 (54.5%)
* TIA	876/1998 (43.8%)	* Fontaine III	184/736 (25.0%)
* stroke	540/1998 (27.0%)	* Fontaine IV	151/736 (20.5%)

Abbreviations: SD = standard deviation, BMI = body mass index, GFR = glomerular filtration rate, MI = myocardial infarction, TIA = transient ischemic attack, CEA = carotid endarterectomy

Response rates

The response rates for the RAND-36 were 68% (n=1374) for CEA and 63% (n=548) for FEA. There were no significant differences between responders and non-responders on baseline characteristics in both groups (data not shown). Event-free survival between responders and non-responders did not differ within CEA or FEA patient groups (data not shown).

Health-Related Quality of Life scores

HRQoL scores did not differ between CEA and FEA patients (figure 1). In both disease groups, no difference in any of the HRQoL subdomains was observed between patients with different cardiovascular disease burden. Firstly, HRQoL did not differ on the basis of symptomatology (figure 2a and b). Data are shown for PF and RE as PF was unexpectedly high even in Fontaine IV and stroke patients whereas RE was unexpectedly low across all groups. Secondly, no difference was found between patients with and without common risk factors such as male sex, age, hypertension, diabetes, smoking, hypercholesterolemia

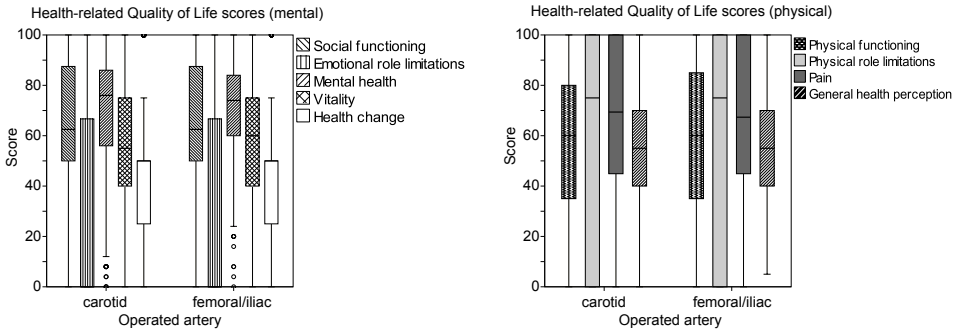

Figure 1. Health-Related Quality of Life scores stratified by operating site.
Carotid: carotid endarterectomy patients, Femoral/iliac: femoral/iliac endarterectomy patients

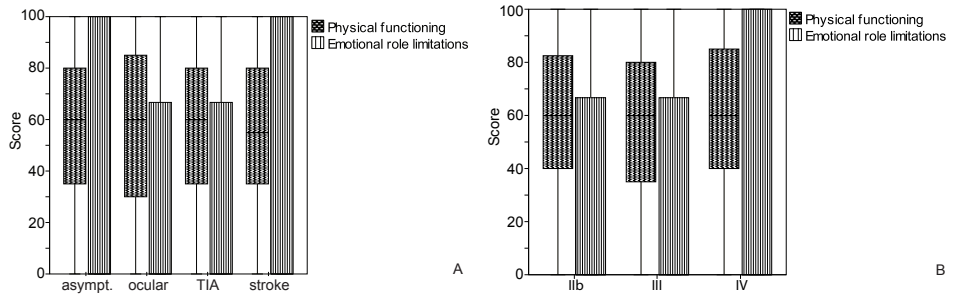

Figure 2. Health-Related Quality of Life scores stratified by symptomatology
A: inclusion diagnosis for carotid endarterectomy patients, **B:** Fontaine classiciation for femoral/iliac endarterectomy patients.
Abbreviations: asympt = asymptomatic, TIA = transient ischemic attack

or obesity (data not shown). Secondly, no difference was found between patient groups with and without a history of cardiovascular disease (supplemental table 2). Correction for potential confounding factors did not alter the results. Event rates were not influenced by below median scores on any of the subdomains of HRQoL in both CEA and FEA patient groups (supplemental table 3). Data are shown in figure 3 for SF and RE because these subdomains differed most from the reference population (see 3.3).

Comparison with general population

Athero-Express patients (both CEA and FEA) report a poor HRQoL compared to healthy Dutch age-matched individuals (table 2). The decline ranges from 5 points (MH, BP, GH, HC) to 50 points (RE) on a scale from 0-100. In the general population, HRQoL declines with age, especially in the physical subdomains. On all subdomains, both CEA and FEA patients in the Athero-Express cohort report a lower HRQoL than 85-year old control subjects. The subdomains that are particularly affected are SF and RE.

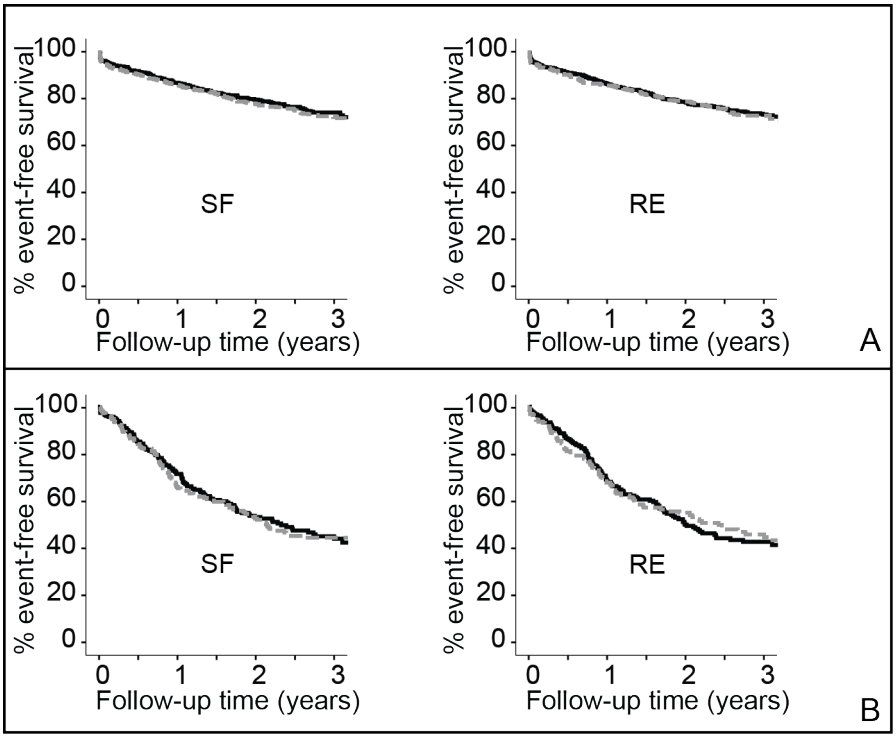

Figure 3. Three year event-free survival for good and poor HRQoL
A: carotid artery endarterectomy patients. **B:** femoral/iliac artery endarterectomy patients; Continuous black line: HRQoL above median, dotted grey line: HRQoL equal to and below median; Abbreviations: SF = social functioning, RE = role limitations due to emotional functioning

Discussion

According to RAND-36 health-related quality of life is poor, but no differences were observed in between subgroups of a severely atherosclerotic population with carotid or femoral stenosis undergoing surgery. Furthermore, HRQoL was not associated with event-free survival during three years of follow-up in both patient groups.

One possible explanation is that all Athero-Express patients have a severe symptomatic type of atherosclerosis, leading to an equally poor HRQoL across the burden groups. Furthermore, Athero-Express patients are all operated on and because of stringent protocols for carotid endarterectomy (surgery within two weeks after the cerebral event) some are only recently confronted with this life threatening disease and the need for vascular surgery, which could lead to emotional distress. This could especially account for the poor scores on functioning due to emotional role limitations in both Athero-Express populations, also when compared to the general population. Depression, known to be of high incidence in patients with CVD, seems to fit this finding less as a cause since

Table 2. Comparison of HRQoL with Dutch general population

	Dutch reference population				**AE carotid artery**		**AE femoral/iliac artery**	
	Age 55-64	**Age 65-75**	**Age 75-85**	**Age 85+**	**mean age: 69**		**mean age: 68**	
	mean (SD)	mean (SD)	mean (SD)	mean (SD)	mean (SD)	median (IQR)	mean (SD)	median (IQR)
Physical functioning	72.7 (24.4)	66.7 (26)	56.0 (26.0)	60.0 (31.8)	55.7 (28.8)*	55 (35-80)	57.7 (28.6)	60 (35-85)
Social functioning	86.6 (21.4)	83.2 (23.7)	82.0 (24.9)	75.1 (31.1)	63.1 (28.6)*	62 (50-87.5)	66 (28.4)*	62.5 (50-87.5)
Role limitations physical	76.5 (38.1)	69.1 (42.5)	60.1 (43.1)	76.6 (35.9)	56 (44)	75 (0-100)	54.6 (44.2)*	75 (0-100)
Role limitations emotional	90.1 (24.5)	82.9 (33.8)	73.7 (40.4)	82.4 (39.3)	33.2 (43.1)*	0 (0-100)	32.6 (43)*	0 (0-66.7)
Mental health	77.1 (18.7)	75.9 (17.3)	76.9 (14.3)	78.3 (15.7)	70 (20.3)	72 (56-84)	70.9 (19)	72 (60-84)
Vitality	67.0 (21.3)	64.2 (22.0)	60.1 (21.3)	67.5 (23.2)	54.6 (22.4)*	55 (40-70)	56.5 (20.7)*	60 (40-70)
Pain	74.7 (25.0)	74.8 (28.0)	72.0 (30.3)	77.3 (26.7)	68.5 (28.7)	69.4 (44.9-100)	69.5 (28)	68.4 (44.9-100)
General health perception	64.4 (22.2)	60.1 (23.9)	59.0 (21.2)	61.4 (21.3)	54.6 (20.1)	55 (40-70)	55.8 (19.5)	55 (40-70)
Health change	48.7 (15.4)	46.8 (20.5)	45.1 (18.7)	50.0 (0.0)	40.1 (27.0)	50 (25-50)	41.8 (25.1)	50 (25-50)

