Risk recognition in patients with atherosclerotic disease

associations of circulating biomarkers
with plaque composition and clinical outcomes

Ian D. van Koeverden

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Risico herkenning in patiënten met slagaderverkalking

associaties van circulerende biomarkers
met plaque samenstelling en klinische uitkomsten
(met een samenvatting in het Nederlands)

Proefschrift

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PART

one

INTRODUCTION AND BACKGROUND

1

Introduction

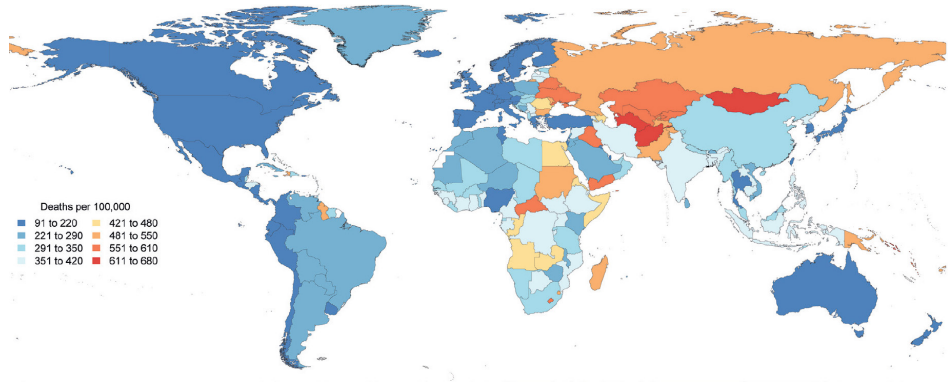
Cardiovascular disease (CVD) is a collective name for different (sub)clinical diseases that are characterized by pathologic changes of the heart or vasculature. One of the major underlying processes in vascular pathology is atherosclerosis, causing clinical manifestations, such as; myocardial infarction, angina pectoris, ischemic stroke and chronic limb ischemia. Atherosclerosis is a lipid driven inflammatory disease, causing narrowing of the arteries due to the buildup of plaque in the vascular wall.[1] Interestingly, atherosclerotic lesion development is not specific for modern-day Western society. Even in ancient times humans already had a predisposition for developing atherosclerotic lesions since considerable atherosclerotic lesions have already been found in the remains of mummified Ancient Egyptians living over three thousand years ago.[2] Whether these lesions also resulted in manifestations of CVD remains uncertain. Interestingly, the lowest recorded levels of coronary artery disease in any population to date were found in an indigenous South American population.[3] Although age-standardized death rates due to CVD are highest in developing countries (Figure 1), this unique group of forager-horticulturalists living of a lifestyle of hunting, gathering, fishing, and farming was protected from CVD.[4] Lifestyle factors such as high physical activity, no smoking, high fruit and vegetable diets correlated with low blood pressure, low cholesterol and low glucose levels. In Western society, it appears that the rise in cardiovascular disease mortality has only been brought to a halt most recently among others through improved medical and preventive therapy combined with government legislation. In the Netherlands today, CVD is no longer the number one killer and has been passed by cancer in 2004 for men and in 2011 for women.[5] Cessation of smoking, public smoking bans and restrictions in dietary fat and salt intake all contribute to these effects. In developing countries, however, the incidence of CVD is still on the rise which is tightly related to the global burden of obesity, smoking and air pollution.[6]

ATHEROSCLEROSIS

Atherosclerosis is firmly linked to blood lipid levels and the progression of atherosclerotic plaques often takes decades to develop.[7,8] This process is often gradual and starts early-on in life with the deposit of fatty streaks on the inner lining of the arteries adjacent to the endothelial layer. Different risk factors such as hypertension, smoking or high lipid levels can activate the endothelial layer and initiate the process of plaque formation. Endothelial cells take up LDL particles and thereby activate an immune response.[9] The inflammatory response starts by blood monocytes entering the subendothelial layer to clean up the debris or fatty streaks. When these monocytes migrate into the vascular wall they are activated to become macrophages and progressively transform into macrophage-derived foam cells.[10]

At first this immune-response is effective and it is possible to clear out the fatty streak, however, due to the ongoing supply of lipids, the accelerated inflammatory response will prove to be detrimental and atherosclerotic lesions start to build up. The inflammatory process progresses and smooth muscle cells eventually migrate to form a cap over the

Figure 1. Global Map, Age-Standardized Death Rate of CVD in 2015.

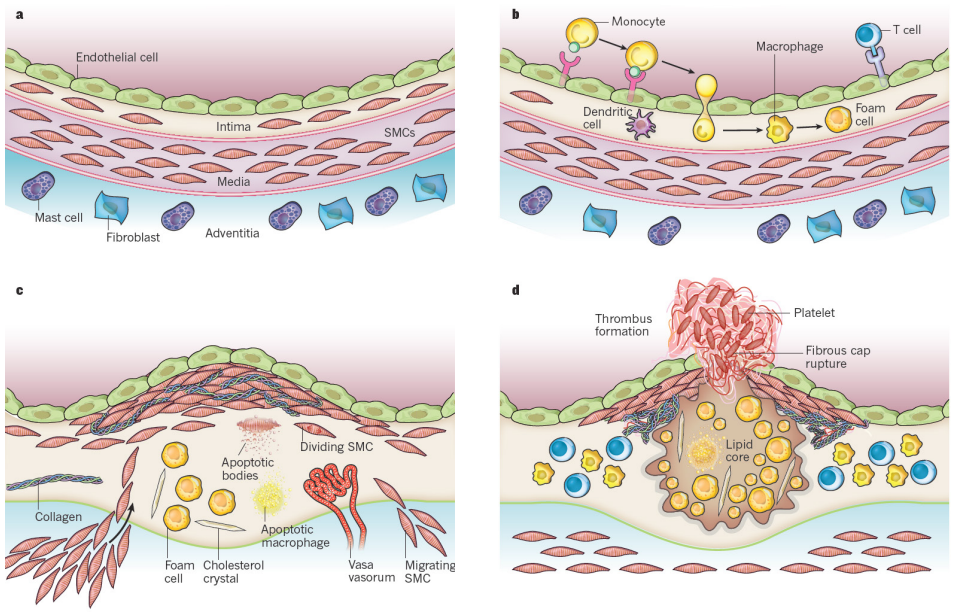


Adapted from: Global, Regional, and National Burden of Cardiovascular Diseases for 10 Causes, 1990 to 2015. Gregrory A. Roth et al. Journal of the American College of Cardiology 78, (2017).

underlying inflammatory culprit. This inflammatory process results in a pool of death lipid-loaded immune-cells all contributing to necrotic core formation. The continuous inflammatory process can lead to hypoxic conditions within the necrotic core, triggering neovascularization of the plaque originating from the vasa vasorum. The neovessels often not mature completely and combined with an unstable extracellular environment due to Matrix metalloproteinases (MMPs) can result in disruption of these vessels. When this happens, erythrocytes enter the plaque further contributing to destabilization and initiating the stage called intraplaque hemorrhage.[11] The presence of intraplaque hemorrhage is considered high-risk and a risk factor for future cardiovascular events in other vascular beds and thereby reflecting the state of systemic vessel wall integrity.[12] Interestingly, while the presence of intraplaque hemorrhage in male plaques predicts future events in other vascular beds, this was not the case for women, likely reflecting underlying sex differences.[13] Possibly plaque erosion, more commonly seen in women, as a substrate for acute events may interfere with this relation.[14] Analyses in this thesis were therefore sex-stratified when appropriate.

The different stages of plaque formation are shown in Figure 2. Due to this slow gradual progression, atherosclerotic plaques often remain asymptomatic for decades but in an advanced stage will develop symptoms in the affected vascular territory. Early symptoms of diminished blood-flow can be relatively mild such as a decreased walking distance or exercise-induced chest pain which passes during rest. The common denominator here is the inadequate arterial blood-flow to the specific target-organ (in this case the muscles of the legs or the myocardium) failing to guarantee proper tissue oxygenation.

Figure 2. Different stages in atherosclerotic lesion development.



a, The normal artery contains three layers. The inner layer, the tunica intima, is lined by a monolayer of endothelial cells that is in contact with blood overlying a basement membrane. The middle layer, or tunica media, contains SMCs embedded in a complex extracellular matrix. Arteries affected by obstructive atherosclerosis generally have the structure of muscular arteries. The adventitia, the outer layer of arteries, contains mast cells, nerve endings and microvessels. **b**, The initial steps of atherosclerosis include adhesion of blood leukocytes to the activated endothelial monolayer, directed migration of the bound leukocytes into the intima, maturation of monocytes (the most numerous of the leukocytes recruited) into macrophages, and their uptake of lipid, yielding foam cells. **c**, Lesion progression involves the migration of SMCs from the media to the intima, the proliferation of resident intimal SMCs and media-derived SMCs, and the heightened synthesis of extracellular matrix macromolecules such as collagen, elastin and proteoglycans. Plaque macrophages and SMCs can die in advancing lesions, some by apoptosis. Extracellular lipid derived from dead and dying cells can accumulate in the central region of a plaque, often denoted the lipid or necrotic core. Advancing plaques also contain cholesterol crystals and microvessels. **d**, Thrombosis, the ultimate complication of atherosclerosis, often complicates a physical disruption of the atherosclerotic plaque. Shown is a fracture of the plaque's fibrous cap, which has enabled blood coagulation components to come into contact with tissue factors in the plaque's interior, triggering the thrombus that extends into the vessel lumen, where it can impede blood flow.

Adapted from: Progress and challenges in translating the biology of atherosclerosis. Peter Libby et al. Nature 473, 317–325 (2011).

ATHEROTHROMBOSIS

The event in which a thrombus occludes an artery is called atherothrombosis or thromboembolic infarction. Its clinical presentation is often acute. There are several pathophysiological phenomena that can lead up to atherothrombosis and one of these is rupture of an atherosclerotic plaque.[15] Plaques most prone to rupture are those plaques with a large necrotic core with a thin overlying fibrous cap, hence the name thin-capped fibroatheroma.[16] These plaques often have abundant inflammation, ingrowth of neovessels and intraplaque hemorrhage, these factors contribute to the destabilization of the smooth

muscle rich fibrous cap and can eventually lead up to plaque rupture. In the case of plaque rupture, the shear stress of the arterial blood flow overcomes the structural integrity of the plaque resulting in rupture of the fibrous cap. This process often takes place in the shoulder regions of the plaque and when plaques rupture the necrotic content and interior of the plaque will be exposed to the bloodstream activating the clotting cascade.[17] Both fragments of the plaque and newly formed thrombi may disembark and travel further downstream in a process called thromboembolic infarction.

Another process that can trigger atherothrombosis is plaque erosion in which the endothelial layer covering the plaque is injured and thereby exposing the extracellular matrix to the bloodstream also activating the clotting cascade. Interestingly, this mechanism of atherothrombosis occurs more frequently in women before menopause.[18]

In this thesis, I mainly focused on thromboembolic infarction caused by atherosclerotic disease of the carotid arteries. In particular I studied methods to predict patients at high-risk for new cardiovascular events after surgical treatment of the carotid artery.

The carotid arteries or carotids are two large arteries branching from the aorta (left carotid) and the brachiocephalic trunk (right carotid) and pass through the front of the neck to the head. The carotids are two of the main arteries responsible for adequate tissue oxygenation of the brain, face, and neck. When atherothrombosis takes place in the carotids a thromboembolism can travel through the cerebral circulation and block an artery supplying the brain from oxygen-rich blood. Due to insufficient blood supply nerve tissue will stop to function and neurological failure ensues. Depending on which part of the brain is depleted from blood, brain location-specific symptoms will occur and depending on the duration of arterial occlusion these symptoms might be transient in a process called transient-ischemic-attack or often permanent in case of stroke.

Recognition of cardiovascular risk

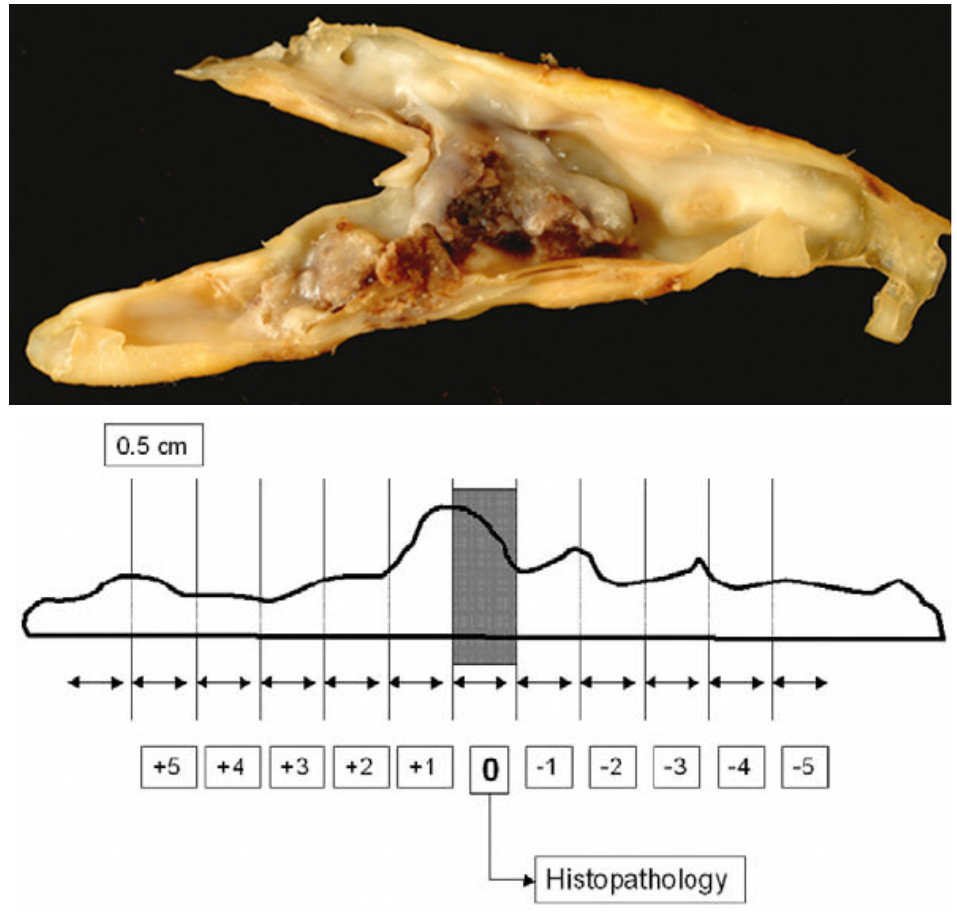
Treatment of atherosclerotic disease of the carotid artery is preferably done by a surgical procedure called carotid endarterectomy (CEA).[19] During this procedure, the affected artery is opened and the atherosclerotic plaque is removed in order to restore adequate blood flow to prevent (new) neurological events. Unfortunately, interventions are hardly ever without risk and a small part of the population will develop a stroke during or shortly after the procedure.[20] Moreover, since atherosclerosis is a systemic condition, other vascular beds such as the coronary or the femoral arteries can induce symptoms in the following years as well. These aspects add to the difficulties of treatment and risk prediction in patients with carotid atherosclerosis. Ideally, high-risk patients undergo stringent preventive therapy, intensive monitoring during procedures, and aggressive medical care in order to attack these potentially preventable cardiovascular events. Supervised programs to improve medication compliance and lifestyle interventions are commenced best in patients at highest need. The identification of these high-risk populations is preferably done with little effort for the treating physician but remains challenging.

Biomarkers can assist in this process by providing insights on inflammation, coagulation and/or specific function of organs. A reliable estimate of kidney function, for instance, can be determined by measuring creatinine levels in a blood sample.[21] Since the kidneys are tightly controlling blood-pressure and fluid homeostasis they are a critical component of the cardiovascular system. Moreover, decreased kidney function is an important risk factor for the occurrence of cardiovascular events.[22] How this interaction between kidney function and the atherosclerotic plaque ensued was partly unknown. By using plaque histology and plaque proteomics we could study important interactions between inflammation and coagulation within the carotid artery and their association with kidney function. Moreover, with the use of clinical follow-up data we could assess how survival was affected by decreased kidney function and thereby providing insights in this important risk factor. Comparable to estimating kidney function, sex-hormonal status can be assessed by measuring testosterone (T) and estradiol (E2) levels. This is particularly important considering the existing controversies on the effects of sex-hormones in cardiovascular disease. Both hormones are expected to exert protective effects on the vasculature.[23] However, hormone replacement therapy trials did not result in the anticipated health benefits. By studying individual hormone levels and the interplay between testosterone and estradiol by use of the Testosterone/Estradiol (T/E2) ratio we provide important insights in the cardiovascular risk associated with hormonal imbalance. We show that T/E2 ratio reflects both systemic and plaque inflammation which likely drives the found increased risk for future cardiovascular events.