Abbreviations: SD = standard deviation, IQR = interquartile range, * = p<0.001 compared to age matched group

scores on mental health were nearly unaffected compared to the general population.
Comparison of outcomes of the HRQoL scores of the Athero-Express biobank study remains suboptimal. Firstly, the RAND-36 HRQoL questionnaire is being replaced by SF-36 in current literature. The SF-36 and RAND-36 are roughly, but not totally comparable (bodily pain and general health are calculated differently). Secondly, researchers tend to display their outcomes in graphs that do not contain the actual scores, making extraction of data a challenging expedition. Thirdly, both RAND-36 and SF-36 scores are often presented as means with standard deviations, which cannot be interpreted correctly since the outcome of both questionnaires is not normally distributed. In this way, information is lost and a proper comparison impossible.
When compared to other (SF-36) CEA patient cohorts, patients of the Athero-Express biobank operated on carotid arteries exhibit poor HRQoL scores on functioning to emotional role limitations, whereas impact on role limitations due to physical functioning is relatively low. Stolker et al.[19] for example report a 20 points lower HRQoL score in the RP subdomain at baseline than the Athero-Express patients. This could be explained by the selection of "high risk" CEA patients in their SAPPHIRE study. However, Cohen et al.[20] (CREST study group) report a 10 points lower HRQoL score on RP and a 40 points higher score on RE. Strikingly, because both cohorts contain more asymptomatic patients than the Athero-Express cohort (70% and 47% respectively), they are thus expected to have a higher HRQoL on the physical subdomains, not lower. Two different European studies exhibit the same pattern. A British study[21] with 13% asymptomatic patients (equal to the Athero-Express cohort) reports RE that is 30 points higher than Athero-Express patients while RP is 20 points lower. The Serbian cohort of Vlajinac et al.[22] with 35% stroke patients reported an excellent PF (71.7) at baseline but the same pattern exists within the role limitation subdomains: lower PF and higher RE than Athero-Express patients (-15 and +15 points respectively). All cohorts, including the Athero-Express cohort, have a mean age around 70.
Mazari et al.[23] recorded SF-36 scores in 178 patients that were about to undergo femopopliteal angioplasty (median age 70) and Van Hattum et al.[24] report HRQoL scores of 1001 patients (mean age 62) before peripheral bypass surgery. Compared to these cohorts, Athero-Express FEA patients differ in the same way as Athero-Express CEA patients do: a poor score on RE with relatively high RP. There is no clear explanation for the differences found in functioning due to role limitations.
A possible limitation of the Athero-Express biobank study regarding HRQoL is the fact that the Athero-Express population consists of only patients that have undergone surgery, leading to selection bias of patients that are physically able to handle the procedure. Even more severely disabled patients are thus excluded, leading to a possible overestimation of the actual HRQoL. However, as inclusion of non-operable patients would further lower the scores, this would probably not affect the already observed 'floor effect'.
Non-response on the RAND-36 is not thought to influence the outcome either, because response rates did not differ with cardiovascular disease burden. Only patients with a second affected peripheral artery had consistently lower response rates in CEA and FEA,

but this difference did not reach significance. This could however implicate that more severely disabled patients may not have filled in the questionnaire. Since survival rates did not differ between responders and non-responders in both CEA and FEA patients, impact on survival is not to be expected.

Another possible confounder is socio-economic status, for it is known that patients with a low socio-economic status experience a significantly lower Health-Related Quality of Life compared to patients with a high socio-economic status[25]. This difference could well be explained by education. Both factors are not measured in the Athero-Express biobank, so could not be corrected for and may have introduced confounding.

Atherosclerotic disease affecting the cerebrovascular and peripheral arteries has a large impact on HRQoL, independent of risk factors or medical history. Considering the systemic nature of the disease and the lack of options to completely abolish atherosclerotic plaque burden in individual patients, treatment should include the improvement of HRQoL. Health care specialists that work with patient groups affected by atherosclerosis therefore need to be aware of the poor quality of life of their patients to be able to offer practical physical and emotional support or detect more severe mental health issues that need attention from psychological experts.

Conclusion

Using RAND-36, HRQoL is poor, especially regarding emotional and social functioning, but cardiovascular disease burden does not influence HRQoL within patients suffering from severe atherosclerotic disease undergoing surgery. In addition, reported quality of life was not associated with incident cardiovascular events during follow-up.

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Supplemental material

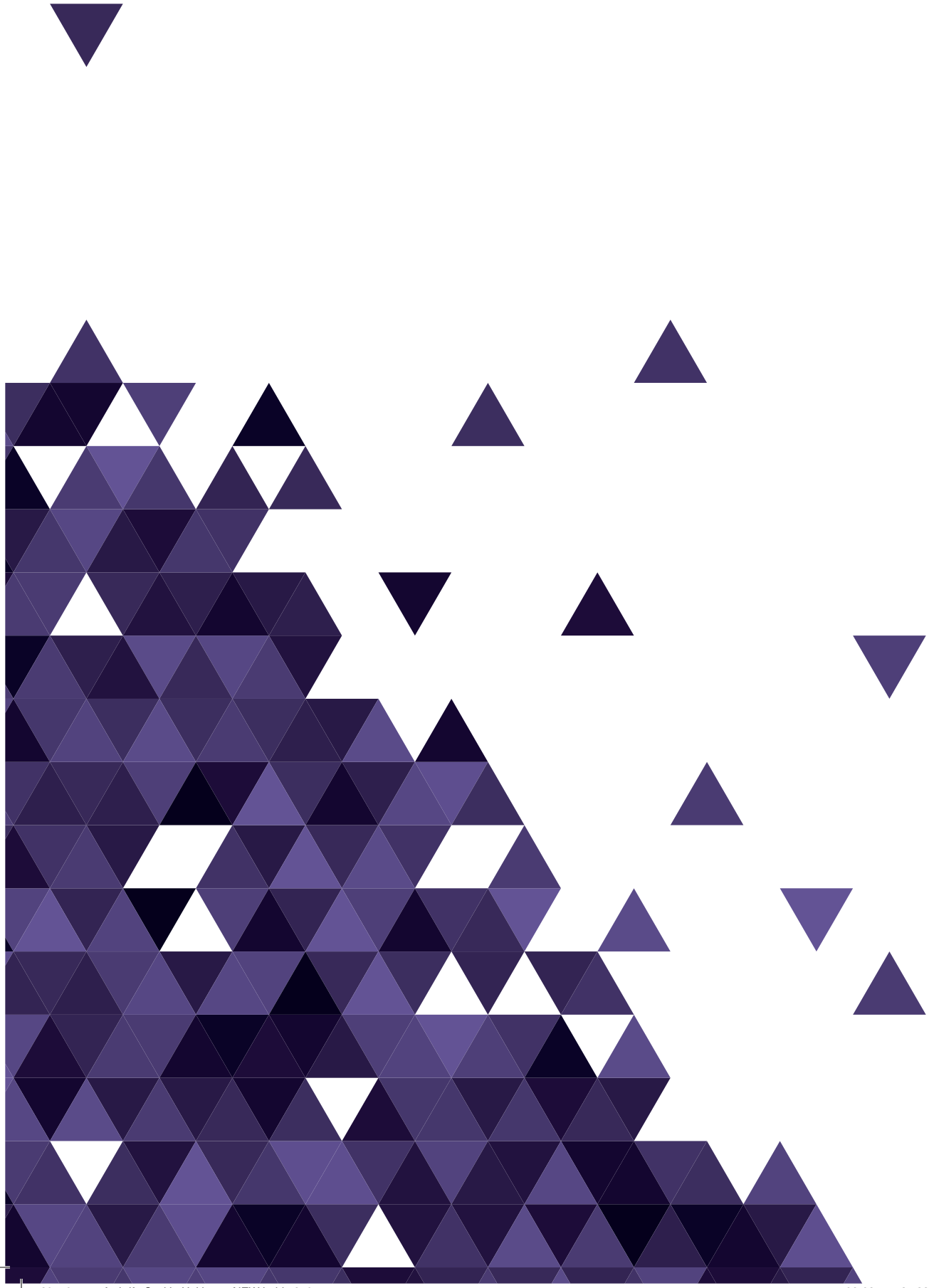
Supplemental figure 1. Histograms of all subdomains of HRQoL for carotid endarterectomy patients (A) and femoral endarterectomy patients (B)

Supplemental figure 2. Three year event-free survival for good and poor HRQoL

Supplemental table 1a. HRQoL for different history of cardiovascular disease for carotid endarterectomy patients

Supplemental table 1b. HRQoL for different history of cardiovascular disease for femoral endarterectomy patients

Supplemental material is omitted because of space limitations



CHAPTER 13

Summary and general discussion

In part I of this thesis models of atherosclerotic disease susceptibility were studied. In **Chapter 2**, all APOE $^{-/-}$ and LDLR $^{-/-}$ mouse models were assessed for translatability to human atherosclerotic disease. For this, the literature for these models was systematically reviewed and genes with a known effect on atherosclerosis in mice were extracted. These were compared to gene-based p-values based on genome-wide association studies in coronary artery disease and stroke in humans. Little overlap was found when looking for the murine genes in the human disease-associated genes. Therefore the conclusion was that the translatability of the most used atherosclerosis model is poor and human evidence should be the starting point of research in atherosclerotic disease. In **Chapter 3**, *Homo sapiens* was used as a model. DNA regulatory elements are thought to exert their effect by bending of the DNA, facilitating physical proximity between the element and the gene it regulates. Within human coronary endothelial cells and human monocytes, using 4C-sequencing such chromatin-chromatin associations were studied from the point of view of DNA regulatory elements that co-localize with GWAS susceptibility loci for coronary artery disease and stroke. This way, these loci could be annotated in an alternative way, enabling the identification of alternative candidate genes for atherosclerotic disease. Several novel genes were identified, some located far away from the locus, and also some genes that are known to be regulated by the genetic variation at the susceptibility locus. These genes should be investigated further for their involvement in atherosclerotic disease.