Studying a changing disease

We recently reported that atherosclerotic plaque features such as large lipid core and intraplaque hemorrhage have become increasingly less prevalent in recent years when compared to earlier years.[24,25] This temporal change in plaque characteristics goes hand in hand with time dependent alterations in the incidence of acute clinical manifestations due to atherosclerotic disease.[14] This stabilization of atherosclerotic plaque composition coincided with an age-adjusted decline in stroke and ST-elevated myocardial infarction in Western society. Over time, large improvements have been made in cardiovascular risk management. For instance; the introduction of novel pharmaceutical agents, public smoking bans and reduced dietary intake of salts and trans-fats likely contributed to this stabilization. The ongoing collection of plaques allowed for a form of track and trace system for this dynamic disease. This not only provides important insights in changes of characteristics of the underlying culprit lesion but also ensures that the disease-process is dynamic over time and that observations in current studies are representative for the disease affecting patients today, and not necessarily of manifestations observed decades ago. These changes over time provided for a valuable research topic. In this thesis, time-dependent effects in clinical outcome were studied in 1684 patients following carotid and in 530 patients following iliofemoral endarterectomy (IFE).[20] Moreover, considering that diabetes is a strong risk determinant in patients undergoing IFE, a study was performed focusing on time-dependent effects on plaque composition and three-year outcome in diabetic patients

Figure 3. Atherosclerotic plaque specimen derived from the carotid artery.



In the top panel a typical example of an atherosclerotic plaque removed during carotid endarterectomy. In the bottom panel a schematic overview on how the plaque was processed after removal.

alone. Lastly it is currently unknown how plaque composition within the same patients could differ over time. By studying a unique sample of patients who are operated at two moments in time (left and right carotid) we could study these changes.

Research approaches

There are various methods to study atherosclerotic disease such as vascular imaging techniques, genetic sequencing, mouse knockout models and plaque histology studies which all have their strengths and limitations. The work presented in this thesis has largely originated from data collected in the Athero-Express study.[26] This biobank was initiated in

2002 and has continuously collected over 3500 plaques derived from carotid or iliofemoral endarterectomy and making it the largest atherosclerotic plaque biobank in the world. Patients included were asked before surgery whether the atherosclerotic plaque and a pre-operative blood-sample could be collected and enclosed in the biobank. A considerable strong point of this research approach is that these plaques are removed by the vascular surgeon as part of best medical treatment making supply a relatively straightforward task. The atherosclerotic plaque is dissected into segments and the culprit lesion will be used for histopathological assessment (Figure 3). Routine histological staining is performed on the culprit lesion for the presence of lipids, calcifications, smooth muscle cells, collagen, microvessels, intraplaque hemorrhage, and macrophages. The other segments of the plaque are dissected into 5mm segments and snap frozen before storing in -80°. These segments are used for protein, DNA and RNA isolations. These biological specimens combined with clinical data and three-year follow-up data offered valuable insights into the complex biology of atherosclerotic disease. This collection not only allowed for the identification of cardiovascular risk factors it helped reveal how these risk factors correlate with specific features of the plaque and other traits of cardiovascular disease.

THESIS OUTLINE

Part I - Introduction and background
Part I provides a general introduction to atherosclerosis, atherothrombosis, clinical risk recognition and strategies for studying cardiovascular disease. The Athero-Express study is outlined and the concept of the vulnerable plaque is exemplified. In **Chapter 2** the current state of carotid artery biobanking is discussed and how it shaped our concepts of disease. We illustrate some important results derived from over a decade of biobanking and describe some of the limitations of extrapolation of results to current clinical practice.

Part II - Studies on clinical outcome after carotid or iliofemoral endarterectomy
In Part II clinical outcomes after surgical revascularization were studied. Carotid endarterectomy is performed to prevent neurological symptoms in patients that either already experienced a neurological event (symptomatic patients) or in patients that never experienced a neurological event but with significant narrowing of the carotid artery (asymptomatic patients). It is important to provide insights into the number of patients that develop new neurological events considering the goal of the procedure is to prevent these secondary events. In patients with iliofemoral atherosclerotic disease surgical endarterectomy is performed to improve walking distance, promote wound-healing and decrease ischemic rest-pain. In **Chapter 3** the number of secondary events after CEA and IFE were studied over a twelve year time-period. This study was performed because patients in the Athero-Express showed strong stabilization of atherosclerotic plaque composition combined with improvements over time in patient characteristics. These improvements made us question whether cardiovascular events during follow-up decreased

over the last decade as well. **Chapter 4** describes the differences in cardiovascular outcomes in the first month after carotid endarterectomy in a cohort of 17699 patients from the United States of America compared to 2318 Dutch patients included in the Athero-Express. In **Chapter 5** we assessed the performance of three risk prediction models used in patients with critical limb ischemia and show that performance was poor to fair. We did this by using three different intervention cohorts and propose how these models can be improved.

Part III - Circulating biomarkers in cardiovascular disease
Biomarkers are characteristics that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. Circulating biomarkers in cardiovascular disease in this thesis refer to blood cell traits, protein concentrations or hormones measured in a blood sample obtained through a venipuncture. In **Chapter 6** the association of kidney function with atherosclerotic plaque characteristics and three-year follow-up were investigated. Moreover, plaque proteomics were performed to assess the correlation of kidney function with the presence in a large array of plaque proteins. In **Chapter 7** the interplay of the sex-hormones Testosterone and Estradiol were studied in a cohort of 610 male Athero-Express patients. We studied the role of these sex-hormones on plaque composition and clinical follow-up. Moreover, we studied the association with several inflammatory markers within the blood and the characteristics of the plaques with these sex-hormones. **Chapter 8** describes the potential clinical usefulness of a routine measured hematological parameter, the red cell distribution width, for prediction of inflammatory adverse outcomes after major cardiovascular surgery. We show this predictive value in a cohort of patients undergoing open aneurysm surgery and in a cohort of patients undergoing coronary artery bypass grafting. Moreover, we provide insight in the relation of this marker and hematopoietic tissue activity.

Part IV - Atherosclerotic plaque studies
Part IV describes two studies on time-dependent effects on atherosclerotic plaque composition in the Athero-Express Study. **Chapter 9** describes the changes in atherosclerotic plaques derived from diabetic patients with iliofemoral artery disease over a twelve-year time-period. In addition, follow-up data over these twelve years was studied and compared to follow-up data from non-diabetic iliofemoral artery disease patients. In **Chapter 10** a group of patients were studied that underwent carotid endarterectomy of both the left carotid and right carotid artery on two separate moments in time. This allowed for the possibility to study time-dependent effects on plaque composition within the same patient.

Part V - Summary and general discussion
In Part V this thesis is summarized combined with a general discussion and future perspective in Chapter 11. Finally, in Chapter 12 a Dutch summary is provided.

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2

Biobanking in carotid artery disease: translation to clinical practice

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ABSTRACT

Biobanking of atherosclerotic tissue samples has contributed to our understanding of vascular occlusive disease. The careful examination of atherosclerotic plaques derived during vascular surgery or autopsies helped shape our minds in understanding the underlying substrate of arterial thrombosis. This review will outline concepts of progression of atherosclerotic disease that have been based on descriptions of human plaque pathology. In addition, we will discuss the current shift in clinical presentation and underlying pathology of acute cerebral and coronary events that asks for a careful consideration of the currently widely applied description of the "vulnerable plaque".

The shift in atherosclerotic plaque characteristics that associate with a thrombotic event reflects the treatment and risk factor management that has undergone major changes in recent times. These changes may influence the value of past biobanking efforts in the current era: many inferences are being made upon sample data from cohorts that have been assembled in previous decades while large shifts in patient demographics and disease substrates over time occurred raises the question if biomarkers validated in historical biobanks can be extrapolated to the current era. As an example of altering profiles of biomarkers in the last decade, a panel of twelve selected plasma proteins was measured in the Athero-express cohort, showing time-dependent trends in serum biomarkers over the last decade. These findings strengthen our hypothesis that the pathogenesis of cardiovascular disease (CVD) is changing and future biobanking is required to successfully keep track of the mechanisms involved in CVD pathogenesis today.

INTRODUCTION

In previous decades major efforts have been made in an attempt to challenge the global epidemic of cardiovascular disease. Treatment strategies focusing on both lipid and tension control with the use of effective pharmacologic agents have made major contributions in bringing a halt to cardiovascular disease progression. These advances combined with the widespread implementation of percutaneous vascular interventions have resulted in a total decline of 50% in age-adjusted cardiovascular mortality of Western societies in the last 3 decades.[1] Biobanking of human liquid and tissue samples improved our knowledge of the underlying causal triggers and substrates of cardiovascular disease. Widespread collections of diseased tissue samples combined with clinical data of patients, including risk factors and co-morbidities, facilitated the exploration of the underlying mechanisms and pathways in cardiovascular disease.

The preservation of biological material for future investigation enables researchers to investigate mechanisms and come to novel biomarkers and treatments for multifactorial complex diseases. Collections of biomaterial may also enable researchers to utilize the material at later time points using analytical methods that were beyond technical feasibility at the moment of inclusion.

As biobank studies may differ in primary objectives, large variations in cohort size can be observed. A biobank can aim at specific tissues for single research group purposes as well as applications up to population based bio banks. As an example, the UK biobank includes a widespread collection of biosamples from 500.000 people aged 40-69 from 2006-2010 across the UK which is accessible for any research purpose.[2] The UK biobank is unique in its sample size and open access structure which enables investigators worldwide to conduct research with data gathered in one single biobank. With the recognition of added value of biobanking, major investments have been made and it is expected that the global biobanking market will increase its worth from $141.2 billion in 2010 up to $183.6 billion in 2015.[3]

This review addresses the value of atherosclerotic tissue biobanking and how it has contributed to our current understanding of arterial occlusive disease. We will also discuss future perspectives how the growing biobank sample sizes will further increase our knowledge by assessment of plaque characteristics that associate with genetic variability in the human population. In extension, the major threats that undermine the value of past biobanking efforts in the current era: "generalizability of old to new" will be discussed.

How atherosclerotic biobanks have shaped our scientific minds and concepts of disease

Biobanking of vascular specimens have contributed to our understanding of the mechanism involved in luminal thrombosis and consequent acute coronary syndrome, stroke and leg claudication that affect our population. Large samples of collected biosamples identified the pathogenesis of plaque pathology, examples of important biobank findings are atherosclerotic plaque rupture, plaque hemorrhage, plaque erosion and arterial remodeling.

Figure 1. Plaque rupture with associated luminal trombosis precipitated by a atheromatous plaque with a large necrotic core infiltrated by macrophages and lymphocytes with an overlying thin fibrous cap.

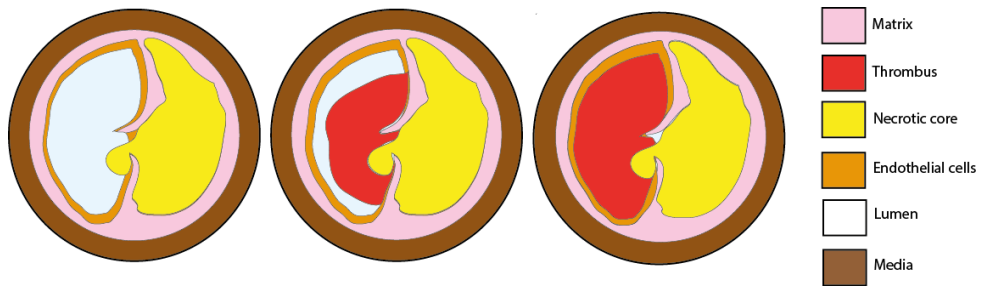
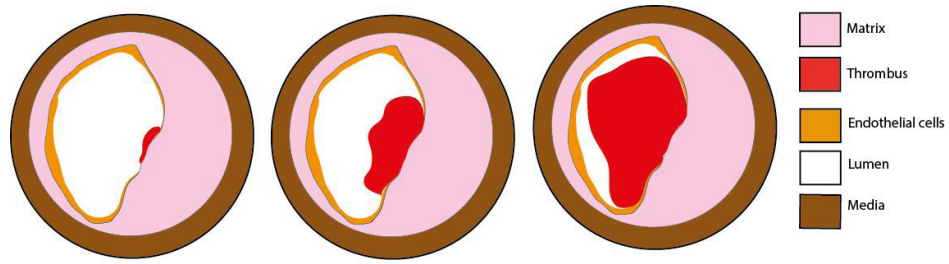


Figure 2. Plaque erosion characterized by relative stable plaque features without tendency to rupture, absence of large lipid core but presence of endothelial cell apoptosis which actively promotes luminal thrombosis.



Plaque rupture and myocardial infarction

The first evidence on how atherosclerotic plaques give rise to arterial occlusive thrombosis have originated from autopsy studies performed in the second halve of last century. The careful examination of coronary samples from patients who died of an acute myocardial infarction provided essential insights in the pathophysiology of acute coronary syndrome and arterial thrombosis.[4] An observation that dominated the research field of vascular biologists is the description of the plaque that underlies the luminal thrombosis: a plaque that is prone to rupture characterized by a large necrotic core infiltrated by macrophages and lymphocytes with an overlying thin fibrous cap as presented in figure 1.[5] These inflammatory and lipid-rich plaques instigated decades of cardiovascular research and major worldwide efforts have been made in an attempt to understand and tackle the lipid and inflammatory driven pathways in cardiovascular disease. This has resulted in a large number of clinically applied drug-therapies that lower serum cholesterol and testing of drugs that alter the inflammatory profile of patients.

Plaque erosion

With plaque rupture originally accounting for the vast majority of all acute coronary syndromes observed in postmortem obtained coronary artery samples, another mechanism of arterial occlusive events is plaque erosion.[6] The histological examination in a biobank of patients who died acutely of an acute coronary syndrome showed that plaques with surface erosions are inherently different from plaques that are vulnerable to rupture. Compared to plaques with large atheroma pools and a collagen depleted thin fibrous cap, eroded plaques are lipid poor but proteoglycan and glycosaminoglycan rich. Plaques with surface erosions have less inflammatory cells and less smooth muscle cell apoptosis. Instead these plaques show secondary recruitment of neutrophils and endothelial cell apoptosis. The term erosion reflects the absence of plaque rupture and it is considered that desquamation of endothelial cells on the luminal surface triggers thrombus formation. The de-endothelialized surface is thought to promote platelet attachment and subsequently promote luminal thrombosis, the mechanism in which arterial thrombus is formed. (Figure 2) Since the underlying lesion characteristics in plaque erosion differ from plaques that are prone to rupture, the mechanism in which plaque erosions triggers luminal thrombosis is also expected to differ from luminal thrombosis triggered by plaque rupture. In autopsy studies the thrombus found is confined to the most luminal portion of the plaque with an absence of plaque rupture or plaque hemorrhage, and a relative low degree of lumen narrowing.[6]

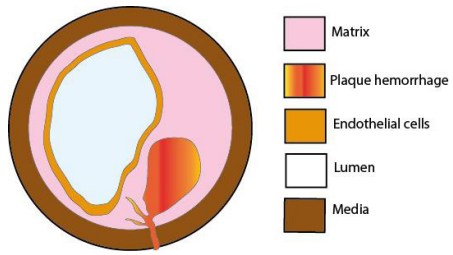
The causal mechanisms for plaque erosion are not well understood and understudied. Plaque erosions are more often found in younger patients, especially in women and seem to be associated with smoking and high levels of LDL. There is an increasing interest in the mechanisms that explain the occurrence of luminal thrombosis on top of a plaque with fibrous characteristics since there is accumulating evidence that the relative rate of these lesions that associate with myocardial infarction and stroke is rapidly increasing as a result of a better primary and secondary prevention.[7]

Plaque hemorrhage

Another example how atherosclerotic plaque biobanking has contributed to the understanding of atherosclerotic lesion progression is the description of plaque hemorrhage as a source of cholesterol accumulation in the vascular wall. In contrast with the achievements and efforts made in targeting the lipid and inflammatory pathways it is important to notice however that there are alternative effectors of plaque vulnerability. With decades of cardiovascular research focused on lipid and inflammation response control, plaque hemorrhage (PH) can trigger plaque expansion and plaque rupture as well. [8] PH is considered to result from disruption of microvessels that are present in plaques in combination with insufficient presence of support tissue such as collagen and other extracellular matrix components produced by smooth muscle cells. The expansive growth of plaques trigger neovascularization of micro vessels from the adventitia towards the plaque and several reports showed positive associations between plaque vessel density, PH and the degree of necrotic core formation (figure 3).[9] This implies that atherogenesis and subsequent PH are associated with growth of the vulnerable plaque.