Within the Athero-Express Biobank, atherosclerotic plaque specimens are stored of around 2300 carotid and 1000 (ilio)femoral arteries. Part II revolves around characteristics of these atherosclerotic plaques. In **Chapter 4**, the association between estradiol, the most abundant female sex-hormone, and characteristics of the carotid atherosclerotic plaque was studied in women below 70 years of age. Interestingly, a positive association between estradiol levels and the amount of intraplaque microvessels was observed, and a trend towards a positive association between estradiol levels and the amount of macrophages. These findings are remarkable, as atherosclerotic plaques obtained from women generally display features that are associated with stable plaque characteristics, such as a large amount of collagen and smooth muscle cells. In young women, the plaque erosion mechanism of atherothrombosis is often observed. Although preliminary, these findings could point towards a more complex pathophysiological mechanism of plaque erosion. In **Chapter 5**, ilio-femoral plaque characteristics of patients with diabetes mellitus were studied. Diabetes mellitus is an important risk factor for peripheral arterial disease and its prevalence is rising. More calcification was observed within plaques of patients with diabetes. Moreover, the clinical outcome of these patients was studied during three years of follow-up. More secondary cardiovascular outcomes were observed in patients with diabetes. Surprisingly, the amount of re-interventions for atherosclerotic disease in the operated vessel ("target vessel revascularizations") did not differ between patients with and without diabetes. Earlier research in carotid endarterectomy specimens revealed a shift in plaque characteristics over the last decade, towards more plaques

with features associated with plaque stability. In **Chapter 6**, it was therefore investigated whether a time-dependent difference in (ilio)femoral endarterectomy specimens could also be detected. Indeed, such an effect was found, independent of patient characteristics that changed in the past decade. Evidence from another vascular bed supports the hypothesis of time-dependent changes in atherosclerotic disease. This finding underlines the need for up-to-date biobanks and a different preventive and therapeutic approach to the atherosclerotic disease of today.

In Part III studies on (epi)genetic variation in the Athero-Express Biobank can be found. For Chapter 7 and 8 only men were studied. In **Chapter 7**, genetic variation on the Y chromosome ("Y chromosomal haplogroups") and its association with characteristics of the atherosclerotic plaque and aneurysmal artery wall was studied. No association was found between any of the haplogroups and characteristics of the diseased vessel wall, nor a different distribution of the haplogroups in our cohorts when compared with a Dutch cohort from the general population. These results make (causal) involvement of the Y chromosome in atherosclerotic disease less likely. In **Chapter 8**, loss of the Y chromosome was studied. Such loss was previously associated with ageing, smoking, and overall-mortality, so a role for cardiovascular disease was hypothesized. No association was found between Y chromosomal loss and atherosclerotic plaque characteristics. However, an association was found between Y chromosomal loss and secondary cardiovascular events during 3-year follow-up. How Y chromosomal loss affects the development of cardiovascular events remains elusive. In **Chapter 9**, differences in DNA methylation between women and men were studied across the genome. Many differences were identified and it was observed that most of them were not associated with atherosclerotic disease risk factors or characteristics of the atherosclerotic plaque. Not many DNA methylation studies investigated diseased tissue. These findings emphasize the need for stratification by sex in epigenetic studies.

Part IV of this thesis contains studies on clinical outcomes in patients. In **Chapter 10**, mortality rates after coronary artery bypass grafting surgery between women and men in the IMAGINE trial were studied. A poorer survival for women in univariable analysis was observed. However, after correction for possible confounders the association was gone. This effect has been observed also in other coronary artery bypass grafting cohorts and warrants further study, preferably in a much larger number of women. In **Chapter 11**, long-term follow-up of both carotid and femoral endarterectomy patients was studied in the Athero-Express Biobank. Patients were matched to a cohort from the general population and their survival was studied. Interestingly, sex-differences were observed in survival after carotid endarterectomy surgery, where women displayed an advantage over men and followed the survival curve of the cohort of age-matched women from the general population. However, such a sex-difference was not observed after femoral endarterectomy surgery. In **Chapter 12**, health-related quality of life was studied in the Athero-Express Biobank. It was hypothesized that severity of disease would impair quality

of life in these patients. Yet, not such effect was observed. Moreover, all patients displayed a poor quality of life compared to the general population. Clinicians may want to address this issue and refer patients to address coping strategies for dealing with atherosclerotic disease.

General discussion

Present-day problems call for present-day approaches

This thesis consists of eleven studies that in some way add a little piece of information to the cloud of existing knowledge on atherosclerosis in the world. In fact, every research that has ever been undertaken somehow added information to the cloud of human knowledge. From the point of view of this cloud, every piece of missing information carries value and helps mankind to better understand the world we live in. However, time and money are limited, and some issues are more pressing than others. This way, some pieces of information may seem more relevant than others. Atherosclerosis is such an unresolved issue. The incidence of atherosclerotic diseases is dropping, but its prevalence continues to put a high burden on healthcare systems. Moreover, the disease is changing. Present-day clinical problems like atherosclerotic disease call for smart translational models that facilitate precision medicine, collaborative efforts that boost scientific progress and open access to the cloud of all existing knowledge in the world for everyone.

Optimization of translatability is key

Life sciences are a challenging research field. Variation, the hallmark of evolution, hampers the extrapolation from biomedical "reality" that is observed in a research setting to "reality" of the seven billion unique (potential) patients in the outside world. Yet, we want to practice precision medicine every day, applying the best evidence-based treatment for each individual. However, the current relative absence of refined evidence prevents this individual treatment.

Sex-stratification aids precision medicine

Sex-stratification in biomedical research is a logical first step towards precision medicine. The differences between women and men, from their sex chromosome constitution to their sex hormone variation, need no clarification. Biomedical researchers should therefore always look for sex-differences in every experiment. This starts with description of the biological sex of the research model and discarding every pooled male-female experiment that may hamper translatability to the patient. Even proof-of-principle studies in mixed-sex models may yield uninterpretable results.

On the short term, this approach may seem more expensive as power may be limited by sample size. However, for example, if a phase III trial in a (mixed-sex) population cannot determine the effectiveness of a drug, a pharmaceutical company may be inclined

to discontinue the drug, whereas it might be beneficial in one sex only and detrimental to the other sex. Sex-stratification will show both effects and may enable the company in question to file for approval for one sex only and thus save money invested in the preclinical research. Moreover, this drug will then benefit patients. The food and drug administration (FDA) indeed has approved drugs for one sex only (e.g. alosetron in women, balsalazide in men).

Research in women raises ethical questions, as they might be pregnant when exposed to experimental drugs and procedures. The fear of repetition of the consequences of exposure of unborn children to drugs such as diethylstilbestrol[1] or thalidomide[2] induced a hesitant approach of research in women. However, pregnant women have the right to precision medicine and should be therefore be included in carefully designed trials[3]. Other groups of women that require special attention because their absence hampers translatability of research results are African American women[4] and post-menopausal women that suffer from polypharmacy. Overall, women are found less likely to participate in clinical trials[5] and thus should be addressed separately.

Sex-differences can also be studied by reanalyzing readily available data. The LOng-Term outcome following coronary arTERy bYpass surgery in women (LOTTERY) study is collecting data on coronary artery bypass grafting (CABG) surgery in collaboration with cohorts from all over the world to be able to properly study long-term outcome in women. Women are severely understudied on this topic because their contribution to the CABG population is limited and stratification within one cohort is thus limited in power.

Geneticists that discard sex-chromosomes from their research (often for practical reasons as the study of X and Y is not as straightforward compared to autosomal chromosomes) thereby eliminate possible interesting sources of sex-differences. Yet, the male-specific Y chromosome contains haplo-insufficient genes that have a gene regulatory role for all other chromosomes. Research within this thesis shows effects on secondary cardiovascular disease prevalence associated with loss of Y chromosomal content. The Y chromosome has been proven an interesting part of the genome that needs scientific attention. Genotyping information on this chromosome needs to be released and new methods should be invented to overcome the problems of analysis of haploid chromosomes. The same applies to the X chromosome, where inactivation leads to (at least partial) haploidy that can affect sex-differences.