On immunohistochemically staining of lipid-rich atherosclerotic plaques strong colocalization of lipids and Glycophorin, a red blood cell membrane marker, has been reported.[10] Since

Figure 3. Plaque hemorrhage depicted in plaque characterized by disrupted micro vessels with insufficient support tissue such as collagen and compounds produced by smooth muscle cells. Plaque hemorrhage contributes to formation of large necrotic core with high tendency to rupture.



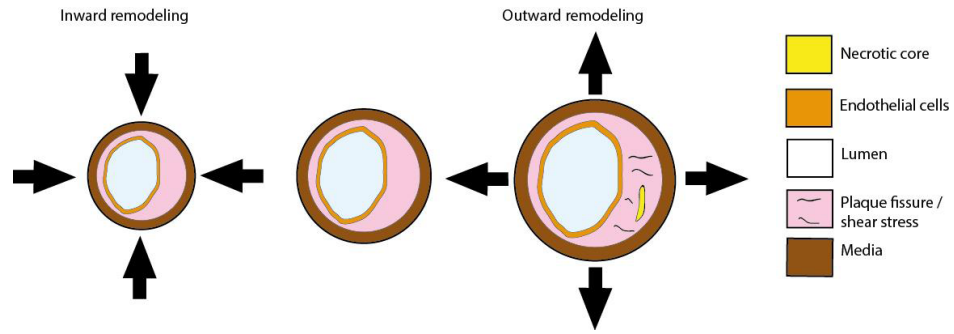
erythrocytes are composed of at least 40% lipids one could speculate that at least part of the lipids found in the necrotic core are derived from erythrocytes and conceivably derived from micro vessels. In plaque pathogenesis this could have large implications for our understanding of plaque progression. These findings suggest that plaque progression is not just an inward-out model with accumulation of lipids, macrophages and foam cells derived from the lumen of the affected vessel but rather a combination with an outward-in model of influx of atherosclerotic progenitor cells originating from disrupted neo vessels. These findings are important for the concept of the vulnerable plaque but do require further investigation.

Interestingly, PH is the only plaque phenotype that has been discovered to predict major adverse cardiovascular events (MACE) during follow-up of patients with PH upon inclusion. Interestingly, PH also predicts events in other vascular beds suggesting it to reflect a systemic instability of the atherosclerotic plaque.[11] Upon further investigation, the capacity of PH to predict outcome was confined to men only.[11] This strengthens the hypothesis that one culprit lesion can reflect the stability of other atherosclerotic plaques in the same arterial system. With the use of high tech imaging modalities such as MRI for visualization of vulnerable plaque characteristics these high-risk patients can now be identified.[12] However, at this moment good consensus regarding MRI-settings that are previously validated with histologic findings is lacking. Biobanking can be of use for validating MRI-findings with plaque histology so predefined protocols for high-tech imaging can be established. Combining state-of-the-art imaging techniques will further aid in the translation of vulnerable plaque to vulnerable patient.

Arterial remodeling

Compensatory mechanisms for lumen preservation in atherosclerotic disease are other important findings that can at least partially be attributed to biobanking of atherosclerotic tissues. In 1987 Glagov et al. discovered in human plaque samples that in patients suffering from coronary disease, arteries can undergo compensatory enlargement to compensate the plaque growth and preserve the lumen. In histological sections of the left main coronary

Figure 4. Arterial remodeling in atherosclerotic disease shows inward remodeling without lumen preservation but stable plaque features. Outward remodeling shows lumen preservation but formation of fissures and subsequently plaque rupture and a more inflammatory phenotype.

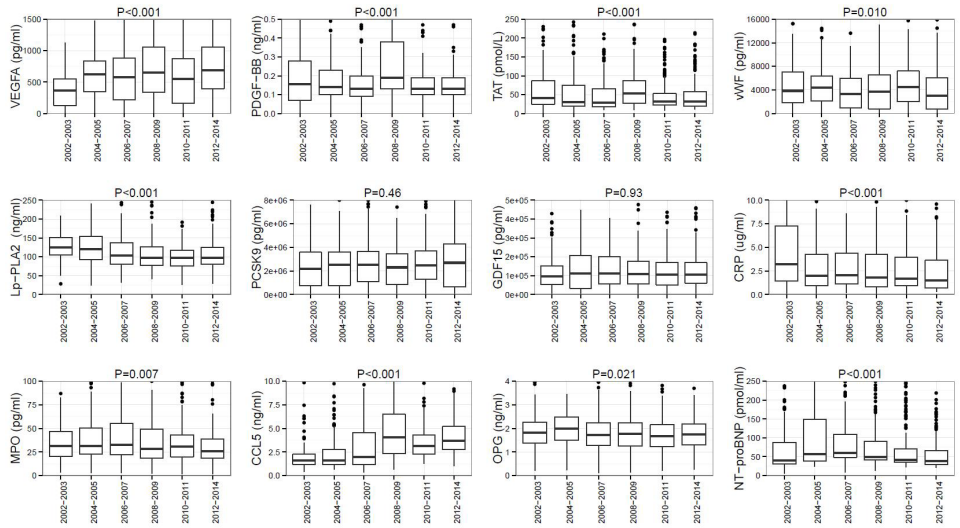


artery from 136 human hearts they showed that the area circumscribed by the internal elastic lamina area directly correlated with the area of the atherosclerotic lesion, thus suggesting that the vessel circumference increased when atherosclerotic disease was present.[13] Outward remodeling does not provide sufficient protection to atherosclerotic lumen narrowing because the amount of remodeling often does not adequately preserve blood flow and furthermore outward remodeling in atherosclerotic vessels can contribute to plaque destabilization due to shear stress and necrotic core formation (figure 4). Controversially, some plaques induce inward remodeling at the site of the atherosclerotic lesion resulting in a further reduction of blood flow to distal tissues.[14] In healthy arteries remodeling occurs as a response to changes in blood flow and circumferential stretch in order to restore wall tension and shear stress. Important effectors in the outward response are nitric oxide and gelatinase matrix metalloproteinases (MMPs), which can inhibit proliferation and promote apoptosis of smooth muscle cells.

Predictive value of plaque markers

A final example of the value of atherosclerotic plaque biobanks is the discovery of lesion characteristics and proteins that have predictive value for outcome. In the search for markers that can be used for risk stratification of patients at risk to suffer from acute events due to atherosclerotic disease, focus should not only be on morphologic or mechanistic traits of the plaque. Proteins that are expressed in one culprit harvested lesion have been hypothesized to contain molecular information that reflects the stability of the entire vascular system. We have executed a study in which we compared the proteome of plaques from patients who underwent carotid surgery that endure MACE in a three year follow-up compared to plaques from patients that remained event-free during a three year follow-up. By comparing protein expression levels in dissected culprit lesions between these two groups we were able to identify multiple proteins that associated with MACE during follow-up and thus could act as plaque biomarkers to predict which patients are at risk for secondary manifestations of cardiovascular disease. Osteopontin is a plaque and

Figure 5. Boxplots with levels of different biomarkers over time. The middle bar within the box represents the median. The lower and upper ends of the box are 25th and 75th percentiles. The lower and upper whisker extend to 1.5 * interquartile range (proportional to the 25th and 75th percentile, respectively). Note that the higher extreme outliers are not completely displayed, as this would impair legibility of the plots. Unadjusted P-values of changes over time (Mann-Whitney U test) are shown above each plot.



serum biomarker that was identified using this approach. Comparing the highest quartile of plaque Osteopontin with the lowest quartile showed a hazard ratio of 3.8 (95% CI, 2.6 –5.9) for MACE in 3-year follow-up.[15] These findings were consistent for MACE in all vascular beds in plaques derived from both the carotid and femoral arteries. This strengthens the hypothesis that local plaque markers can predict future events in different vascular beds and therefore supports the hypothesis that one single plaque can be reflective of systemic changes in the arterial system.

Other identified plaque markers that are associated with increased risk for MACE during follow-up are MIF, FABP-4 and MMP-8.[16] Surprisingly, plaque protein markers did not always associate with local plaque destabilizing features such as lipid content or macrophage presence. Consistent predictive values for outcome of the investigated proteins remained, irrespective of the expression of fibrous or lipid compounds in the plaque. This observation revealed that plaque markers predictive for adverse outcome do not necessarily associate with plaque morphology.

The prognostic value of these plaque markers is therefore independent of known mechanisms that are involved in plaque rupture and subsequent arterial thrombosis. These findings suggested that certain plaque markers are involved in other pathways and behold prognostic value over known mechanisms of arterial thrombosis.

Changes in plaque characteristics over time

A recent conceptual finding that has been observed in human plaque biobanks is the change in plaque characteristics over time. By monitoring a diseased population over time, shifts in disease presentation, progression or disease prognosis can be made. Long term biobanking provides valuable information on temporal changes in disease status. The Athero-Express biobank hosted a large number of human atherosclerotic plaques collected over the last 14 years without major changes in indication for surgery. At present, the biobank comprises over 2300 carotid and 1000 ileofemoral derived atherosclerotic plaques.

One of the major findings within the Athero-Express biobank is that plaque characteristics that are deemed unstable are significantly declining in both the carotid (CEA) and ileofemoral (IFE) patient cohorts. We found a significant reduction in the percentage of atheromatous plaques from 33.2% in 2002-2003 to 14.4% in 2010-2011 (p<0.001), plaque hemorrhages declined from 74.4% in 2002-2003 to 37.6% in 2010-2011 (p<0.001), the percentage of median macrophage staining declined from 0.41% in 2002-2003 to 0.09% in 2010-2011 (p<0.001) and heavy calcifications where only present in 24.0% of patients operated on in 2010-2011 compared to 52.0% of patients operated on in 2002-2003 (p<0.001).[17] This decline was consistent over time and observed in both cohorts. For CEA patients this decline was observed in all patient subgroups presenting with different index events such as stroke, transient ischemic attack, ocular symptoms, and in asymptomatic patients.

Together with stabilization of the atherosclerotic plaque we found improvements in risk factor management and secondary prevention strategies among both CEA and IFE patients. We found more favorable lipid profiles, lower systolic and diastolic blood pressures and an increase in the amount of anti-hypertensive and lipid-drugs used in most recent years.[17] While the retrospective nature of the biobank cannot prove causality, the synergistic effect of plaque stabilization and improved risk factor management does support such an effect. Since these changes occur in all patient subgroups and in both carotid and iliofemoral derived plaques we believe this change is systemic in nature.

The limitation of extrapolation of results obtained from historical biobanks in the current patient domain

In light of current findings that the incidence of primary manifestations of atherosclerotic disease are declining with in addition changes in the underlying pathological substrate we should take into account that traditional risk factors show time-dependent trends as well. In the last decades improvements have been made in drug-therapy, lifestyle adjustments and legislation which resulted in reduced dietary salt intake, public smoking bans and a cutback in the intake of trans-fats. In translation of findings in historical biobanks to clinical practice one could question whether these measurements still hold the same value. Inflammatory biomarkers in present populations, with different atherosclerotic plaque phenotype and inflammatory profiles compared to observations made more than one decade ago, one could argue that biomarker levels may have changed resulting in different cutoff values for patients at high risk. This emphasizes the need for investments in ongoing

biobank activities including repeated validation of biomarkers in order to successfully track and treat evolving diseases. In clinical decision making this can also have consequences for the diagnose-treatment trajectory. Since patients now have more favorable risk factors than patients operated on three decades ago this could result in older patients still eligible for surgery today. One could argue that evolving disease mechanisms demand evolving clinical decision making.

As an example of altering profiles of biomarkers in the last decade, a panel of twelve selected plasma proteins was measured in the Athero-express cohort. All selected proteins have previously been established as relevant biomarkers in CVD or are involved in different pathways of atherogenesis. Markers we measured in our biobank have been associated with angiogenesis (vascular endothelial growth factor A (VEGF-A) and plaletet derived growth factor-BB (PDGF-BB). Endothelial/coagulation markers studied were thrombin-antithrombin complex (TAT) and von Willebrand factor (vWF). Lipid markers were lipoprotein-associated phospholipase A2 (Lp-PLA2) and Proprotein convertase subtilisin/kexin type 9 (PCSK9). Growth differentiation factor 15 (GDF15), high-sensitivity C-reactive protein (hs-CRP), myeloperoxidase (MPO), chemokine ligand 5 (CCL5), and osteoprotegerin (OPG) were measured as markers of inflammation. N-terminal-pro Brain Natriuretic Peptide (NT-proBNP) represents heart function/neurohumoral activity.

In this biomarker study 6 out of 12 biomarkers showed significant temporal changes (figure 5). After correcting for confounding and multiple testing, VEGF-A and CCL5 increased over time while Lp-PLA2, hsCRP, OPG, and NTproBNP significantly decreased over time. This shows that not only the primary pathological substrate of CVD is shifting but also other causal and non-causal markers of CVD-pathways reveal temporal changes in a diseased population. The increase in VEGF-A and CCL5 was unexpected. The role of VEGF-A in atherogenesis is actually still ambiguous.[18] On one hand, it has been associated with plaque progression and destabilization, while on the other hand, VEGF-A gene transfer was shown not to have an effect on clinical events and gene polymorphisms associated with a higher VEGF-A expression were even protective for coronary artery disease.[16,17,22] For CCL5 the current literature on the association with CVD is also conflicting. Some studies suggest that CCL5 is protective for CVD progression in patients with coronary artery disease, while others show that high levels are associated with increased mortality in these patients. [23] [24] In a population-based cohort, there was no relationship of CCL5 serum levels with incident coronary events.[25] Therefore, it is unclear how the observed increases in VEGF-A and CCL5 levels should be interpreted in terms of altered pathophysiology leading to arterial occlusion. Moreover, we cannot explain why only half of the markers significantly changed over time, and others (within the same pathway) did not.

It has to be noted that changes in 6 markers were recognized significant while being quite conservative with a P-value threshold that was adjusted for multiple testing. When using the conventional cut-off of 0.05, two additional proteins would be identified; MPO and GDF15 in men (a decrease of 4.5% and 5.2% per 2 years in time, respectively, both with a P= 0.0059).

This study shows that levels of several biomarkers in patients with manifest atherosclerosis show independent changes over the last decade, implying that not only the main underlying substrate of CVD is changing namely the plaque but also its markers. These findings strengthen our hypothesis that the pathogenesis of CVD is changing with a decline in vulnerable plaque characteristics and its associated lipid and inflammation derived biomarkers.

Circumstantial evidence of plaque stabilization in coronary artery disease

In coronary artery disease (CAD) similar changes are occurring in clinical presentation in patients diagnosed with a myocardial infarction, with a decline in ST-elevated myocardial infarctions (STEMI) and a relative increase in Non-STEMI incidence (NSTEMI).[26] It seems that the underlying effectors in acute coronary syndrome (ACS) are changing in primary prevention (statin) treated societies.

Postmortem coronary plaque studies revealed that vulnerable plaque characteristics with subsequent plaque rupture were significantly associated with STEMI while NSTEMI cases were more often caused by plaque erosions.[5] We believe that this shift in underlying disease presentation can at least partially be explained by stabilization of the atherosclerotic plaque and that the lesion underlying the occlusive thrombus may be erosive.

Future perspectives: genetics

As biobanks may become relevant for the scientific community at time points far beyond the date of inclusion when technology in sample analysis evolves and allows high throughput screening of the genome, transcriptome or proteome new added value can be discovered. The sequencing of the human genome in 2001 is such a milestone that led us to understand more about associations between genetic variants and the occurrence of complex diseases such as clinical manifestations of atherosclerosis. An important example of combining state-of-the-art research and biobanking is the discovery of new target drug therapy modalities such as proprotein convertase subtilisin–kexin type 9 (PCSK9) inhibitors. In this success story investigators discovered that in people with a rare variant in the gene encoding for PCSK9 low levels of LDL-cholesterol and heart disease were found. These first-in-class human antibodies that successfully inactivate PCSK9 received approval for use in patients by the FDA as of August 2015.

In discovering the influence of genetic variation on determinants of atherosclerotic disease understanding atherosclerotic biology is crucial. Genome Wide Association studies (GWAS) are critical in further discovery of future drug-targets but can also help us understand the association of genetic variations with substrates of atherosclerotic disease. In the Athero-Express 1439 patients have been genotyped and investigation of previously identified CAD and large artery stroke (LAS) susceptibility loci are currently associated with plaque characteristics. Combing GWAS result with unique determinants of disease such as atherosclerotic plaque morphology supports the hypothesis that genetic variation can influence atherosclerotic plaque characteristics.

Clinical implications and the importance of the evolving mechanisms of carotid artery stenosis

It seems that in the process of battling against the vulnerable atherosclerotic plaque, major progress has been made. We see a decline in all manifestations of acute atherosclerotic disease that are associated with the vulnerable plaque and subsequent plaque rupture. This triumph, however not proven to be causal, seems to go hand-in-hand with improvements made on a population-based level with more stringent lifestyle adjustments and better and more widespread use of therapeutic drug agents. Without the use of biobanks these underlying changes of stabilization of atherosclerotic plaques would be hard to objectify, are not fully understood and more importantly these changes would quite possibly go unnoticed. The use of well-built and well maintained biobanks facilitates to successfully track, trace and intervene in complex multifactorial diseases.

The battle is far from over, the residual risk of the new or remaining vulnerable plaque still poses a substantial risk for the general population and efforts should continue to be made into understanding the underlying biomechanics of atherosclerotic disease. Furthermore these findings are relevant because hypotheses that are built around the concept of the vulnerable plaque together with biomarkers that are labelled as valuable in CAD were carried out over two decades ago. One could question whether the prognostic value of earlier validated biomarkers and mechanisms still holds in this stabilized, less inflammatory atherosclerotic phenotype.

In conclusion both plaque and plasma biomarkers measured in CEA patients show independent temporal changes over the last decade. These temporal changes show us that the underlying lipid-driven and inflammatory pathogenesis of atherosclerotic disease is changing and our findings suggest that other risk factors such as plaque erosion have become more important.

Future biobanking is required to continue tracking the mechanisms of a complex disease such as atherosclerosis and discover new plaque phenotypes or biomarkers and validate non-invasive imaging tools which will hopefully result in a further reduction of the burden of atherosclerotic disease.

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PART

two

STUDIES ON CLINICAL OUTCOME AFTER CAROTID OR ILIOFEMORAL ENDARTERECTOMY

3

Time-dependent trends in cardiovascular adverse events during follow-up after carotid or iliofemoral endarterectomy

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ABSTRACT

Background

Recent observations have suggested a decline in vulnerable carotid artery and iliofemoral atherosclerotic plaque characteristics over the past decade. The aim of this study was to determine whether, in the presence of clinically manifest carotid or peripheral artery disease, secondary adverse cardiovascular events decreased over this period.

Methods

Patients included in the Athero-Express biobank between 2003 and 2012 were analysed. During 3-year follow-up, composite cardiovascular endpoints were documented yearly, including: myocardial infarction, coronary interventions, stroke, peripheral interventions and cardiovascular death. The major cardiovascular endpoint consisted of myocardial infarction, stroke and cardiovascular death.

Results

Some 1684 patients who underwent carotid endarterectomy (CEA) and another 530 who had iliofemoral endarterectomy (IFE) were analysed. In total, 405 (25.2 per cent) and 236 (45.9 per cent) patients had a composite cardiovascular endpoint within 3 years after CEA and IFE respectively. Corrected for possible confounders, the percentage of patients with a secondary cardiovascular event after CEA did not change over time (hazard ratio (HR) 0.91, 95 per cent c.i. 0.65 to 1.28; $P = 0.590$, for 2011–2012 *versus* 2003–2004). In patients who had IFE, the incidence of secondary cardiovascular events significantly decreased only in the last 2 years (HR 0.62, 0.41 to 0.94; $P = 0.024$), owing to a decrease in peripheral (re) interventions in 2011–2012 (HR 0.59, 0.37 to 0.94; $P = 0.028$). No decrease in major cardiovascular events was observed in either group.

Conclusion

In patients who had undergone either CEA or IFE there was no evidence of a decrease in all secondary cardiovascular events. There were no differences in major cardiovascular events.

INTRODUCTION

In recent years the rate of myocardial infarction and stroke has significantly declined in Western society.[1–3] Improved medical treatment and lifestyle adjustments have contributed to this decline.[4–6] Typically, patients with carotid and/or peripheral atherosclerotic disease suffer from a higher incidence of (secondary) cardiovascular events than the general population, despite best preventive care.[4,7]

The pathophysiological mechanisms that could explain the decline in myocardial infarction and stroke due to cardiovascular disease in the general population may relate to changes in nutritional status, lifestyle changes, cessation of smoking, a steady decline in the percentage of smokers in the Dutch population, and medication use (statins, antihypertensive and antiplatelet therapy).[8,9] Furthermore, several studies have investigated plaque characteristics to determine which vascular lesions are more prone to rupture, one of the underlying mechanisms in stroke and myocardial infarction.[10–18]

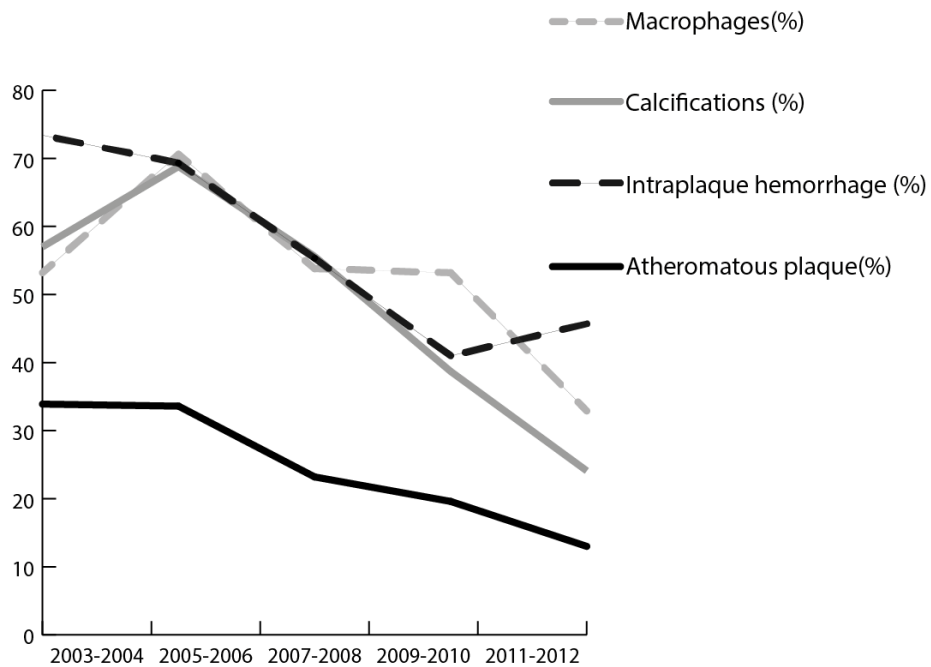
Interestingly, recent studies within the Athero-Express biobank revealed a significant decline in what are considered to be vulnerable plaque characteristics in patients undergoing carotid endarterectomy (CEA) or iliofemoral endarterectomy (IFE) over the past decade (Figures 1 and 2).[19,20] These findings are important in light of the present follow-up study as it has been established previously that patients with plaque characteristics reflecting a measure of instability, such as intraplaque haemorrhage (IPH), have a poorer outcome during 3-year follow-up than patients with plaques that do not contain intraplaque haemorrhage.[15]

With the incidence of primary acute manifestations of cardiovascular diseases decreasing, it is unknown whether the same trend is observed for secondary manifestations in patients who already suffer from established atherosclerotic disease and who mostly receive optimal treatment. Patients with carotid and peripheral artery disease have a high risk of secondary atherosclerotic manifestations in the coronary, cerebral and peripheral circulation. In the Athero-Express biobank patient cohorts, a significant temporal improvement in blood pressure and lipid profile control was observed. Together with the observation that the culprit lesion characteristics are stabilizing over time, the hypothesis of this study was that the incidence of secondary manifestations would decrease over time. To investigate this hypothesis, the incidence of secondary cardiovascular events during follow-up was evaluated in patients who underwent CEA or IFA between 2003 and 2012.

METHODS

The Athero-Express is an ongoing, prospective biobank study, collecting atherosclerotic plaques from patients undergoing either CEA or IFA in two Dutch tertiary referral hospitals: University Medical Centre Utrecht and St Antonius Hospital Nieuwegein.[21] All patients recorded in the Athero-Express biobank between 2003 and 2012 were included for analysis in the present prospective observational cohort study. The year 2012 was chosen as a

Figure 1. Changes in plaque characteristics over time after carotid endarterectomy. Plaques dissected from patients operated on in 2011–2012 show less heavy staining for macrophages than those from patients operated on in earlier years, with fewer heavy calcifications ($P = 0.001$), intraplaque haemorrhages ($P = 0.001$) and large lipid cores ($P < 0.001$).



cut-off point for inclusion, so that 3-year follow-up data could be analysed for every included patient. The whole cohort was divided into 2-year strata based on time of inclusion. Indication for surgery was based on international guidelines for carotid and iliofemoral atherosclerotic disease, and standardized treatment protocols and operative techniques were applied.[22–25] The medical ethics committees in both participating centres approved the study. All patients provided written informed consent.

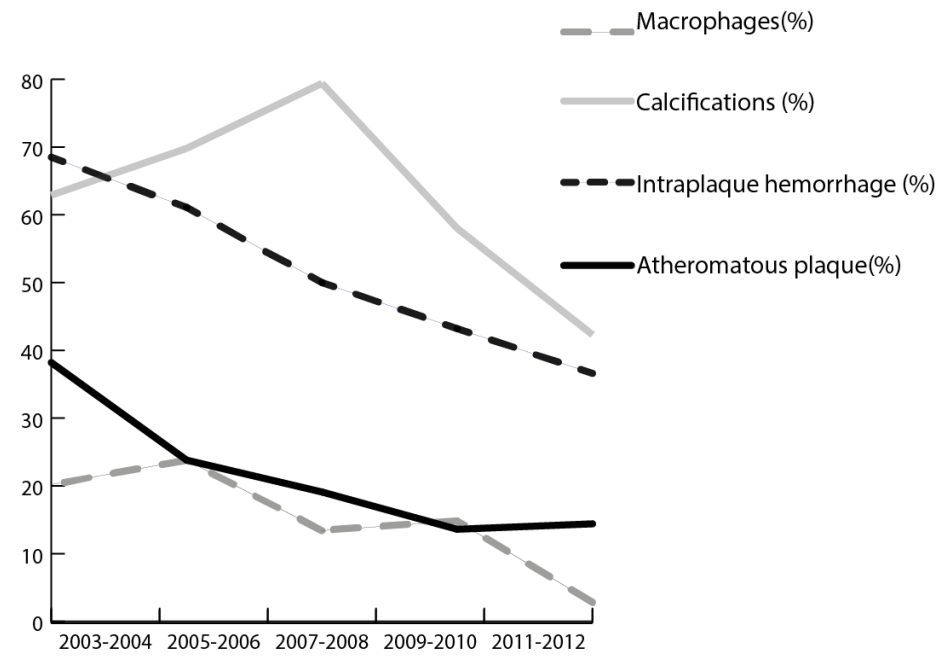
Inclusion and exclusion criteria

All patients who underwent carotid or iliofemoral artery plaque removal and who were included in Athero-Express biobank between 2003 and 2012 were eligible for inclusion. Recent research has shown that restenotic lesions have different plaque characteristics compared with *de novo* lesions.[26,27] Therefore, all patients who had surgery to treat restenotic lesions were excluded.

Follow-up

All patients were followed for 3 years after the initial procedure with a follow-up questionnaire at 1, 2 and 3 years after intervention. If the patient indicated that a cardiovascular event had occurred, this was validated through health records kept by the

Figure 2. Changes in plaque characteristics over time after iliofemoral endarterectomy. Plaques dissected from patients who had surgery in 2011–2012 show less heavy staining for macrophages than those from patients operated on in earlier years, with fewer heavy calcifications ($P = 0.001$), intraplaque haemorrhages ($P < 0.001$) and large lipid cores ($P < 0.001$).



general practitioner. If the questionnaire was not returned, the general practitioner was contacted directly for follow-up information.

Endpoints

A composite cardiovascular endpoint for the outcome analysis of patients or underwent CEA or IFE was used. This consisted of: (sudden) cardiovascular death, stroke, myocardial infarction, coronary intervention (coronary artery bypass grafting or percutaneous coronary intervention), peripheral (re)intervention or leg amputation. For patients who reached multiple endpoints during follow-up, only the first manifestation of a cardiovascular event was used for analysis of the composite endpoint.

Subanalyses

An additional analysis of all major cardiovascular manifestations alone was performed, to emphasize the most clinically relevant endpoints, and to exclude (re)interventions (such as procedures for a restenotic lesion or endarterectomy on another location), which were subject to protocol and guideline changes over time. The major cardiovascular endpoint included myocardial infarctions, stroke and cardiovascular death.

Statistical analysis

Patient characteristics at baseline across the different time cohorts were compared using Pearson's chi-squared test for dichotomous variables and one-way ANOVA for normally distributed continuous variables. The Mann–Whitney *U* test and Kruskal–Wallis test were applied for continuous variables that showed a non-parametric distribution. Possible confounders that were added to the multivariable analyses were based on both empirical evidence and changes occurring in baseline characteristics. For patients who had CEA, the following variables were added to the model: age, sex, kidney function and contralateral stenosis. For those who had IFE, age, sex, type of operation, Fontaine classification, kidney function and diabetes were added. These confounders were added to the multivariable model of operation year strata and secondary cardiovascular outcomes, in which $P < 0.050$ was deemed significant. To avoid the limitation of complete-case analyses, single imputation with R computing platform version 3.0.2 (R Project for Statistical Computing, Vienna, Austria) was performed to calculate missing values. SPSS® version 21.0 (IBM, Armonk, New York, USA) was used for all statistical analyses.

RESULTS

Some 1801 patients undergoing CEA and 802 having IFE were included in the Athero-Express biobank between 2003 and 2012, of whom 1684 and 530 respectively were eligible for the present analysis. Seventy-four patients who had CEA (4.4 per cent) and 16 who underwent IFE (3.0 per cent) were lost to follow-up (Figure S1, supporting information). Baseline characteristics of patients undergoing CEA or IFE included in this study represent the typical vascular patient, with a high prevalence of diabetes (23.0 and 31.3 per cent for CEA and IFE groups respectively), hypertension (70.8 and 70.2 per cent) and hypercholesterolaemia (60.8 and 60.8 per cent). A substantial proportion had a history of cardiovascular disease manifestations, such as coronary artery disease, myocardial infarction, stroke or peripheral intervention (Tables 1 and 2).

Patient characteristics over time

The time to CEA surgery after the index event decreased from a median of 92 (range 34–145) days in 2003–2004 to 16 (9–30) days in 2011–2012. In most recent years, fewer asymptomatic patients had surgery than in earlier years: 9.4 per cent of patients in 2011–2012 compared with 16.2 per cent in 2003–2004. The percentage of symptomatic patients presenting with a stroke increased from 27.8 per cent in 2003–2004 to 34.7 per cent in 2011–2012, and those with ocular symptoms from 12.2 to 19.3 per cent, respectively. The number of patients presenting with a transient ischaemic attack remained stable throughout the years. The percentage of patients with a contralateral stenosis, an important predictor of cardiovascular events during follow-up[28], did not change over time.

In patients having IFE, the Fontaine class was worse in recent years (23.5 per cent Fontaine IV in 2011–2012 *versus* 16 per cent in 2003–2004). Conversely, the stenosis grade of the

Table 1. Baseline characteristics of patients undergoing carotid endarterectomy in 2-year cohorts.