Animal models need human supportive evidence

Smart research also means using animal models the right way. Mice can be a valuable model for human atherosclerotic disease, if researchers bear in mind their limitations. For example, when genetically modifying a mouse for atherosclerosis research, human evidence for the involvement of the to be investigated gene(s) should always be used as a starting point. Mice do not exhibit atherosclerotic disease by themselves. Consequently, inducing it is the first step away from translatability to the human situation. Translational biomedical scientists that study their favorite pathway that consists of murine genes with

no orthologue in man study a disease that simply does not exist in nature, and should thus be halted. It is immoral to use living creatures for research that serves no human need. In the interest of both the animals and the translation of results, animal ethical committees should make sure human evidence is the starting point in every biomedical experiment aimed at targeting human disease.

Translatability starts at the bench

Basic *in vitro* or *in silico* research sometimes seems far away from the patient. However, measures to improve refinement of research can be undertaken that ultimately will benefit the patient. Genetic background can be taken into account in precision medicine, yet is not commonly incorporated in daily practice. Indeed, genetic burden for disease, ranging from monogenic rare diseases to polygenic common diseases can be directly determined and used in patient care. Research has proven life-style changes to limit genetic risk to be effective and vice versa[6].

Unravelling the biological mechanism of genetic susceptibility should be approached with caution. For example, DNA susceptibility loci that are found within DNA regulatory elements are thought to act via regulation of gene expression, either in time, place or absolute levels. Regulation of gene expression, by means of physical interaction, chromatin looping and recruitment of transcription factors, has been proven much more complicated than previously thought. This complexity entails effects that rely on cell-type, cell-differentiation, or environmental stimuli. These complex mechanisms are difficult to study and research settings may not mimic the patient situation. It remains to be determined if, how and at what scale epigenetic mechanisms can eventually influence clinical practice. Taking multiple snap-shots of epigenetic or metabolic signatures of tissues throughout the human lifespan may soon be reality and offers the opportunity to use these signatures as a biomarker. However, the interpretation of biological mechanisms within these data remains a challenge. It is therefore important to systematically unravel chromatin organization and epigenetic mechanisms in a sex- and cell-type-specific manner. This way, translatability to the patient is enhanced. As an example, the TWIn Endothelial Cell Epigenetics (TWIECE) study investigates endothelial cells from twin newborns, aiming to clarify whether sex-differences already exist in the epigenetic profiles of endothelial cells of boys and girls. The use of a pure primary cell population of newborns that shared a similar environmental exposure enables the researchers to study of the most 'clean' sex-differences available.

A moving target needs a flexible archer

Variation and adaptation give rise to change, which continuously challenges biomedical paradigms that are posed as 'truth'. For example, within the Athero-Express Biobank Study, it was recently discovered that the atherosclerotic plaque is changing[7,8]. The plaque characteristics that are associated with the classic plaque rupture are less frequently observed, while the process of plaque erosion is now more common. Moreover, an association was seen with a shift from ST elevation myocardial infarction

to non-ST elevation myocardial infarction and from fatal stroke to less invalidating cerebral events[9]. This shift may be the effect of current (preventive) treatment and for example limitation of exposure to passive smoking. In another example, the optimal BMI values have shifted[10]. To be able to facilitate precision medicine, research needs to shift too, to facilitate translatability to the current disease. Mechanisms of plaque erosion need to be studied and *in vivo* and *in vitro* models should be pre-treated with what is currently considered optimal preventive treatment. Scientific efforts in general should be flexible to new developments and paradigm shifts. Moreover, there is a continuous need for up to date, preferably longitudinal, patient material and patient data to be able to provide knowledge that underlies evidence-based care for the current patient.

Shaking up the biomedical community boosts scientific progress for patients

When looked at from some distance, the scientific community (in academia) sometimes seems hopelessly inefficient. At numerous places around the world, scientists perform exactly the same experiments, hoping to publish their paper first in a high impact journal. Replication articles are considered less important and negative studies are not reported on, creating so-called publication bias. Biomedical breakthroughs are no longer pursued for the benefit of the patient, but seem to be necessary life goals to facilitate a successful career for the researcher. These wrong incentives can lead research groups to areas of research that score well in terms of impact in scientific journals, but do not benefit the patient within a reasonable timeframe. Initiatives such as Science in Transition aim to address these problems[11]. However, without financial stimuli and explicit policies, change will be slow and the frontrunners will be at a demotivating disadvantage.

Collaborative efforts bring together scientists that agree upon a certain research strategy. Data are shared to facilitate replication or meta-analysis and, if there is a sponsor, the party providing financial support can demand publication of negative studies and push for proposals with sufficient societal impact and translational possibilities. The way the genetic community has embraced collaboration, including data sharing and replication, serves as an example for the rest of the scientific community[12,13]. These initiatives are not meant to wipe out fundamental biomedical research areas that are (by definition) or seem (by current standards) untranslatable to patients. The current challenge is to facilitate fundamental biomedical research without forcing fundamental biomedical scientists into making false translational claims. Unfortunately, emphasizing translational research in grant applications may encourage the latter.

Sharing is caring

Even more appalling is the fact that outcomes of research paid by public money disappear behind pay walls of large publishers that make enormous profits. The purpose of writing papers is spreading the knowledge one gained, not hiding it. Scientists should claim their right to spread the knowledge of the people among the people. All knowledge should

be available to everyone so it can benefit mankind as a whole (the same goes by the way for intellectual property, but disassembling the private domain is more challenging to accomplish). For example, all available knowledge in the open access cloud could be data-mined by algorithms and outsmart every single doctor when it comes to precision medicine.

Last but not least, evidence-based medicine should be accessible for everyone. Either by facilitating health care insurance or by means of free healthcare for all citizens of a country[14]. The little piece of evidence that I added to the cloud of scientific knowledge is anyhow meant for everyone.

Saskia Haitjema, November 2016

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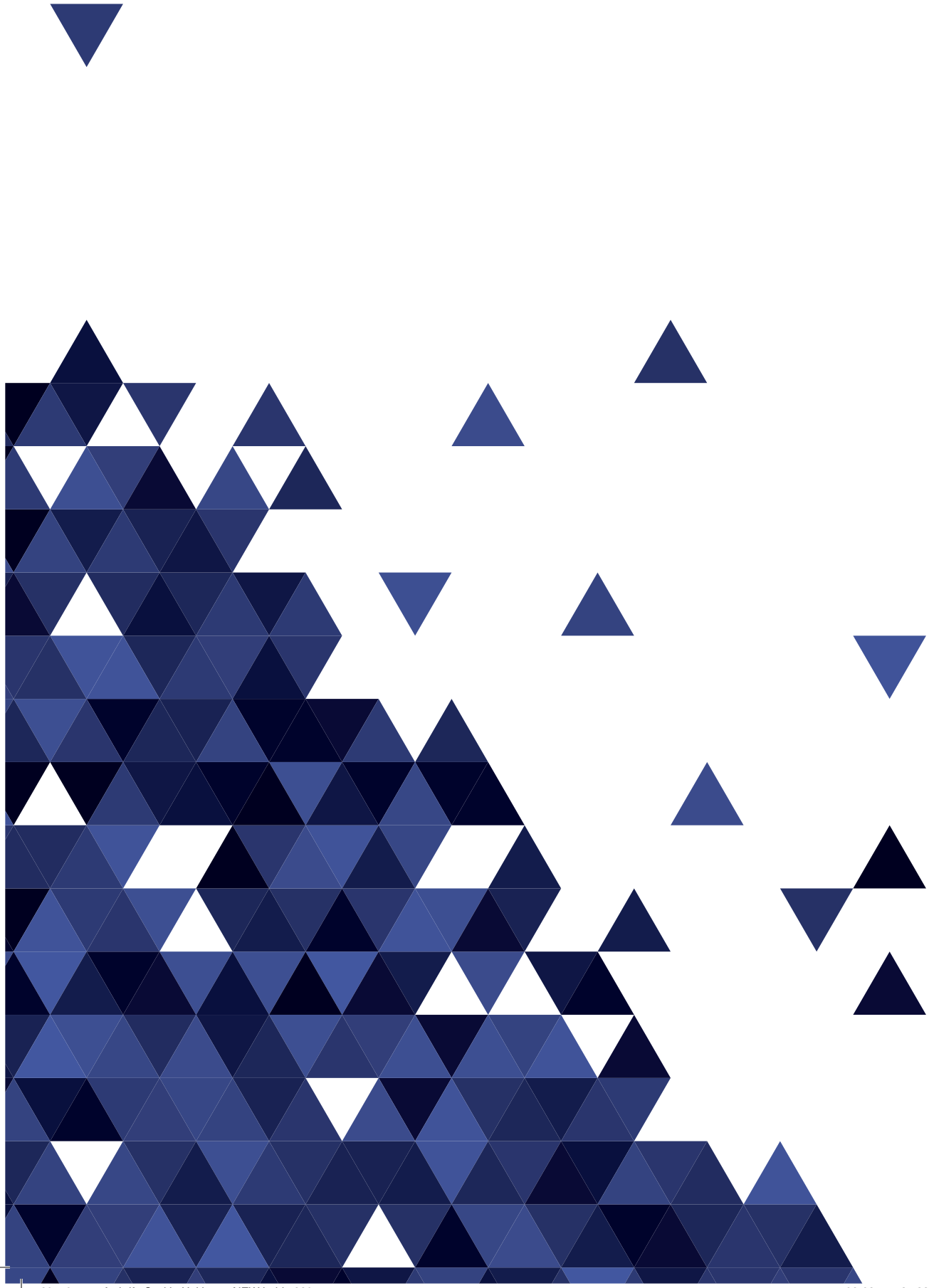


CHAPTER 14

Nederlandse samenvatting

Slagaderverkalking

Slagaderverkalking of "atherosclerose" is een ontstekingsziekte van de bloedvatwand van slagaders die gedreven wordt door vet. De ziekte begint als een verdikking van de binnenste laag van het bloedvat (endotheel) die voornamelijk plaatsvindt op punten waar de slagaders vertakken. Kleine beschadigingen van de bloedvatwand lokken witte bloedcellen naar binnen om het vet dat zich onder het endotheel heeft opgehoopt op te ruimen. Deze ophoping van ontstekingscellen en vet, die bij elkaar gehouden wordt door een dekseltje van gladde spiercellen wordt een atherosclerotische plaque genoemd.