	2003–2004 (*n* = 345)	2005–2006 (*n* = 382)	2007–2008 (*n* = 294)	2009–2010 (*n* = 326)	2011–2012 (*n* = 337)	*P*‡
Age (years)	68 (60–73)	70 (63–76)	71 (64–76)	70 (62–78)	71 (65–78)	< 0.001§
Men	242 (70.1)	259 (67.8)	198 (67.3)	218 (66.9)	234 (69.4)	0.875
BMI (kg/m²)*	26.1 (24.0–28.4)	25.9 (23.9–28.3)	25.8 (24.1–28.4)	26.0 (23.9–28.7)	25.7 (23.7–28.2)	0.998§
Current smoker†	142 (41.9)	121 (32.4)	84 (29.9)	108 (33.5)	109 (32.9)	0.017
Diabetes	76 (22.0)	91 (23.8)	60 (20.4)	82 (25.2)	78 (23.1)	0.680
Hypertension†	241 (70.5)	295 (77.8)	196 (73.1)	232 (73.4)	228 (70,2)	0.137
Hypercholesterolaemia†	212 (61.8)	250 (66.1)	162 (67.5)	192 (69.6)	208 (69.8)	0.190
History of CAD†	109 (31.6)	108 (28.3)	86 (29.5)	94 (28.8)	11 (32.9)	0.644
History of PAD†	69 (20.0)	77 (20.2)	49 (16.8)	62 (19.0)	71 (21.1)	0.712
Contralateral stenosis (%)†						0.265
0–49	193 (56.8)	205 (55.0)	136 (56.2)	142 (52.4)	133 (48.5)	
50–99	87 (25.6)	116 (31.1)	65 (26.9)	91 (33.6)	99 (36.1)	
100 (occlusion)	60 (17.6)	52 (13.9)	41 (16.9)	38 (14.0)	42 (15.3)	
Symptoms†						< 0.001
Asymptomatic	56 (16.2)	51 (13.4)	38 (12.9)	32 (9.9)	31 (9.4)	
Ocular	42 (12.2)	52 (13.6)	53 (18.0)	39 (12.0)	64 (19.3)	
TIA	151 (43.8)	194 (50.8)	131 (44.6)	150 (46.3)	121 (36.6)	
Stroke	96 (27.8)	85 (22.3)	72 (24.5)	103 (31.8)	115 (34.7)	
Time between last event and operation (days)*†	92 (34–145)	52 (21–95)	35 (16–63)	25 (13–48)	16 (9–30)	< 0.001§
eGFR (ml per min per 1.73 m²)*	71 (59–81)	72 (57–88)	74 (58–88)	71 (58 –85)	74 (61–88)	0.062§
Systolic BP (mmHg)*†	155 (140–173)	152 (135–170)	154 (140–173)	150 (135–173)	149 (134–166)	0.003§
Diastolic BP (mmHg)*†	85 (75–90)	80 (74–90)	81 (75–90)	80 (70–90)	80 (70–86)	< 0.001§
Triglycerides (mg/dl)*†	1.6 (1.2–2.3)	1.2 (0.9–1.7)	1.3 (1.0–1.8)	1.4 (1.0–2.0)	1.4 (1.0–2.0)	< 0.001§
Total cholesterol (mg/dl)*†	4.9 (4.1–5.6)	4.4 (3.7–5.3)	4.3 (3.6–5.2)	4.4 (3.7–5.3)	4.8 (3.7–5.8)	< 0.001§
HDL (mg/dl)*†	1.1 (1.0–1.4)	1.2 (1.0–1.4)	1.1 (0.9–1.3)	1.1 (0.9–1.4)	1.2 (1.0–1.4)	< 0.001§
LDL (mg/dl)*†	2.8 (2.2–3.5)	2.6 (2.0–3.3)	2.5 (1.9–3.3)	2.6 (2.0–3.3)	2.8 (2.0–3.8)	0.001§
Statin use†	235 (68.7)	295 (77.2)	226 (76.9)	261 (80.1)	271 (80.4)	0.001
Antiplatelet use†	311 (90.9)	342 (89.5)	254 (86.4)	291 (89.3)	290 (86.6)	0.276
Anticoagulant use†	45 (13.2)	42 (11.0)	39 (13.3)	31 (9.5)	35 (10.4)	0.461
Dual antiplatelet use†	173 (50.6)	134 (35.1)	172 (58.5)	210 (64.4)	224 (66.9)	< 0.001
RAAS medication use†	161 (47.1)	203 (53.1)	145(49.3)	168 (51.5)	178 (52.8)	0.461
Beta-blocker use†	154 (45.0)	159 (41.6)	134 (45.6)	145 (44.5)	133 (39.5)	0.454
Oral glucose inhibitor use†	53 (15.5)	62 (16.2)	45 (15.3)	54 (16.6)	58 (17.2)	0.965
Insulin use†	20 (5.8)	30 (7.9)	9 (3.1)	25 (7.7)	24 (7.1)	0.091

Values in parentheses are percentages unless indicated otherwise; values are median (i.q.r.). †Some data were missing for these variables. CAD, coronary artery disease; PAD, peripheral artery disease; TIA, transient ischaemic attack; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; RAAS, renin–angiotensin–aldosterone system. ‡Pearson _² test, except §one-way ANOVA and ¶Kruskal–Wallis test.

Table 2 Baseline characteristics of patients undergoing iliofemoral endarterectomy in 2-year cohorts

	2003–2004 (*n* = 93)	**2005–2006** (*n* = 128)	**2007–2008** (*n* = 73)	**2009–2010** (*n* = 104)	**2011–2012** (*n* = 132)	***P*‡**
Age (years)*	67 (60–74)	68 (59–75)	68 (62–75)	69 (63–74)	70 (64–75)	0.269§
Men	67 (72)	100 (78.1)	51 (70)	67 (64.4)	94 (71.2)	0.245
BMI (kg/m²)*†	26 (23–29)	26 (24–28)	26 (23–29)	25 (23–28)	27 (23–28)	0.925§
Current smoker†	45 (48)	56 (44.4)	25 (36)	46 (45.1)	53 (40.5)	0.322
Diabetes†	26 (28)	44 (34.4)	24 (33)	32 (31.1)	40 (30.3)	0.968
Hypertension†	66 (72)	76 (59.4)	51 (72)	78 (77.2)	101 (79.5)	0.006
Hypercholesterolaemia†	63 (68)	78 (61.4)	47 (72)	52 (65.0)	82 (70.7)	0.455
History of CAD†	35 (38)	52 (40.6)	30 (41)	33 (32.0)	56 (42.4)	0.885
History of stroke	4 (4)	10 (7.8)	2 (3)	6 (5.8)	3 (2.3)	0.059
History of peripheral intervention†	40 (43)	51 (39.8)	29 (40)	44 (42.7)	53 (40.2)	0.868
History of amputation†	3 (3)	7 (5.5)	1 (1)	2 (2.3)	4 (3.1)	0.495
Fontaine class						0.044
IIb	60 (65)	78 (60.9)	42 (58)	58 (55.8)	66 (50.0)	
III	18 (19)	27 (21.1)	16 (22)	31 (29.8)	35 (26.5)	
IV	15 (16)	23 (18.0)	15 (21)	15 (14.4)	31 (23.5)	
Stenosis grade (%)						< 0.001
0–49	2 (2)	12 (9.4)	4 (5)	2 (1.9)	33 (25.0)	
50–99	28 (30)	31 (24.2)	23 (32)	31 (29.8)	34 (25.8)	
100 (occlusion)	63 (68)	85 (66.4)	46 (63)	71 (68.3)	65 (49.2)	
Contralateral stenosis 50–100%	58 (62)	82 (64.1)	48 (66)	71 (68.3)	77 (58.3)	0.598
Operated artery						0.001
Femoral	80 (86)	110 (85.9)	67 (92)	98 (94.2)	126 (95.5)	
Iliac	13 (14)	18 (14.1)	6 (8)	6 (5.8)	6 (4.5)	
Operation type						< 0.001
REA	44 (47)	47 (36.7)	19 (26)	19 (18.3)	20 (15.2)	
TEA	49 (53)	81 (63.3)	54 (74)	85 (81.7)	112 (84.9)	
eGFR (ml per min per 1.73 m²)*†	74 (54–91)	78 (59–103)	84 (60–107)	73 (59–96)	78 (61–107)	0.310§
Systolic BP (mmHg)*†	150 (140–170)	144 (130–163)	145 (130–167)	145 (130–154)	147 (132–167)	0.137§
Diastolic BP (mmHg)*†	80 (75–90)	80 (70–85)	80 (71–87)	76 (70–85)	76 (69–85)	0.052§
Triglycerides (mg/dl)*†	1.8 (1.2–3.0)	1.6 (1.0–2.4)	1.8 (1.0–2.5)	1.7 (1.0–2.2)	2.0 (1.4–3.2)	0.044§
Total cholesterol (mg/dl)*†	5.1 (4.3–5.7)	4.5 (3.9–5.2)	4.8 (3.8–5.5)	4.8 (4.0–5.5)	4.8 (4.0–5.6)	0.023§
HDL (mg/dl)*†	1.2 (0.9–1.5)	1.2 (0.9–1.4)	1.2 (0.9–1.3)	1.2 (1.0–1.5)	1.1 (0.9–1.3)	0.198§
LDL (mg/dl)*†	2.9 (2.1–3.5)	2.5 (1.8–3.1)	2.7 (1.9–3.3)	2.6 (2.1–3.2)	2.6 (1.9–3.2)	0.083§
Statin use†	59 (63)	90 (70.3)	59 (81)	72 (69.9)	105 (79.5)	0.018
Antiplatelet use†	79 (85)	101 (78.9)	57 (78)	95 (93.1)	113 (85.6)	0.137
Anticoagulant use†	17 (18)	30 (23.4)	16 (22)	7 (6.8)	13 (9.8)	0.001
Dual antiplatelet use†	30 (32)	11 (8.6)	11 (15)	18 (17.6)	21 (15.9)	0.115
RAAS medication use†	56 (60)	76 (59.4)	51 (70)	61 (59.2)	79 (59.8)	0.934
Beta-blocker use†	37 (40)	60 (46.9)	31 (42)	37 (35.9)	67 (50.8)	0.409
Oral glucose inhibitor use†	18 (19)	29 (22.7)	13 (18)	24 (23.3)	33 (25.0)	0.341
Insulin use†	12 (13)	14 (10.9)	8 (11)	8 (7.8)	12 (9.1)	0.262

Values in parentheses are percentages unless indicated otherwise; values are median (i.q.r.). †Some data were missing for these variables. CAD, coronary artery disease; REA, remote endarterectomy; TEA, thromboendarterectomy; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; RAAS, renin–angiotensin–aldosterone system. ! ‡2 test, except §one-way ANOVA.

Table 3. Clinical outcomes within 3 years after carotid endarterectomy in 2-year cohorts

	2003–2004 (*n* = 333)	**2005–2006** (*n* = 357)	**2007–2008** (*n* = 283)	**2009–2010** (*n* = 317)	**2011–2012** (*n* = 320	**Total cohort** (*n* = 1610)
Composite* endpoint	90 (27.0)	100 (28.0)	63 (22.3)	82 (25.9)	70 (21.9)	405 (25.2)
Major endpoint†	44 (13.2)	59 (16.5)	34 (12.0)	33 (10.4)	39 (12.2)	209 (13.0)
Death from any cause	28 (8.4)	41 (11.5)	40 (14.1)	37 (11.7)	38 (11.9)	184 (11.4)
Cardiovascular death	19 (5.7)	21 (5.9)	15 (5.3)	13 (4.1)	10 (3.1)	78 (4.8)
CAD	27 (8.1)	29 (8.1)	15 (5.3)	18 (5.7)	20 (6.3)	109 (6.8)
Myocardial infarction	12 (3.6)	22 (6.2)	10 (3.5)	9 (2.8)	13 (4.1)	66 (4.1)
Coronary intervention	21 (6.3)	15 (4.2)	9 (3.2)	14 (4.4)	16 (5.0)	75 (4.7)
Stroke	26 (7.8)	36 (10.1)	17 (6.0)	18 (5.7)	20 (6.3)	117 (7.3)
Peripheral intervention	42 (12.6)	42 (11.8)	33 (11.7)	51 (16.0)	31 (9.7)	199 (12.4)

Values in parentheses are percentages. *Cardiovascular death, stroke, myocardial infarction, coronary interventions and peripheral interventions. †All cardiovascular death, and all cerebral and myocardial infarctions. CAD, coronary artery disease.

culprit lesion decreased in the same interval, especially in the last 2-year cohort. In more recent years, surgical procedures were increasingly performed in the femoral arteries instead of the iliac arteries, and thromboendarterectomy (TEA) was more often undertaken instead of remote endarterectomy (REA) (TEA from 53 to 84.9 per cent, and REA from 47 to 15.2 per cent, in 2003–2004 and 2011–2012 respectively).

Measures of established risk factors and medication use over time

The mean age of patients undergoing CEA increased from 68 years (2003–2004) to 71 years (2011–2012). A statistically significant decrease in both systolic and diastolic BP was observed in the CEA population. Prescription of statins increased in both CEA and IFE groups. Patients undergoing IFE presented with an increased prevalence of hypertension, but a decrease in measured systolic and diastolic BP.

Secondary cardiovascular outcome

In total, 405 (25.2 per cent) and 236 (45.9 per cent) patients had a composite cardiovascular endpoint within 3 years after CEA and IFE respectively. Of all patients, 209 (13.0 per cent) in the CEA group and 49 (9.5 per cent) in the IFE group had a major cardiovascular event during follow-up. In total, 78 (4.8 per cent) and 33 (6.4 per cent) died from cardiovascular causes within 3 years after CEA and IFE respectively (Tables 3 and 4).

Secondary cardiovascular outcome over time

Corrected for possible confounders, the percentage of patients with a secondary cardiovascular event during follow-up after CEA did not significantly decrease over time (hazard ratio (HR) 0.91, 95 per cent c.i. 0.65 to 1.28; $P = 0.590$) (Table S1, supporting information). Secondary cardiovascular events within 30 days of CEA occurred in 89 patients (5.5 per cent), including a cardiovascular death rate of 1.0 per cent and 3.9 per cent stroke risk for the whole cohort. No major differences in 30-day events over time were observed (Table 5).

Table 4. Clinical outcomes after iliofemoral endarterectomy in 2-year cohorts.

	Within 3 years						
	2003–2004 (*n* = 92)	**2005–2006** (*n* = 125)	**2007–2008** (*n* = 71)	**2009–2010** (*n* = 99)	**2011–2012** (*n* = 127)	**Total cohort** (*n* = 514)	**Total cohort by 30 days**
Composite* endpoint	50 (54)	58 (46.4)	34 (48)	48 (48)	46 (36.2)	236 (45.9)	11 (2.1)
Major endpoint†	9 (10)	7 (5.6)	7 (10)	13 (13)	13 (10.2)	49 (9.5)	6 (1.2)
Death from any cause	13 (14)	23 (18.4)	14 (20)	17 (17)	13 (10.2)	80 (15.6)	4 (0.8)
Cardiovascular death	7 (8)	6 (4.8)	4 (6)	8 (8)	8 (6.3)	33 (6.4)	4 (0.8)
CAD	7 (8)	6 (4.8)	9 (13)	7 (7)	11 (8.7)	40 (7.8)	3 (0.6)
Myocardial infarction	1 (1)	2 (1.6)	4 (6)	4 (4)	4 (3.1)	15 (2.9)	3 (0.6)
Coronary intervention	7 (8)	4 (3.2)	8 (11)	4 (4)	8 (6.3)	31 (6.0)	1 (0.2)
Stroke	3 (3)	0 (0)	1 (1)	3 (3)	2 (1.6)	9 (1.8)	0 (0)
Peripheral events							
Amputation	4 (4)	5 (4.0)	7 (10)	7 (7)	5 (3.9)	28 (5.4)	2 (0.4)
PTA or TEA	41 (45)	49 (39.2)	24 (34)	36 (36)	33 (26.0)	183 (35.6)	4 (0.8)

Values in parentheses are percentages. *Cardiovascular death, stroke, myocardial infarction, coronary interventions and peripheral interventions. †All cardiovascular death, and all cerebral and myocardial infarctions. CAD, coronary artery disease; PTA, percutaneous transluminal angioplasty; TEA, thromboendarterectomy.

Table 5. Thirty-day clinical outcomes after carotid endarterectomy in 2-year cohorts.

	2003–2004 (*n* = 333)	**2005–2006** (*n* = 357)	**2007–2008** (*n* = 283)	**2009–2010** (*n* = 317)	**2011–2012** (*n* = 320)	**Total cohort** (*n* = 1610)
Composite* endpoint	19 (5.7)	26 (7.3)	12 (4.2)	13 (4.1)	19 (5.9)	89 (5.5)
Major endpoint†	17 (5.1)	24 (6.7)	11 (3.9)	10 (3.2)	16 (5.0)	78 (4.8)
Cardiovascular death	3 (0.9)	3 (0.8)	3 (1.1)	4 (1.3)	3 (0.9)	16 (1.0)
CAD	3 (0.9)	6 (1.7)	4 (1.4)	3 (0.9)	5 (1.6)	21 (1.3)
Stroke	16 (4.8)	19 (5.3)	8 (2.8)	7 (2.2)	13 (4.1)	63 (3.9)
myocardial infarction	1 (0.3)	6 (1.7)	3 (1.1)	2 (0.6)	4 (1.3)	16 (1.0)
Coronary intervention	2 (0.6)	3 (0.8)	1 (0.4)	2 (0.6)	2 (0.6)	10 (0.6)

Values in parentheses are percentages. *Cardiovascular death, stroke, myocardial infarction, coronary interventions and peripheral interventions. †All cardiovascular deaths, and all cerebral and myocardial infarctions. CAD, coronary artery disease.

The percentage with a composite endpoint after IFE remained stable until 2010, but in the last 2-year cohort there was a decline, to 36.2 per cent in 2011–2012 from 54 per cent in 2003–2004 (HR 0.62, 0.41 to 0.94; $P = 0.024$) (Table S2, supporting information). This decrease in rate of composite endpoints was mainly explained by fewer peripheral (re) interventions in 2011–2012 compared with 2003–2004 (26.0 *versus* 45 per cent; HR 0.59, 0.37 to 0.94; $P = 0.028$).

Subgroup analyses: major cardiovascular endpoint

Some 209 patients (13.0 per cent) in the CEA group and 49 (9.5 per cent) in the IFE group had a major cardiovascular event during follow-up (Tables 3 and 4). Correcting for possible

confounders, this did not decline significantly between 2003–2004 and 2011–2012 in patients who underwent CEA (HR 0.95, 95 per cent c.i. 0.59 to 1.52; $P = 0.833$) (Table S1, supporting information). The number of events was too small in the IFE population to determine changes over the 2-year strata statistically, but the percentage of patients reaching a major cardiac endpoint did not change over time (Table 4).

DISCUSSION

This study revealed no time-dependent decrease in secondary cardiovascular events over the past decade during 3-year follow-up of patients undergoing CEA. In patients undergoing IFE, only a recent decrease in secondary cardiovascular events was observed, mainly as a result of a decline in peripheral reinterventions in 2011 and 2012. Of greater clinical importance was the observation that no changes in the amount of major secondary cardiovascular events occurred in either group.

Several studies have investigated population-wide trends in atherosclerotic disease and its variety of manifestations over time.[1,2,6] Most pointed towards a decrease in major primary manifestations of atherosclerotic disease, such as myocardial infarction and stroke, although this has not yet resulted in a decline in the total disease burden from cardiovascular disease.[3,7] It has been suggested that the decrease in major cardiovascular events may have resulted from improved medical therapy in patients with known cardiovascular disease.[2,5,6] Yet, no decrease in the incidence of major secondary cardiovascular events following CEA or IFE for atherosclerotic disease was observed here.

Among patients who underwent CEA more recently there were a lower percentage of smokers, more favourable lipid profiles, and lower diastolic and systolic BPs than among those treated in the early years of the study. In addition, earlier research showed a decrease in plaque characteristics associated with a vulnerable atherosclerotic plaque, such as intraplaque haemorrhage, large lipid core and inflammation.[19] Surprisingly, these temporal changes in plaque characteristics and cardiovascular risk profiles did not coincide with a decrease in secondary cardiovascular events during 3-year follow-up in the present population.

Among patients who had IFE, the observed decrease in composite secondary cardiovascular events in the 2011–2012 cohort was mainly due to a decrease in peripheral reinterventions during follow-up and not to a decrease in major secondary cardiovascular events, which remained stable over time. After correcting for possible confounders, this decrease in peripheral reinterventions was observed only in the last 2-year cohort. A possible explanation could be that collagen-rich plaques were observed less often in recent years. A recent publication reported a significant decline in collagen-rich plaques over time. As high collagen content is associated with restenosis after endarterectomy, this could explain the decrease in peripheral interventions in the last 2 years (from 36 per cent in 2009–2010 to 26.0 per cent in 2011–2012).[29] Another explanation for the decrease in peripheral reinterventions could be that guidelines for IFE treatment have changed over the course of this study. This

could have resulted in altered indications for surgical treatment, or improved postprocedural care, such as improved medical treatment or structured exercise therapy.[30] It was not possible to test for these effects in the present study. In further analyses, no changes in the incidence of other possible markers of more advanced atherosclerotic disease, such as ankle : brachial pressure index, duration of symptoms or history of atherosclerotic disease in other vascular beds, were found over time among patients undergoing IFE.

In patients with established manifest atherosclerotic disease, it is not fully understood what risk determinants explain the occurrence of subsequent cardiovascular events. A possible explanation for the lack of decline in major secondary cardiovascular events over time could be that, with current optimal preventive care, the average patient at cardiovascular risk less often develops symptomatic atherosclerotic plaques considered for surgery. This indicates that over time there has been natural selection of patients least responsive to current medical treatment. In patients who do develop symptoms despite best preventive care, the risk of secondary cardiovascular events may be less likely to change. Still, major changes in the underlying pathological substrate of the dissected culprit lesions have been observed.[19,20] The changes in atherosclerotic plaque characteristics that underlie symptomatic disease could thus point to the selection of a poorly understood patient group with a different risk profile, and for whom different risk prevention and treatment strategies may have to be considered.[18,31]

The number of patients in the general population developing major manifestations of atherosclerotic disease is becoming smaller, perhaps as a result of improving best medical therapy.[1,2,7] This may mean that the remaining symptomatic patients could suffer from a different type of vulnerable plaque, with characteristics such as erosions on the plaque surface, which in turn could cause acute thromboembolic events during follow-up.[11,32-35] These patients with altered plaque characteristics and a different cardiovascular risk profile pose both challenges and possibilities for future diagnostic and therapeutic developments, and a focus for future research.

This study has some limitations. Over time, treatment options and operative procedures have improved, and in-hospital treatment protocols have changed, which could have influenced the results. Patients now receive CEA rapidly after the index event.[36] Preventive care in patients with peripheral artery disease has improved with supervised exercise therapy and more stringent lifestyle adjustments.[30] Unfortunately, these treatment modalities could not be assessed in either cohort in the present study. All measures taken in recent years to improve preventive care could have resulted in selection bias, in which patients operated most recently – despite better preventive care – still developed atherosclerotic lesions that required surgical intervention. Therefore, patients treated in most recent years could have had a more severe form of atherosclerosis with more widespread disease. It is also possible that the single culprit plaque lesion characteristics may not necessarily reflect similar changes in the total vascular system. Owing to the division of the total cohort into 2-year time frames, the total number of patients included per interval was smaller than the substantial size of the total cohort for both procedures. This may have resulted in a type II error. Only the first cardiovascular event in each patient

was used in the analyses. This approach was chosen with a focus on the percentage of patients affected by a secondary cardiovascular event, rather than the total number of events in each cohort. This led to an anticipated underestimation of the total disease burden in both cohorts. However, this underestimation is only relevant to the group of patients who experienced multiple secondary cardiovascular events during follow-up.

In two separate cohorts of patients undergoing CEA or IFE, no consistent decrease in secondary cardiovascular events was observed despite improved measures of risk factors and stabilized atherosclerotic plaque characteristics. In particular, no differences in clinically important major cardiovascular events were observed.

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SUPPLEMENTAL MATERIAL

Supplemental Figure 1. Study flow chart. CEA, carotid endarterectomy; IFE, iliofemoral endarterectomy; FU, follow-up

Supplemental Table S1. Multivariable analysis of influence of operative cohort on risk of composite endpoint and major endpoint after carotid endarterectomy

Supplemental Table S2. Multivariable analysis of influence of operative cohort on risk of composite endpoint after iliofemoral endarterectomy

Supplemental material is omitted because of space limitations

4

Overtreatment or Undertreatment of Carotid Disease: A Transatlantic Comparison of Carotid Endarterectomy Patient Cohorts

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ABSTRACT

Background
Proportionally, more asymptomatic patients undergo carotid endarterectomy (CEA) in the United States of America (USA) than in the Netherlands. However, how this impacts patient characteristics and their outcomes remains unknown.

Objective
We compared baseline characteristics and perioperative outcomes for patients undergoing CEA between American and Dutch patient cohorts, with 30-day MACE (death, stroke, MI) as our primary endpoint.

Methods
We identified all CEAs in the American Targeted-Vascular National Surgical Quality Improvement Program (NSQIP) registry and the Dutch Athero-Express (AE) registry (2003-2015) and stratified patients on preprocedural symptom status.

Results
We included 20,017 CEA patients; 17,699 US; of whom 10,092 (57%) were asymptomatic. Of the 2,318 Dutch patients, 311 (13%) were asymptomatic. Notably, compared to American patients, Dutch patients more often had contralateral carotid occlusion (CCO) (asymptomatic: 32% vs. 5.2%; symptomatic: 13% vs. 4.2%) and underwent concomitant cardiac surgery (3.0% vs. 0.2%). Thirty-day MACE was comparable between Dutch and American patients, for asymptomatic (3.7% vs. 3.0, P=.5), and symptomatic patients (4.9% vs. 4.4%, P=.5). However, Dutch asymptomatic patient experienced higher 30-day strokes rates than American asymptomatic patients (3.4% vs. 1.3%, P<.01).

Conclusion
Dutch asymptomatic CEA patients have a higher prevalence of procedural risk factors, illustrative of the differential selection of asymptomatic patients for CEA in the Netherlands and the USA. Dutch asymptomatic patients experienced higher 30-day stroke rates after CEA than American asymptomatic patients, whereas outcomes between American and Dutch symptomatic patients were comparable.

INTRODUCTION

Outcomes after both carotid revascularization and medical therapy in randomized controlled trials (RCTs) have improved over time,[2,3] however their generalizability to general practice has been questioned, further fueling the ongoing debate on the treatment of carotid stenosis. The resulting disparity is most apparent in the large international differences in the proportion of asymptomatic patients selected for carotid endarterectomy (CEA).[4] For example, in 2016, asymptomatic patients comprised a mere 2.8% of all patients undergoing revascularization in the Netherlands, compared to 66% in the United States of America (USA)[5,6] This difference in the selection of patients for revascularization is clearly visible in the annual number of CEA procedures performed per 100,000 population, which was 32.3 for the US, compared to 13.0 for the Netherlands.[5,7] Yet interestingly, in 2009, health care visits related to stroke per 100,000 population were comparable, with 31.7 for the USA,[8] and 28.4 for the Netherlands (Dutch Central Bureau for Statistics: http://statline.cbs.nl). However, despite this clear difference in the proportion of asymptomatic patients between the USA and Netherlands, it is unknown how this impacts the type of patients selected for revascularization or their outcomes. Thus, we aimed to compare baseline patient characteristics and perioperative outcomes for patients undergoing CEA between an American and a Dutch database.

METHODS

Registries
For this transatlantic comparison, we used the Targeted-Vascular module of the American College of Surgeons (ACS) National Surgical Quality Improvement Program (NSQIP) registry and the Dutch Athero-Express biobank study registry (AE).
The Vascular-Targeted NSQIP is a prospective database collecting data on over 270 predefined variables including baseline patient characteristics, intraoperative disease-specific variables, and outcomes up to 30 days. The validity of data entry has been published before (https://www.facs.org/quality-programs/acs-nsqip). In 2015, 89 hospitals contributed data on 4,258 CEA procedures to the Targeted NSQIP, which is roughly 5% of all CEAs performed in the USA annually.[7]
The AE registry is world's largest carotid plaque biobank, which prospectively collects plaque material, baseline characteristics, and outcomes up to 3-years from two large Dutch vascular referral centers, one academic and one community. These two centers combined collect data on >10% of all CEAs performed in the Netherlands annually.[5]

Patients
We included all CEA patients in the Targeted NSQIP from 2011 to 2015 (N = 18,045) and from 2003 to 2015 in the AE (N = 2318). From both cohorts we excluded patients with an unknown preoperative symptom status (NSQIP: N = 346, 1.9%, AE: N = 22, 0.9%).
The resulting sample included 20,017 patients, with 17,699 USA and 2,318 Dutch patients.

Variables

Thirty-day major adverse cardiovascular events (MACE) was our primary endpoint, and included any stroke, myocardial infarction (MI), or death, for both cohorts. Secondary endpoints were the individual components of our primary endpoint.

Stroke was defined as any new acute ipsilateral neurological dysfunction lasting > 24 hours, or a postoperative radiologic finding indicative of new cerebral infarction. MI included the documentation of one or more of the following ECG changes: >1mm ST elevation, Q-wave in >2 leads, or left bundle branch block, or was diagnosed in patients with a threefold elevation of troponin levels. All variables were non-modifiable, as these were predefined by the NSQIP and AE registries prior to data collection and can be found in the NSQIP online user guide, www.facs.org/quality-programs/acs-nsqip/program-specifics/participant-use and previously published papers.[9]

Analysis

After stratification on symptom status, we compared American to Dutch CEA patients. We presented categorical variables as counts and percentages and continuous variables as mean ± standard deviation (SD), or median and interquartile range (IQR) and used Chi-square, Fisher's exact, Student's T-test, or Mann-Whitney U-test where appropriate.

To quantify how the American selection of asymptomatic patients would affect the Dutch patient cohort we extrapolated the American proportion of symptomatic vs. asymptomatic patients to the Dutch patient cohort, thereby increasing the number of Dutch asymptomatic patients while keeping the Dutch symptomatic patients constant.

Due to the inherently small sample of Dutch asymptomatic patients, we were unable to perform adjusted analyses. We performed all analyses with SPSS version 22.0 statistical software (IBM, Chicago, USA).

RESULTS

We included 17,699 American patients, of whom 10,092 (57%) were asymptomatic. Of the 2318 Dutch patients, 311 (13%) were asymptomatic. In general, regardless of symptom status, American patients, compared to Dutch patients, were more often over 80 years old (asymptomatic: 18% vs. 6.1%, symptomatic: 21% vs. 16%, Table 1), female (asymptomatic: 40% vs. 25%, symptomatic: 37% vs. 32%), obese, anemic, more commonly had diabetes mellitus (DM) and renal insufficiency (i.e. eGFR < 60 ml/min), and more likely presented with an ipsilateral stroke (45% vs. 32%) among symptomatic patients. However, American patients were less often smokers, were less likely to receive patch closure, and less commonly had chronic obstructive pulmonary disease (COPD) or contralateral carotid occlusion (CCO), especially pronounced among asymptomatic patients, with a CCO prevalence of 4.9% in American vs. 32% in Dutch patients. Additionally, American asymptomatic patients less often underwent concomitant cardiac surgery.

Table 1. Differences in 30-day outcomes after carotid endarterectomy between American and Dutch patients.

	USA		Netherlands		USA		Netherlands	
	Asymptomatic				Symptomatic			
	N	%	N	%	N	%	N	%
N	10092	57	311	13	7607	43	2007	87
Age Years (Means, SD)	71.2	8.7	68	10	70.7	9.9	70	8.9
Age ≥ 80 years	1844	18	19	6.1	1567	21	314	16
Women	4035	40	79	25	2800	37	648	32
Preprocedural neurological symptom								
Ipsilateral Amauroris fugax	-	-	-	-	1305	17	378	19
Ipsilateral TIA	-	-	-	-	2875	38	985	49
Ipsilateral stroke	-	-	-	-	3427	45	644	32
Smoker	2565	25	93	30	2176	29	677	35
Obese (BMI>30)	3444	34	47	16	2472	34	293	16
Diabetes mellitus (DM)	3166	31	69	22	2153	28	474	24
Insulin dependent DM	1142	11	23	7.5	868	11	141	7.0
Hypertension	8698	86	232	77	6094	80	1455	75
CHF	120	1.2	10	3.2	92	1.2	56	2.8
COPD	1009	10	45	15	779	10	269	13
Anemia (HCT: ♂<39%, ♀<36%)	3073	32	70	24	2435	33	498	26
eGFR < 60 ml/min	3510	37	89	30	2281	32	573	32
Preoperative medication								
Any antiplatelet therapy	9051	90	284	93	6709	89	1755	88
Statin	8249	82	254	83	5985	79	1528	76
Betablocker	5879	59	158	51	3804	51	858	43
Ipsilateral stenosis								
Mild (USA: 0-50%, NL: 0-70%)	63	0.6	11	3.5	139	1.9	166	8.3
Moderate (USA: 50-79%, NL: 70-90%)	2459	25	148	48	2746	37	982	49
Severe (USA: 80-99%, NL: 90-99%)	7308	74	143	46	4477	60	794	40
Occluded (USA: 100%, NL: 100%)	60	0.6	1	0.3	132	1.8	17	0.8
Contralateral carotid occlusion	430	4.9	93	32	276	4.1	226	13
Operative Details								
Emergency case	31	0.3	5	1.7	425	5.6	117	6.1
Concomittant cardiac surgery	17	0.2	10	3.0	5	0.1	5	0.3
General anesthesia	8509	84	311	100	6652	88	2007	100
CEA with patch	7784	86	278	91	5817	83	1820	92

USA: United States of America, NL: Netherlands, COPD: chronic obstructive pulmonary disease, eGFR: estimated glomerular filtration rate, calculated by the Cockcroft-Gault equation.

Perioperative outcomes

American asymptomatic patients experienced comparable rates of 30-day MACE (3.0% vs. 3.7%, P=.5, Table 2), 30-day mortality (0.6% vs. 1.4%, P=.1), and 30-day MI (1.2% vs. 0.7%, P=.5) compared to Dutch asymptomatic patients, however 30-day stroke rate was

Table 2. Differences in baseline characteristics between American and Dutch carotid endarterectomy patients.