De ontwikkeling van een atherosclerotische plaque, die uiteindelijk de diameter van het bloedvat verkleint, duurt vele jaren. Over de tijd groeit de ophoping van dode cellen en groeien er kleine bloedvaatjes de plaque in om het ontstekingsproces van zuurstof te voorzien. Zulke bloedvaatjes kunnen knappen, waardoor er een bloeding ontstaat in de plaque. Tegelijkertijd verzwakt zo'n bloeding, samen met enzymen die gepaard gaan met ontsteking, de stabiliteit van het plaqueweefsel.

Uiteindelijk kan dit op twee manieren leiden tot een verstopping van het bloedvat (atherothrombose). Bij de eerste manier ontstaat er een scheur in de deksel van de plaque, waardoor de kern met dode cellen wordt blootgesteld aan de bloedstroom. Dit zorgt onmiddellijk voor stolling in het bloedvat, waardoor een bloedprop (trombus) ontstaat. Bij de tweede manier ontstaat er een soort schaafwondje op de endotheellaag van de deksel van de plaque. Hierdoor komt de onderliggende stabiliserende laag bloot te liggen. Ook het bloot liggen van deze laag zorgt voor stolling en het ontstaan van een trombus, maar dit gaat waarschijnlijk minder snel dan bij de scheur. Bovendien ontstaan er bij het schaafwondje ook veel kleine bloedpropjes die stroomafwaarts van de plaque kunnen zorgen voor verstopping van kleinere bloedvaatjes.

Hart- en vaatziekten

Hart- en vaatziekten (HVZ) die gepaard gaan met atherosclerose en atherothrombose kunnen op twee manieren voor symptomen zorgen. Ten eerste kan het verkleinen van de diameter van een slagader zorgen voor te weinig toevoer van zuurstofrijk bloed naar het achterliggende orgaan, in het bijzonder wanneer zo'n orgaan het bloed het meeste nodig heeft. Zo wordt pijn op de borst (angina pectoris) veroorzaakt door atherosclerose van de kransslagaderen. Die slagaders, die het hart van bloed voorzien, kunnen dan te weinig zuurstofrijk bloed naar het hart vervoeren op momenten dat het hart dit juist het meeste nodig heeft, bijvoorbeeld bij inspanning. Etalagebenen (claudicatio intermittens) worden veroorzaakt door atherosclerose in de beenslagaders, waardoor de beenspieren niet genoeg bloed krijgen tijdens het lopen en patiënten vaak even blijven staan (alsof ze in de etalage kijken) om de pijn weer af te laten zakken. De tweede mogelijkheid waarop atherosclerose kan leiden tot symptomen is door atherothrombose. De bloedprop leidt hierbij tot plotseling zuurstoftekort (ischemie) van het achterliggende orgaan, waardoor het begint af te sterven. Op deze manier kan een bloedprop vanuit de

halsslagader tot zuurstoftekort van een gebied in de hersenen leiden (beroerte) en een bloedprop in de kransslagader tot zuurstoftekort in het hart (myocardinfarct). De plaats waar de bloedprop uiteindelijk "vastloopt" in de slagader en tot een blokkade van de bloedstroom leidt, bepaalt hoe groot het stuk van het achterliggende orgaan is dat afsterft. Zo kan een bloedprop in de hoofdstam van de kransslagaders zelfs zorgen voor plotselinge dood.

De behandeling van hart- en vaatziekten bestaat momenteel uit grofweg twee dingen. 1) het voorkomen van risicofactoren (vetwaardes in het bloed verlagen, bloedstolling bestrijden, bloeddruk verlagen, suikerwaardes verlagen en stoppen met roken om meer endotheelschade te voorkomen) en 2) het openen van geblokkeerde bloedvaten (door via een katheter in het bloedvat met een ballonnetje het bloedvat weer open te duwen (dotteren), met een operatie de atherosclerose te verwijderen of een omleiding aan te leggen om de vernauwing heen). Ondanks deze aanpak concurreren hart- en vaatziekten elk jaar weer met kanker om de nummer-1 positie van de ziekte waar de meeste mensen aan doodgaan. Daar bovenop komt nog eens dat hart- en vaatziekten zorgen voor veel mentale en fysieke beperkingen, wat leidt tot verloren arbeidsjaren en een verlies van kwaliteit van leven.

Genetische variatie

We hebben de afgelopen decennia veel geleerd over hart- en vaatziekten door de kenmerken van mensen met en zonder de ziekten te vergelijken. Zo hebben we ontdekt dat roken en suikerziekte (diabetes) een risicofactor zijn voor het krijgen van hart- en vaatziekten. Verder hebben we ook ontdekt dat er families zijn waarin hart- en vaatziekten heel veel voorkomen. Een speciaal geval is een ziekte waarbij er enorm veel vet in de bloedbaan zit, familiaire hypercholesterolemie. Deze ziekte wordt veroorzaakt door een mutatie in één gen (monogenetische ziekte) en zorgt voor atherosclerose op heel jonge leeftijd. Een nieuwere methode om naar genetische invloed te kijken is door het bestuderen van genetische variatie op het hele DNA tegelijk en deze genetische variatie te vergelijken tussen zieke en gezonde mensen.

Het DNA van mensen bestaat uit drie miljard paren van bouwblokjes, waarbij bouwblokje A altijd een verbinding heeft bouwblokje T en bouwblokje C altijd een verbinding heeft met bouwblokje G. De volgorde van de bouwblokjes vormt een code voor genen, of eiwitrecepten. Deze code wordt door onderdelen van de cel afgelezen tot RNA en vervolgens vertaald in eiwit. Intussen weten we dat het merendeel van de DNA-code helemaal niet wordt omgezet in eiwit. Sommige gedeeltes van DNA zorgen ervoor dat genen vaker worden afgelezen, andere gedeeltes worden alleen maar afgelezen en niet omgezet in eiwit.

In de drie miljard paren van bouwblokjes vinden we veel variatie. Op sommige plekken heeft de helft van de bevolking bijvoorbeeld een AT-paartje, waar de andere helft van de

bevolking een CG-paartje heeft. We denken nu dat ziektes zoals atherosclerose waarbij heel veel genen betrokken zijn (polygene ziektes) beïnvloed worden door zulke genetische variatie, maar dan op een veel minder heftige manier dan de manier waarop een mutatie zorgt voor familiaire hypercholesterolemie. Door genetische variatie van bouwblokjes (single nucleotide variants) te vergelijken tussen mensen met en zonder ziekte in zogenoemde "genoombrede associatiestudies" (GWAS), kunnen we de ingewikkelde genetische vatbaarheid voor de ziekte onderzoeken. Een voordeel van deze manier van onderzoek doen is dat er van te voren geen selectie wordt gemaakt naar welke genen gekeken gaat worden, we kijken over het hele DNA verspreid naar genetische variatie. Omdat het effect van een variant zo klein is, zijn er hele grote aantallen van zieke en gezonde mensen nodig om zo'n effect te vinden. Grote initiatieven zijn gestart om tienduizenden tot honderdduizenden mensen te verzamelen van wie genetische informatie beschikbaar is om hart- en vaatziekten te onderzoeken. Intussen zijn er tientallen varianten bekend die de vatbaarheid voor hart- en vaatziekten verhogen. Het probleem is nu dat die varianten overal op het DNA kunnen voorkomen, dus ook op plaatsen waar geen gen ligt en waarvan de functie onduidelijk is. Het is dus vaak niet meteen duidelijk hoe de variant uiteindelijk zorgt voor een verhoogde vatbaarheid voor de ziekte.

Epigenetische oorzaken of gevolgen

Het menselijk lichaam bestaat uit heel veel verschillende soorten cellen. Die zien er allemaal anders uit, doordat het aflezen van het DNA tussen de cellen verschilt waardoor in elke cel andere eiwitten voorkomen. Deze "regulatie van de genexpressie" maakt het ook mogelijk voor een cel om te reageren op prikkels vanuit de omgeving.

Regulatie van de genexpressie wordt mogelijk gemaakt doordat het DNA flexibel is. Als het opgerold ligt om eiwitten, kan het niet afgelezen worden. Daarnaast kan het voor het aflezen van een gen belangrijk zijn dat een ander, verderop gelegen, gedeelte van het DNA in de buurt komt te liggen van het gen. Het DNA kan zo buigen dat dit gebeurt. Naast het buigen van DNA kan genexpressie ook beïnvloed worden doordat er chemische groepen worden vastgeplakt aan het DNA. De meest bestudeerde chemische verandering van DNA is DNA methylatie.