	USA		Netherlands			USA		Netherlands		
	Asymptomatic					Symptomatic				
	N	%	N	%	*P-value*	N	%	N	%	*P-value*
30-day MACE	228	2.9	11	3.7	.5	278	4.8	81	4.4	.5
30-day Stroke/Death	138	1.8	10	3.4	.1	229	4.0	66	3.6	.5
30-day Mortality	48	0.6	4	1.4	.1	62	1.1	17	0.9	.6
30-day Stroke	94	1.2	10	3.4	.004	191	3.3	62	3.4	.9
30-day MI	103	1.3	2	0.7	.6	63	1.1	20	1.1	1.0

MACE: major adverse cardiovascular event (death, stroke, MI), MI: myocardial infarction.

lower in American asymptomatic patients (1.3% vs. 3.4%, P<.01). Among symptomatic patients, rates of 30-day outcomes were comparable between American and Dutch patients, for MACE (4.9% vs. 4.4%), stroke (3.3% vs. 3.4%), mortality (1.2% vs. 0.9%), and MI (1.0% vs. 1.1%, (all P>.1). On logistic regression CCO was associated with MACE in Dutch asymptomatic patients (OR:5.8 [95% C.I. 1.5-23.1] P=.01), whereas CCO was not associated with MACE in American asymptomatic patients (OR:1.3 [0.8-2.2] P=.3).

Impact of the American selection of asymptomatic patients in the Dutch cohort

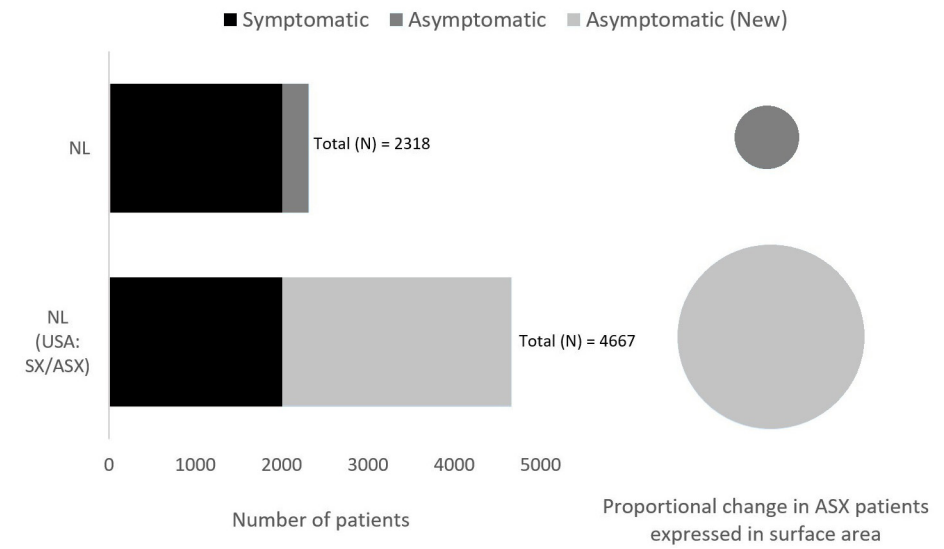
When projecting the American asymptomatic vs. symptomatic patient ratio to Dutch patients, in relation to the 2,007 Dutch symptomatic patients, the Dutch asymptomatic patient sample increased from 311 to 2,660 patients, i.e. from 13% to 57% of CEA patients (Figure 1).

DISCUSSION

In our transatlantic comparison of patients with carotid stenosis selected for CEA, the proportion of asymptomatic patients undergoing CEA in the USA was over 4 times higher than in the Netherlands. The high prevalence of CCO and lower proportions of women and octogenarians among Dutch asymptomatic patients are indicative of the differential selection of asymptomatic patients for CEA in the Netherlands, compared to the USA. Compared to Dutch asymptomatic patients, American asymptomatic patients experienced lower rates of 30-day stroke after CEA, whereas outcomes were comparable between American and Dutch symptomatic patients.

However, interpretation of the differences in 30-day outcomes between American and Dutch asymptomatic patients should be done with caution. The more limited selection of Dutch asymptomatic patients for CEA has arguably resulted in a population with a higher procedural risk, illustrated by differences in the prevalence of CCO among asymptomatic patients with a recorded MACE, as 7 of the 11 (64%) Dutch asymptomatic patients with a recorded MACE had CCO, compared to 16 of 258 (6.2%) among American asymptomatic

Figure 1. Impact of the American asymptomatic to symptomatic ratio in Dutch carotid endarterectomy patients.



patients. Therefore, it is likely that a proportion of low risk patients, who were not selected for CEA in the Netherlands, did undergo CEA in the USA, or vice versa, thus likely contributing to their better overall performance.

This, however, this highlights our main concern with regard to current research into the management of asymptomatic patients, namely, the general lack of data regarding outcomes in asymptomatic patients who, after the initial diagnosis of carotid stenosis, are not selected for CEA. The few studies that did assess outcomes of medical therapy in asymptomatic patients since the publication of ACAS were limited by small sample sizes and lack a control group, i.e. asymptomatic patients who did undergo primary revascularization.[10–12] The ongoing CREST-2 trial compares intensive medical treatment to revascularization. However, patients enrolled in RCTs are not readily generalizable to clinical practice due to strict in- and exclusion criteria for patients and randomizing surgeons. To answer that, we must first have a thorough understanding of the outcomes and risk factors of asymptomatic patients with a diagnosed carotid stenosis who, either by patient or physician preference, are primarily medically managed.

One explanation for our findings could be guideline disparities. Upon review of the European and the North American guidelines, we found that although both supported CEA for asymptomatic patients with ≥70% stenosis, the European guidelines discourage primary revascularization for patients over age 75, especially in women, which correlate with the differences we identified between American and Dutch asymptomatic patients. In addition, a recent review identified unwarranted global differences in guideline recommendations for the treatment of carotid stenosis in asymptomatic patients.[1] Of 34 reviewed guidelines,

7 recommened CEA alone as the primary treatment for asymptomatic patients, while one recommended just medical therapy. This guideline ambiguity, reflecting the ongoing debate between surgeons, interventionists, and neurologists, paves the way for non-medical factors such as provider-induced demand to influence the selection of patients for carotid surgery.[15]

One limitation of our study is that the small sample of Dutch asymptomatic patients precluded a multivariable analysis of outcomes between American and Dutch asymptomatic patients to account for the inherent bias in the Dutch asymptomatic sample due to differential selection. Moreover, the Dutch cohort may not be generalizable to the entire Dutch population, as it reflects clinical practice of two centers. However, it does represent over 10% of all CEAs performed in the Netherlands annually. Aditionally, due to differences between the registries, the degree of carotid artery stenosis was stratified differently, impeding any direct comparison. As, the Dutch registry started in 2003, compared to 2011 for NSQIP, trends over time could have affected the Dutch patients more than the American patients, however we found no trend over time in outcomes after CEA among Dutch patients. Despite the limitations of our current study, we believe it highlights the ongoing need to elucidate the natural history of medically managed carotid artery disease, without which the true value of carotid revascularization remains unknown.

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5

Validation of Randomized Controlled Trial-derived Models for the Prediction of Post-intervention Outcomes in Chronic Limb-Threatening Ischemia

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ABSTRACT

Background

Chronic Limb-Threatening Ischemia (CLTI) represents the most severe form of peripheral artery disease (PAD) and has a large impact on quality of life, morbidity, and mortality. Interventions aim at improving tissue perfusion and averting amputation with an optimal risk-benefit ratio as well as optimal cardiovascular risk management. Several prediction models regarding post-procedural outcomes in CLTI patients have been developed based on Randomized Controlled Trials (RCT) to improve clinical decision-making. We aimed to determine model performance in predicting clinical outcomes in selected CLTI cohorts.

Methods

This study validated the BASIL, FINNVASC and PREVENT III models in data sets from a PAD registry study (Athero-Express) and two RCT's in CLTI in The Netherlands (JUVENTAS and PADI). Receiver-operating characteristics (ROC) curve analysis was used to calculate their predictive capacity. The primary outcome was amputation-free survival (AFS); secondary outcomes were all-cause mortality and amputation at 12 months after intervention.

Results

The BASIL and PREVENT III models showed high predictive values regarding post-intervention mortality in the JUVENTAS cohort with an area under the ROC-curve (AUC) of 81% and 70% respectively. Prediction of AFS was poor to fair (AUC ROC 0.60-0.71) for all models in each population, with the highest predictive value of 71% for the BASIL-model in the JUVENTAS population. The FINNVASC showed the highest predictive value regarding amputation risk in the PADI population with AUC of 78% at 12 months. Adding diabetes to the PREVENT model significantly improved risk prediction for AFS(p=0.003) and amputation(p= 0.0014) at 12 months after intervention.

Conclusions

In general all models performed poor to fair on predicting mortality and amputation. The FINNVASC seems the least suitable to predict mortality. Since, the BASIL model performed best in predicting AFS we propose to use the BASIL model to aid the clinical-decision process in CLTI.

INTRODUCTION

Chronic limb-threatening ischemia (CLTI), defined as rest pain or tissue necrosis with ulceration or gangrene attributable to peripheral artery disease (PAD), is estimated to develop in 500-1,000 individuals per million per year in Western society.[1] CLTI is associated with a high risk for cardiovascular events.[2–4] Amputation rates up to 40% at six months have been reported in CLTI and from the incident diagnosis mortality rates of 20% at six months, and exceeding 50% at five years are not uncommon.[5–8]

A recent nationwide registry study has shown promising results with consistent decreases in mortality and morbidity over time in CTLI-patients.[4] Proposed mechanisms for these improvements are advancements in preventive, medical and interventional treatment. However, despite these improvements, the relative mortality risk of CTLI-patients remained 2-5 times higher than in the general population. This high risk can make risk assessment and clinical decision making a challenging task.

Reliable CLTI risk prediction models could aid in clinical decision making. Over the last decade, three risk prediction models for CLTI have been developed but validation and replication studies are limited. Models based on particular study populations often result in overfitting and produce over-optimistic estimates. Moreover, the large variation in disease severity in PAD, and even in CLTI-cohorts, contributes to mixed results. In that context it is important to validate those models in different clinical cohorts.

In order to assess validity and usefulness of these tools we scored three different CTLI-cohorts in three commonly used CLTI prediction models. We aimed to determine how comorbidities can influence model performance and thus which CLTI prediction models are best used in clinical decision-making.

METHODS

This study was conducted in accordance with the declaration of Helsinki. The medical ethics board in the participating hospitals approved the studies. All patients provided written consent. All patients who completed one year follow-up or reached an endpoint in the first year were considered eligible for analysis.

Study design and initial study cohorts

We assessed the performance of three commonly used CLTI prediction models (Bypass versus Angioplasty in Severe Ischemia of the Leg [BASIL], Risk-scoring Method for Prediction of 30-Day Postoperative outcome after Infrainguinal Surgical Revascularization for Critical Lower-Limb Ischemia in the Finland National Vascular registry [FINNVASC], and the Prevention of Infrainguinal Vein Graft Failure [PREVENT III], in three different cohorts of CLTI patients.[9–11] The specifics of the initial study cohorts are presented in Table 1.

Table 1. Specifics of initial study cohorts.

Risk model	Design	Inclusion	Study outcome	Initial model performance
BASIL: (Bypass versus Angioplasty in Severe Ischaemia of the Leg)	Multicentre, randomised controlled trial comparing infrainguinal bypass versus angioplasty N=452	Rutherford 4-6	No significant differences in amputation-free survival at 3 years	AUC 1 year survival: 65.1%
PREVENT III: (Project of Ex-vivo vein graft Engineering via Transfection III)	Prospective, randomized, double-blinded, multicenter phase III trial of ex vivo vein graft treatment with edifoligide N=1404	Rutherford 4-6	Ex vivo treatment of lower extremity vein grafts with edifoligide did not confer protection from reintervention for graft failure	AUC 1 year AFS: 63.4%
FINNVASC: (Finland National Vascular registry)	Nationwide vascular registry for patients undergoing femoral endarterectomy, femoropopliteal or infrapopliteal bypass N=3925	Fontaine III + IV	-registry-	AUC 1 year AFS: 63.0%

PADI

The PADI trial is a randomized controlled trial (RCT) (2007-2013) in which percutaneous transluminal angioplasty (PTA) was compared to drug-eluting stents (DES) for treatment of infrapopliteal lesions in CLTI.[12] The primary outcome was therapy success, defined as 6-month patency of treated lesions (maximum of 50% restenosis on CTA). Secondary outcomes were Rutherford classification, minor and major amputation, and mortality. Six-month patency rates were 35.1% for PTA and 48.0% for DES.

Athero-Express biobank

The Athero-Express study (AE) is an ongoing tissue biobank collecting atherosclerotic plaques derived during surgical endarterectomy in two large Dutch tertiary referral centers. The AE started on March 24th, 2002 in the University Medical Center Utrecht (UMCU) and the St. Antonius Hospital Nieuwegein in The Netherlands.[13] Indication for surgery was made by an internal review board based on international guidelines and treatment was performed using standardized treatment protocols. All patients completed an extensive questionnaire. Any incomplete data was collected using in-hospital patient data records. After the surgical procedure, all patients were followed-up annually for a three-year time-period for the occurrence of adverse cardiovascular events. In the case of a reported event or no response data records of the hospital were reviewed and the general practitioner was contacted for additional information.

JUVENTAS

The JUVENTAS trial is a randomized, double-blind, placebo-controlled trial that included 160 patients with severe limb ischemia, who were no candidate for revascularization interventions (NO-CLTI), between 2006 and 2012.[14] The aim was to investigate the effect of repetitive intra-arterial infusion of bone marrow mononuclear cells in NO-CLTI. Inclusion criteria were: age > 18 years, severe PAD (Fontaine class IIb, III, IV), ankle-brachial index (ABI) < 0.6 or unreliable, no-option status and written informed consent. The primary

Table 2. Critical limb ischemia prediction models.

Models	PREVENT III	Score	FINNVASC	Score	BASIL
Risk factors	CAD	1	CAD	1	CAD
	Age>75 years	2	Diabetes mellitus	1	Age
	Ht<0.3	2			
	Tissue loss	3	Gangrene	1	Tissue loss
	Dialysis	4	Emergency procedure	1	Creatinine
					Smoking
					Bollinger score
					Ankle pressure
					BMI
					History of stroke/TIA
Model Specifics:	Risk categories cutoff at 3 and 8 points		Score indicates risk		Online risk calculator

Abbreviations: CAD; coronary arter disease, BMI; Body mass index, TIA; Transient ischemic attack.

outcomes were the incidence of major amputation and all-cause mortality with a median follow-up of 36 months. No significant differences in outcomes were observed between treatment arms.

Endpoints

Our primary endpoint was amputation-free survival (AFS) while amputation and all-cause mortality served as secondary outcomes. The total length of follow-up was chosen at 1-year for a cut-off in the current analyses. Amputation was defined as any major amputation of the lower extremity, through or above the ankle joint.

Model specifics

In Table 2 an overview of the different risk prediction models is presented. The FINNVASC and PREVENT model both use a scoring method based on the presence of four to five risk factors present in the CLTI patient. All models require information on coronary artery disease status and presence of tissue loss/gangrene. BASIL and PREVENT include renal function and age. FINNVASC is the only model that requires information on diabetic status. FINNVASC stratifies risk from 1-4 on the sum of points.[15] PREVENT uses three risk categories for AFS; low (<4 points), medium (4-7 points) and high risk (>7). The BASIL prediction model calculates a continuous score.

Initial model cohorts

All models were named after the initial cohort on which they were developed (Table 1). The BASIL trial consisted of 452 patients from the United Kingdom with CLTI. Patients were randomized for revascularization intervention using either balloon angioplasty (BAP) or bypass surgery (BSX). AFS after three years was 38% and mortality rates were reported at 55% after this period. Vein-BSX was associated with significantly higher long term AFS

and overall survival. Furthermore, through stratifying significant baseline factors, 2-year survival rates could be categorized ranging from 50% to 90%.[9] The PREVENT III study is a RCT conducted in the United States in which a new molecular therapy (edifoligide) was tested for the prevention of vein graft failure in patients undergoing infrainguinal revascularization for CLTI. 30-day mortality occurred in 2.7% of all patients. Major amputation occurred in 1.8% of all patients. Additionally, the molecular therapy did not offer protection for reintervention.[16] FINNVASC is a registry-study from Finland including data on 3,925 infrainguinal surgical revascularization procedures. 30-day major amputation rate was reported at 6.3% and the mortality rate after 30 days was 3.1%.[10]

Statistical analysis

To compare baseline characteristics between cohorts one-way ANOVA was used for continuous variables showing a normal distribution. Kruskal-Wallis test was used for continuous non-normally distributed variables. Chi-square test was used to compare categorical baseline characteristics between cohorts. Survival curves were constructed by use of Cox regression analyses for the different risk scores and the three risk categories. The accuracy of the FINNVASC, PREVENT and BASIL models in prediction of twelve-month AFS, mortality and amputation rate was determined using receiver-operating characteristics (ROC) curves. (IREF) The area under the curve (AUC) of all three prediction models will then be compared within every cohort. Comparisons between related models were performed using the Net Reclassification Improvement (NRI). SPSS 21.0 (SPSS Inc, Chicago, Illinois, USA) was used for all statistical analyses.