Al deze mechanismen die het aflezen van genen beïnvloeden (epigenetica), zorgen niet voor verandering van de bouwblokken van het DNA. Daarnaast kunnen ze overgedragen worden als de cel deelt. Het lastige van het bestuderen van epigenetica is dat je nooit weet of je kijkt naar de oorzaak of het gevolg van een ziekte, omdat omgevingsfactoren kunnen zorgen voor de veranderingen. Hoge vetwaardes of suikerziekte zijn bijvoorbeeld gelinkt aan DNA methylatie veranderingen in DNA methylatie. Maar komen die hoge waardes dan door bepaalde DNA methylatie of zorgen ze er juist voor dat de DNA methylatie verandert? Van roken is ook een effect bekend op DNA methylatie. Omdat het onwaarschijnlijk is dat DNA methylatie ervoor zorgt dat mensen gaan roken,

verwachten onderzoekers dat het roken hierbij de óórzaak is van de verandering in DNA methylatie. DNA methylatie kan gemeten worden met een chip, net als genetische variatie. Maar de architectuur en organisatie van de vouwingen van het DNA zijn moeilijker om te onderzoeken.

Sekse- en geslachtsverschillen

In het Nederlands wordt het woord "geslacht" gebruikt voor zowel het biologische onderscheid tussen mannen en vrouwen als voor het sociale concept in de samenleving. In het Engels zijn daar twee woorden voor, namelijk "sex" en "gender". In het Nederlands wordt officieel voor het biologische verschil tussen mannen en vrouwen het woord "sekse" gebruikt, maar bijna niemand houdt zich hieraan. In het Engels worden sex en gender overigens ook vaak door elkaar gebruikt. Vooral omdat mensen het op de een of andere manier liever over gender hebben dan over sex. Het moge duidelijk zijn dat in dit proefschrift de sekseverschillen (of "sex-differences") in hart- en vaatziekten zijn onderzocht.

De uitgangssituatie van een mens is vrouwelijk. Er is een bepaalde regio op het Y chromosoom nodig om van een vrouw een man te maken. Uiteindelijk zorgt dit verschil voor een verschil in hormoonwaardes en een verschillend uiterlijk voor mannen en vrouwen. Doordat de hormoonwaardes in vrouwen een maandelijkse schommeling vertonen (om zwanger te kunnen worden) maar ook omdat deze hormonen de stofwisseling van de cel en daarmee het functioneren van het vrouwelijke lichaam beïnvloeden, zijn vrouwen moeilijkere proefpersonen om ziekte en gezondheid in te bestuderen. Daarbij komt ook nog eens dat vrouwen zwanger kunnen zijn en hun ongeboren kind daarmee blootgesteld kan worden aan het experiment. Dit alles zorgt ervoor dat er minder vrouwen meedoen aan klinische studies. Daarnaast zijn er ook nog vrouwen voor wie deze hormoonschommelingen niet meer van toepassing zijn, namelijk vrouwen na de overgang. Deze vrouwen moet gezien worden als weer een hele andere groep. Het minder bestuderen van vrouwen heeft geleid tot het toepassen van de bevindingen in mannen (die immers makkelijker te bestuderen zijn) op de lichamen van vrouwen en de onderwaardering van de verschillen tussen mannen en vrouwen in ziekte en gezondheid.

Voor hart- en vaatziekten heeft het feit dat ze vaker voorkomen bij mannen op jonge leeftijd ervoor gezorgd dat onderzoek vaker werd gedaan in mannen. Maar intussen is duidelijk dat verschillen tussen mannen en vrouwen overal in hart- en vaatziekten terug te vinden zijn. Van het ontstaan in het lichaam, tot de uiting van symptomen. Zo zien we in vrouwen die een hartinfarct hebben vaker geen bloedprop die de bloedtoevoer blokkeert. En ziet atherosclerose er in vrouwen anders uit. Ze hebben ook vaker een schaafwondje dan een scheur in hun plaque, als ze zijn overleden aan een plotseling hartinfarct.

Het zou kunnen dat de verschillen in hormonen tussen mannen en vrouwen een verklaring zijn voor de verschillen tussen mannen en vrouwen in hart- en vaatziekten. Vrouwelijke oestrogenen lijken beschermend te werken tegen hart- en vaatziekten omdat vrouwen na de overgang (als de oestrogeenwaardes spectaculair zijn gedaald) een groter risico hebben op hart- en vaatziekten. Helaas lijkt de invloed van oestrogenen op hart- en vaatziekten nogal ingewikkeld. Onderzoek met oestrogenen als medicijn voor vrouwen na de overgang heeft geen verbetering opgeleverd voor het risico op hart- en vaatziekten en het lijkt ook nog uit te maken vanaf hoelang na de overgang de oestrogenen dan worden gebruikt.

Zwangerschap wordt wel gezien als een test voor het vrouwelijke lichaam als het op hart- en bloedvaten aankomen. Daarnaast kan de zwangere haar epigenetische veranderingen doorgeven aan het kind. Vrouwen hebben risicofactoren die niet bij mannen voorkomen omdat ze gerelateerd zijn aan de zwangerschap, zoals zwangerschapssuikerziekte, hoge bloeddruk tijdens de zwangerschap of zwangerschapsvergiftiging. Ook vrouwen die vroeg in de overgang komen hebben een verhoogd risico. Als je naar de sekseverschillen tussen alle risicofactoren kijkt, lijkt een van de grootste verschillen te bestaan in roken, waarbij roken in vrouwen veel schadelijker is dan in mannen.

De geslachtschromosomen, onafhankelijk van hormonen, worden intussen ook bestudeerd als belangrijke speler in de sekseverschillen in ziekte. Het Y chromosoom wordt helaas vaak niet bestudeerd in GWAS, omdat dit technische problemen oplevert in de analyse (alle andere chromosomen komen immers voor in paren en het Y chromosoom is in z'n eentje). Bovendien werd vroeger vaak gedacht dat behalve de geslachtsbepalende regio het Y chromosoom verder niet zoveel voorstelde. Het is maar klein en er liggen niet zoveel genen op. Recent kwam hier verandering in, toen bleek dat er op het Y chromosoom genen liggen waarvan je er twee nodig hebt, en waarvan het andere, vergelijkbare gen, ligt op het X chromosoom. Daarnaast werden er studies gepubliceerd waarbij genetische variatie op het Y chromosoom werd gelinkt aan bloeddruk en kransslagaderverkalking, ook onafhankelijk van geslachtshormonen. Het X-chromosoom wordt helaas ook vaak uit de GWAS analyses gegooid (omdat mannen er maar één hebben en dit de analyses bemoeilijkt). Dit is zo mogelijk nog erger dan het niet meenemen van het Y chromosoom, aangezien het X chromosoom heel groot is, het bevat ongeveer 5% van alle genen! Omdat vrouwen twee X chromosomen hebben en mannen maar eentje, zetten alle cellen in het vrouwelijke lichaam één X chromosoom uit. De cel kiest daarbij willekeurig een van de twee X chromosomen. Dit proces is erg interessant, aangezien nog niet zo heel lang geleden werd ontdekt dat sommige tweede X chromosomen in vrouwen helemaal niet compleet uit staan. Er "ontsnappen" als het ware genen. Op het X chromosoom liggen genen die te maken hebben met ontsteking en die betrokken zijn bij auto-immuunziektes die vaker voorkomen in vrouwen. Er wordt inderdaad gedacht dat het hebben van een tweede X chromosoom zorgt voor een sterkere ontstekingsreactie in vrouwen dan in mannen.

Invalshoeken

Over muizen en mensen

Het moge duidelijk zijn, hart- en vaatziekten kunnen bestudeerd worden vanuit vele gezichtspunten. Allereerst kan de vatbaarheid voor ziekte bestudeerd worden. Omdat het ontstaan van atherosclerose zo lang duurt (namelijk een groot deel van een mensenleven) worden er muizen gebruikt om de ziekte te onderzoeken. Muizen krijgen van zichzelf geen atherosclerose, en atherosclerose is een ingewikkelde ziekte, dus er moet veel gebeuren om muizen atherosclerose te laten krijgen: je moet tenminste één gen uitschakelen en vervolgens de muizen op een dieet zetten met veel vet. Daarmee ontwikkelen ze atherosclerose in een paar weken. Een andere manier om vatbaarheid voor ziekte te bestuderen is te kijken naar kennis die we hebben over hoe de ziekte ontstaat in mensen. Dit kun je bijvoorbeeld doen door te kijken naar genetica van mensen, zoals met genoombrede studies.

Staan op de schouders van reuzen

Je kunt atherosclerose natuurlijk ook onderzoeken door naar de atherosclerotische plaque van mensen te kijken. Als je wilt weten welke cellen er allemaal in zo'n plaque zitten, dan moet je hem in plakjes snijden en onder de microscoop leggen. Chirurgen verwijderen atherosclerotische plaques die voor symptomen zorgen, bijvoorbeeld doordat ze een beroerte hebben veroorzaakt, of doordat ze zorgen voor etalagebenen. Zulke plaques kun je samen met de gegevens van de geopereerde patiënt opslaan in een zogenaamde "biobank", om zo meer te weten te komen over atherosclerose in mensen.

Vijftien jaar geleden, op 24 maart 2002, werd de plaque van de eerste patiënt bewaard in de Athero-Express Biobank Studie (AE). De AE verzamelt mensen van wie een plaque tijdens een operatie is verwijderd. De patiënten werden tot 2014 verzameld in zowel het St. Antonius Ziekenhuis in Nieuwegein, als in het UMC Utrecht. Vanaf 2014 worden alleen nog plaques uit het UMC Utrecht verzameld. De plaque wordt opgeslagen samen met bloed (als dit beschikbaar is) voor elke patiënt. Daarnaast vult de patiënt een vragenlijst in met daarin vragen die gaan over de ziektegeschiedenis van de patiënt en zijn familie, de aanwezigheid van risicofactoren van hart- en vaatziekten en kwaliteit van leven van de patiënt. Na de operatie wordt de patiënt vervolgens drie jaar gevolgd waarbij wordt gekeken of er opnieuw symptomen van hart- en vaatziekten voorkomen. Tot nu toe zijn er 3433 patiënten opgenomen in de AE, 2377 mensen die geopereerd zijn aan hun halsslagader en 1056 mensen die geopereerd zijn aan een beenslagader. Van elke plaque zijn plakjes gesneden die zijn gekleurd om te kijken welke cellen er precies in zitten. Daarnaast zijn er stukjes van de plaque bewaard om de aanwezigheid van bepaalde eiwitten te bestuderen en om DNA of RNA uit te halen. Informatie over genetische variatie is aanwezig voor 1526 patiënten in de AE, van 1217 patiënten hebben we informatie over het Y chromosoom. Informatie over DNA methylatie in de plaque is aanwezig voor 488 patiënten. Er zijn al ruim 100 artikelen verschenen over de Athero-Express. Het is de grootste atherosclerotische-plaque-biobank in de wereld.

Waar het de patiënt om gaat

Studies die gaan om het vinden van factoren die het risico op HVZ verhogen, of de mechanismen achter het ontstaan van de ziekte bestuderen, hebben op zichzelf weinig zin voor de mensen die de ziekte al hebben. Het is daarom belangrijk om ook de uitkomsten van de ziekten die de patiënt direct aangaan te bestuderen, zoals kans op overlijden en de kwaliteit van leven.

Er zijn nog veel meer manieren om onderzoek te doen naar hart- en vaatziekten, maar het onderzoek in dit proefschrift wordt beschreven vanuit deze drie invalshoeken.

Samenvatting van de studies in dit proefschrift

DEEL I

Modellen van vatbaarheid

Deel I gaat over vatbaarheid voor HVZ, bekeken vanuit het standpunt van muizenstudies en vanuit mensen. Muizen zijn geen mensen, ze hebben van nature andere vetwaardes in hun bloed en hun ontstekingsreactie verloopt anders. Het is dus niet vreemd dat de atherosclerotische plaque van een muis er anders uitziet dan de plaque van een mens. Bovendien ontwikkelt een muis vaak wel een plaque, maar krijgt hij geen symptomen van hart- en vaatziekten. De vraag is dus hoe waardevol het muismodel eigenlijk is voor hart- en vaatziekten, want het is de bedoeling dat de bevindingen die we doen in muizen weer terug te vertalen zijn naar mensen. We zijn er immers niet op uit om de niet-bestaande hart- en vaatziekten in muizen uit te roeien. In **Hoofdstuk Twee** hebben we bestudeerd hoe goed de vertaalbaarheid van atherosclerose-onderzoek van muizen naar mensen is. We hebben alle muismodellen die ooit zijn gemaakt voor HVZ onderzocht en vervolgens gekeken of voor de genen die daarbij gevonden werden ook aanwijzing is dat die in mensen zorgen voor een verhoogd risico op ziekte. De uitkomst van het onderzoek was teleurstellend, want er bleek helemaal niet zoveel overlap te zijn tussen muizen en mensen. We concluderen daarmee dat muizen niet handig zijn om als uitgangspunt te gebruiken voor onderzoek naar atherosclerose. Ze kunnen wel handig zijn om genen te bestuderen waarvoor al bewijs is in mensen dat ze iets te maken hebben met de ziekte. In **Hoofdstuk Drie** hebben we een poging gedaan om meer van dat soort genen te ontdekken door met een andere bril op naar de resultaten van GWAS te kijken. Hierbij hebben we gebruik gemaakt van de kennis die we intussen hebben over het buigen van DNA en het feit dat stukken van het DNA die geen recept voor een eiwit bevatten, wel de genexpressie kunnen beïnvloeden van het stuk DNA waar ze naartoe gebogen zijn. We hebben van de plaatsen waar genetische variatie gelinkt was met ziekte (in GWAS) bekeken welke andere stukken van DNA hier (in 3D) aan vastgeplakt zaten. Omdat de vouwing van het DNA afhangt van de soort cel waarin je kijkt, hebben we dit gedaan in endotheelcellen uit de kransslagaderen en in witte bloedcellen. Van deze cellen weten we immers dat ze betrokken zijn bij atherosclerose. Op deze manier hebben we beschreven welke (nieuwe) genen wij denken dat betrokken zijn bij atherosclerose. Zulke genen kunnen dan vervolgens weer verder worden onderzocht.

DEEL II

Atherosclerotische-plaque-studies in de Athero-Express Biobank

Deel II gaat over het bestuderen van kenmerken van de plaque in de Athero-Express Biobank. In **Hoofdstuk Vier** hebben we gekeken naar de link tussen estradiol, het meest voorkomende vrouwelijke geslachtshormoon, en kenmerken van de plaque uit de halsslagader van vrouwen onder de 70 jaar. In deze groep vrouwen is de afname van sterfte door hart- en vaatziekten de afgelopen tijd namelijk veel minder sterk dan in andere vrouwen en dan in mannen. Interessant genoeg vonden we een link tussen

estradiolwaardes in het bloed en het aantal kleine bloedvaatjes in de plaque. Verder vonden we aanwijzingen voor een link met meer witte bloedcellen in de plaque. Dit is zo interessant omdat vrouwen vaak juist een plaque hebben met kenmerken die horen bij een meer 'stabiele' plaque, zoals veel gladdespiercellen. Die vonden we ook wel, maar we vonden dus ook kenmerken die eigenlijk horen bij een meer 'instabiele' plaque. In vrouwen wordt vaker een schaafwondje op de plaque gezien dan een scheur in de plaque. Juist die plaque met een schaafwondje wordt vaker een 'stabiele' plaque genoemd. Wij laten zien dat het misschien nog net iets ingewikkelder is dan dat. In **Hoofdstuk Vijf** deden we onderzoek in plaques uit beenslagaders en keken we naar de link tussen suikerziekte en kenmerken van de plaque. Ook hebben we gekeken naar het voorkomen van hart- en vaatziekten in deze patiënten drie jaar na de operatie. We weten namelijk dat suikerziekte een risicofactor is voor atherosclerose in de beenvaten en suikerziekte komt steeds vaker voor. We vonden meer kalk in de bloedvaten van mensen met suikerziekte. Daarnaast vonden we ook dat ze meer hart- en vaatziekten hadden in de drie jaar na de operatie. Interessant genoeg vonden we niet meer operaties aan hetzelfde vat als waarvoor de patiënt in eerste instantie werd geopereerd. Dat is zo interessant omdat vaak wordt gezegd over patiënten met suikerziekte dat ze slechter genezen. Eerder onderzoek in plaques uit halsslagaders liet zien dat de plaques de afgelopen tien jaar zijn veranderd naar meer plaques met kenmerken van stabiliteit. In **Hoofdstuk Zes** hebben we gekeken of dit ook in beenslagaders het geval was. We vonden inderdaad dat de plaque veranderd was, onafhankelijk van de patiëntkenmerken die de afgelopen tien jaar zijn veranderd. Dit wijst op een verandering van atherosclerose als ziekte. De bevinding onderstreept ook het belang van biobanken die bestaan uit weefsel van mensen van nu, zodat op basis van bewijs uit die biobanken ook een behandeling bedacht kan worden voor de huidige ziekte (en niet de ziekte van veertig jaar geleden, toen de eerste grote studies naar hart- en vaatziekten begonnen). We denken overigens dat de veranderende ziekte wordt veroorzaakt doordat we nu eerder ingrijpen met medicijnen, bijvoorbeeld medicijnen die vetwaardes in het bloed verlagen. Of door minder passief roken, doordat het beleid voor roken in uitgaansgelegenheden is veranderd.

DEEL III

(Epi)genetische studies in de Athero-Express Biobank

In Deel III van dit proefschrift staan studies beschreven die gaan over (epi)genetica in de Athero-Express Biobank. In **Hoofdstuk Zeven** hebben we variatie op het Y chromosoom bestudeerd en de relatie met kenmerken van de plaque en van de wand van mensen met een vaatuitbolling (aneurysma). Dit leek ons interessant, aangezien er recent artikelen verschenen waren die een link lieten zien tussen variatie op het Y chromosoom en kransslagaderverkalking. Wij wilden weten of dit kwam door een andere soort atherosclerotische plaque. Jammer genoeg vonden we geen enkele aanwijzing voor een link tussen kenmerken van de plaque of kenmerken van de vaatwand van patiënten met een aneurysma. Daarnaast vonden we ook geen andere verdeling van de genetische

variatie ten opzichte van een gezonde Nederlandse controlepopulatie. Deze resultaten maken een oorzakelijk verband tussen Y chromosomale variatie en atherosclerose minder waarschijnlijk. In **Hoofdstuk Acht** hebben we een ander fenomeen dat te maken heeft met het Y chromosoom beschreven, namelijk verlies van het Y chromosoom. Als mannen ouder worden komen er steeds meer cellen in hun bloed voor waarin geen Y chromosoom meer aanwezig is. Dit verlies van het Y chromosoom is al eerder gelinkt aan roken, wat een risicofactor is voor hart- en vaatziekten. Wij hebben daarom onderzocht of we een relatie konden vinden met kenmerken van de atherosclerotische plaque. Die vonden we helaas niet. Wel vonden we een link met hart- en vaatziekten in de drie jaar na de operatie: hoe minder Y chromosoom in het bloed, hoe meer hart- en vaatziekten in de drie jaar na de operatie. Hoe dit precies werkt, blijft de vraag. In **Hoofdstuk Negen** hebben we gekeken naar de verschillen in DNA methylatie tussen mannen en vrouwen in de atherosclerotische plaque. We vonden er heel veel en we vonden geen relatie tussen de DNA methylatie en kenmerken van de plaque of risicofactoren van de patiënten. Er zijn nog niet veel studies die DNA methylatie bestuderen in ziek weefsel. Deze uitkomsten onderstrepen het belang van het apart bekijken van mannen en vrouwen als men DNA methylatie bestudeert.

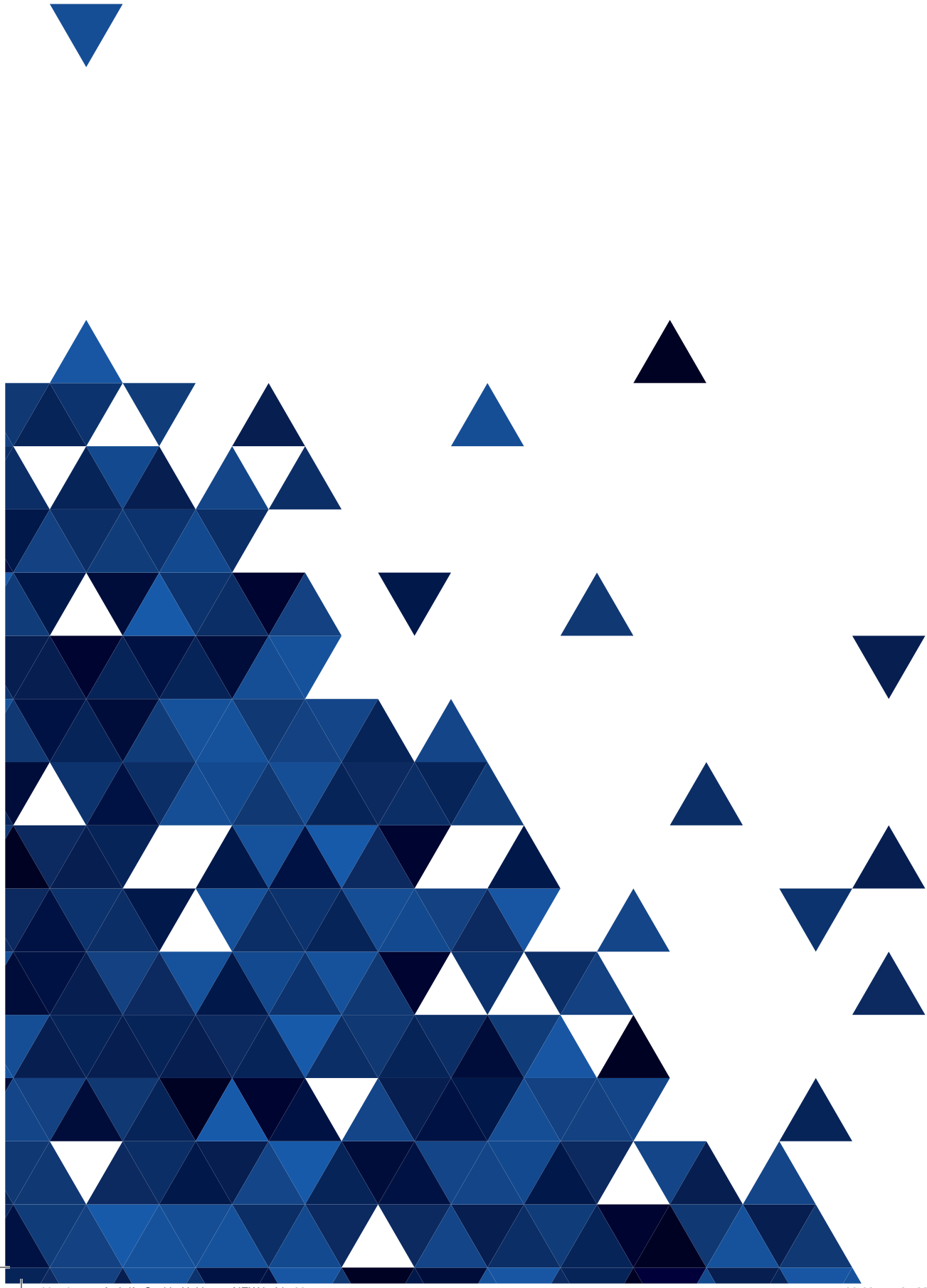
DEEL IV

Studies over klinische uitkomsten

In Deel IV van dit proefschrift beschrijven we het terugkeren van symptomen, het risico op sterfte en de kwaliteit van leven van patiënten met atherosclerose. In **Hoofdstuk Tien** hebben we gekeken naar de verschillen tussen mannen en vrouwen in sterfte na een omleidingoperatie van de kransslagaders. Er zijn namelijk aanwijzingen dat vrouwen na zo'n operatie een slechtere overleving hebben dan mannen. Wij vonden dat in eerste instantie ook, maar toen we beter gingen kijken zagen we dat het verschil kwam door verschillen in risicofactoren. Om een harde uitspraak te kunnen doen over waar het nou precies door komt: enkel sekse, of toch de risicofactoren, is een veel grotere groep vrouwen nodig. Zo'n groep zijn we momenteel aan het verzamelen. In **Hoofdstuk Elf** hebben we ook naar sterfte gekeken, maar dan na de operaties die we bestuderen in de Athero-Express. We hebben onze mannen en vrouwen vergeleken met mannen en vrouwen die even oud waren en die geen operatie ondergingen. Na een halsslagaderoperatie zagen we een verschil tussen mannen en vrouwen: mannen gingen vaker dood dan vrouwen en vaker dood dan gezonde mannen van dezelfde leeftijd. Vrouwen deden het juist heel goed, zij hadden een even grote kans op sterfte als gezonde vrouwen van dezelfde leeftijd. Interessant genoeg zagen we het verschil tussen mannen en vrouwen niet terug in de patiënten die geopereerd werden aan hun beenslagaders. Bij die patiënten gingen zowel de mannen als de vrouwen eerder dood dan hun leeftijdgenoten die niet geopereerd waren. Ten slotte hebben we in **Hoofdstuk Twaalf** een studie beschreven naar kwaliteit van leven. We dachten dat onze patiënten in de Athero-Express een slechte kwaliteit van leven zouden hebben. Daarbij dachten we dat mensen met meer klachten, bijvoorbeeld patiënten die een heftige beroerte hadden

gehad en daar veel beperkingen aan over hadden gehouden, ook een slechtere kwaliteit van leven zouden hebben. Onze eerste gedachte bleek juist: al onze patiënten hadden een slechte kwaliteit van leven ten opzichte van mensen van dezelfde leeftijd uit de algemene bevolking. Maar gek genoeg werd deze kwaliteit van leven helemaal niet beïnvloed door het hebben van een ernstigere vorm van ziekte. Dokters zouden kwaliteit van leven kunnen bespreken met hun patiënten en hen kunnen verwijzen als ze behoefte hebben aan handvatten om om te kunnen gaan met hun hart- en vaatziekte.

Hoofdstuk Dertien bevat de discussie en **Hoofdstuk Veertien** heb je net gelezen.



APPENDIX

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doi: 10.1371/journal.pgen.1005378.

Awards

Abstract prize for best abstract, poster and TedMED talk
Papendal course, Dutch Heart Association, 2015

Best parallel presentation prize
Dutch CardioVascularConference, 2016

Curriculum Vitae

Saskia Haitjema was born on the 4th of February 1988 in Delft, The Netherlands, as the first child of Margreeth Broens and Han Haitjema. Three years later, her brother Jarich was born. Saskia started dancing when she was four and playing the piano when she was six. When she was nine, the family moved to Eindhoven. During her high school period there, Saskia was among others member of the advisory board, played in the school band and was editor-in-chief of the school paper. She graduated gymnasium (cum laude) at the Lorentz Casimir Lyceum in 2006 after which she moved to Utrecht to study Medicine at Utrecht University. In 2007, Saskia started combining Medicine with a bachelor of Linguistics and Phonetics at the same university. In the years that followed, Saskia started volunteering at Vereniging Anderwijs, chaired several polling stations during Dutch elections and joined the Nederlandse Vereniging voor Dans- en Muziekgeneeskunde. She obtained her bachelor's degree in Linguistics and Phonetics at the same time as her master's degree in Medicine in 2012. Saskia gained clinical experience as a surgical resident (not in training) at the TweeSteden Hospital in Tilburg before starting as a PhD candidate at the Laboratory of Experimental Cardiology of the University Medical Center Utrecht in 2013. Under supervision of prof. Gerard Pasterkamp and prof. Folkert Asselbergs, she studied sex-differences in atherosclerotic disease. During her time as a PhD candidate, Saskia was an active member of the Research and Education Council of the UMC Utrecht. Her research resulted in a PhD thesis titled "Sex matters to the arteries" that she will defend in March 2017. In January 2017, Saskia started working at the Utrecht Patient-Oriented Database (UPOD) under dr. Imo Höfer and prof. Wouter van Solinge. Saskia is anxious to know what the rest of her life will bring, and will probably never stop dancing or playing the piano.