RESULTS

Patient characteristics

Baseline characteristics of the three cohorts are presented in Table 3. Patients were oldest in PADI (73.6 ± 12.0) when compared to AE (68.5 ± 8.7) and JUVENTAS (64.9 ± 13.3). In all cohorts more males were treated than females with an overall male to female ratio of 2:1. Diabetes was present in 63% of all patients in PADI compared to approximately 37% in AE and JUVENTAS. Incidence of extra-peripheral vascular disease was high in all cohorts, with around 40% of all patients suffering from ischemic heart disease and 17% of all patients suffering from cerebrovascular disease. PAD severity strongly differed across the three cohorts (<0.001) illustrated by the percentage of patients with PAD Fontaine grade 4. PAD severity, graded by Fontaine classification, was highest in PADI with 86.3% Fontaine class 4 patients compared to 66.0% in JUVENTAS and 43.7% in AE. Cardiovascular risk factors such as history of smoking, hypertension and hypercholesterolemia were highly prevalent in all cohorts.

Table 3. Baseline characteristics CLI-cohorts.

	JUVENTAS (*n*=150)	**PADI** (*n*=131)	**AE** (*n*=398)	**P-value**
Age, years, mean (SD)	64.9(13.3)	73.6(12.0)	68.5(8.7)	<0.001
Men*	102(68.0)	91(69.5)	270(67.8)	0.940
BMI, kg/m², mean (SD)	26.5(5.5)	Unknown	26.3(4.2)	-
Smoking status				
Ex-smoker	87(58.0)	29(22.1)	232(58.3)	<0.001
Current smokers	41(27.3)	31(23.7)	160(40.6)	<0.001
Diabetes	56(37.3)	82(62.6)	146(36.7)	<0.001
Hypertension(medication)	133(88.7)	Unknown	335(84.4)	<0.001
Hyperlipidemia	136(90.7)	Unknown	232(65.7)	-
Coronary disease	60(40.0)	49(37.4)	167(42.0)	0.644
Cerebrovascular disease**	23(15.3)	24(18.3)	63(16.8)	0.799
Impaired renal function***	29(19.3)	35(26.7)	46(11.6)	0.001
(prev.) Hemodialysis	4(2.7)	13(9.9)	Unknown	-
Fontaine classification				<0.001
3	51(34.0)	18(13.7)	224(56.3)	
4	99(66.0)	113(86.3)	174(43.7)	

* All variables in n(%), unless stated otherwise. ** Previous stroke or transient ischemic attack. *** eGFR <45 mL/min per 1.73 m²

Follow-up

Outcomes after one-year follow-up are presented in Table 4. As anticipated the AE-population showed best outcomes when compared with PADI and JUVENTAS. This is particularly true for AFS and amputation. However, regarding all-cause mortality, the percentage is comparable to JUVENTAS. Patients in the PADI cohort had the poorest outcomes with only 66% reaching 12 months AFS, in particular due to high mortality rates. Amputation rate was highest in JUVENTAS with 23% compared to 14% and 6% in PADI and AE, respectively. In PADI 26% of all patients had died within one-year follow-up.

Prediction models performance

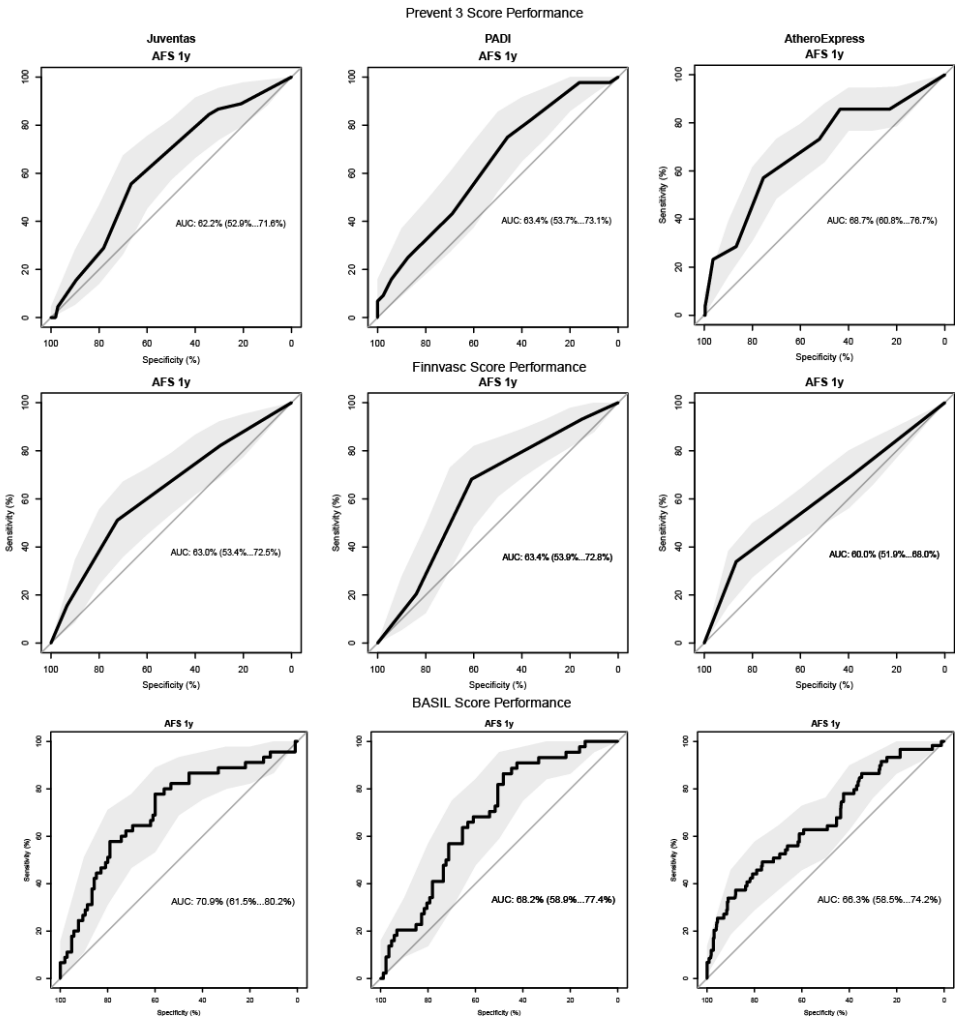
In Table 5 an overview of the different AUC's stratified by prediction model and outcome are presented. In Figure 1 the ROC-curves of twelve month AFS are shown. Overall, twelve-month AFS was best predicted by BASIL with AUC ranging from 66.3% to 70.9%. Both PREVENT and FINNVASC models showed comparable model performance ranging from an AUC of 60.2 to 68.7%. General performance of all models was moderate for prediction of twelve-month AFS. Prediction model performance was stable over time, with ROC curves stabilizing after 4 weeks (Figure 2). In Table 6 the group stratification and outcomes according to PREVENT and FINNVASC are shown. Additionally, in Figure 3 the Cox regression curves are presented for these risk classifications. Since BASIL does not operate with risk categories but with an absolute score such survival analyses could not be performed.

Table 4. One-year clinical outcomes CLI-cohorts.

	JUVENTAS (*n*=150)	**PADI** (*n*=131)	**Athero-Express** (*n*=398)
Amputation free survival 12 months	105(70.0)	87(66.4)	339(85.2)
Amputation	35(23.3)	18(13.7)	25(6.3)
All-cause mortality	15(10.0)	34(26.0)	39(9.8)

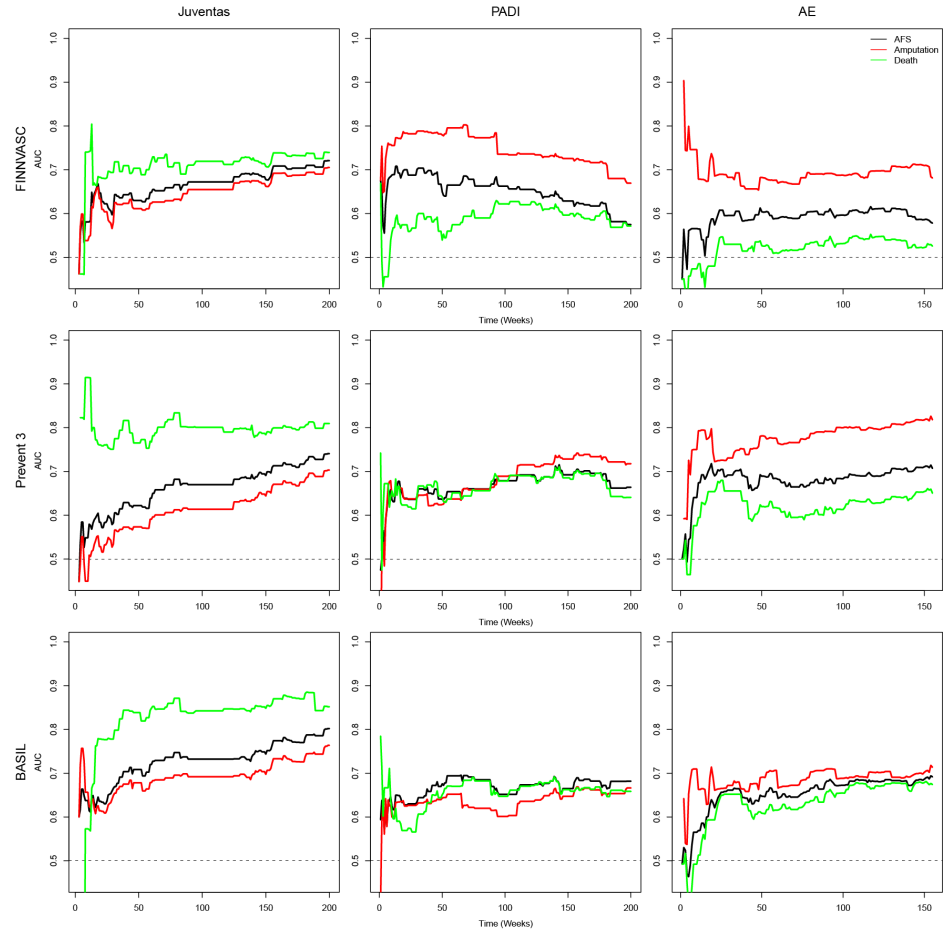
One-year clinical outcomes of the three different CLI-cohorts

Figure 1. ROC curves of the three different prediction models in three cohorts.



ROC-curves for one year amputation free survival in the three different cohorts with the thee different risk scores.

Figure 2. Area under the curve plotted at different moments in time.



AUC plotted per cohort for amputation free survival (black line), amputation (red line) and death (green line) at different moments during follow-up.

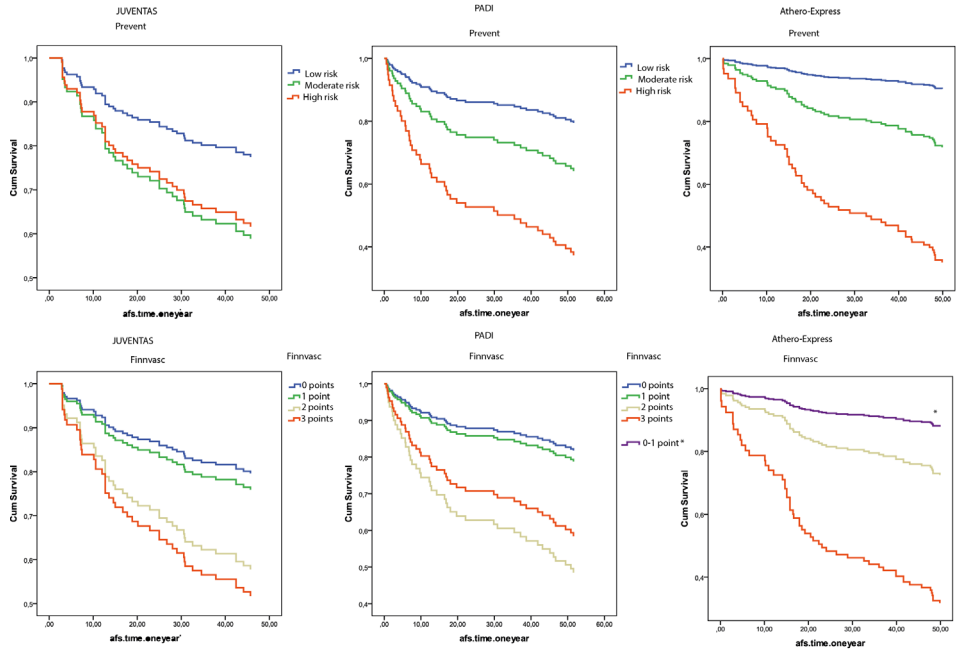
While models performed consistently over the different cohorts for the composite outcome of AFS, performance differed more notably between cohorts in the composite outcomes death and amputation. Which component outcome was better predicted, depended both on the cohort and the prediction model. In JUVENTAS all models performed poorly on amputation, but well on mortality for instance. Interestingly, the FINNVASC model performed well on amputation in PADI and AE, and reasonable in JUVENTAS. This is especially remarkable considering the simplicity of the model, which consists of a simple, unweighted point system. While the risk factors that constitute the model scores largely overlap, FINNVASC is the only model that includes the presence of diabetes mellitus (DM) in the score.

Table 5. ROC analysis for three different outcomes in CLI cohorts.

	AFS 12 months	Amputation 12 months	Mortality 12 months
PREVENT III	(95% CI)	(95% CI)	(95% CI)
JUVENTAS	62.2% (52.9-71.6)	54.5% (43.8-65.3)	70.1% (55.9-84.4)
PADI	63.4% (53.7-73.1)	58.9% (43.0-74.9)	60.9% (50.5-71.4)
AE	68.7% (60.8-76.7)	70.6% (59.2-82.1)	60.6% (50.6-70.7)
FINNVASC			
JUVENTAS	63.0% (53.4-72.5)	59.5% (48.5-70.5)	66.9% (53.8-79.9)
PADI	63.4% (53.9-72.8)	78.1% (67.2-89.1)	54.5% (44.2-64.7)
AE	60.0% (51.9-68.0)	67.9% (56.0-79.7)	52.4% (42.0-62.8)
BASIL			
JUVENTAS	70.9% (61.5-80.2)	64.5% (54.4-74.6)	80.9% (68.1-93.7)
PADI	68.2% (58.9-77.4)	60.0% (46.1-73.9)	66.5% (56.6-76.4)
AE	66.3% (58.5-74.2)	67.8% (57.7-77.9)	63.3% (53.6-72.9)

CI, confidence interval

Figure 3. Survival curves of the different risk prediction models.



Survival curves based on the FINNVASC and PREVENT III risk prediction scores plotted per cohort.

Table 6. Group stratification and outcomes

		JUVENTAS		PADI		Athero-Express	
		Groupsize	Amputation	Groupsize	Amputation	Groupsize	Amputation
FINNVASC	0	39	8 (20.5%)	16	3 (18.8%)	125	15 (12%)
Score	1	59	14 (23.7%)	51	11 (21.6%)	156	19 (12.2%)
	2	38	16 (42.1%)	41	21 (51.2%)	65	18 (27.7%)
	3	14	7 (50.0%)	23	9 (39.1%)	1	1 (100%)
PREVENT III	Low risk	90	20 (22.2%)	40	8 (20.0%)	251	24 (9.6%)
	Moderate risk	55	23 (41.8%)	79	29 (36.7%)	101	29 (28.7%)
	High risk	5	2 (40.0%)	12	7 (58.3%)	5	3 (60.0%)

Outcomes of the different cohorts stratified by the FINNVASC or PREVENT III risk score.

We therefore investigated whether addition of DM to the PREVENT and BASIL models would improve classification for amputation and AFS. In order to maximize statistical power, we pooled the JUVENTAS, PADI and AE datasets for this purpose. The ORs for AFS at 12 months of the component variables in the Prevent 3 score were similar in rank to the original, with dialysis being the most important (OR=13.1) followed by tissue loss (OR=3.2) and Age (OR=2.1). History of CAD (OR=1.4) and Ht (OR=0.8) proved non-significant predictors for AFS. DM had a mutually adjusted OR of 2.0 (p=0.028) and therefore proved an independent predictor of AFS in the pooled cohorts. We next assigned DM 2 points on the Prevent 3 scale, on account of similarity in OR to Age. The thus expanded score indeed performed better than the original with an NRI of 0.28 (p=0.003) for AFS at 12 months and an NRI of 0.44 (p= 0.0014) for amputation at 12 months.

DISCUSSION

We have assessed three commonly used prediction models for CLTI in three different cohorts. Although our cohorts differ from the initial validation cohorts in terms of lesion localization, patient characteristics and type of intervention, performance was reproducible. Overall the BASIL model showed slightly better performance over the PREVENT and FINNVASC models for twelve months AFS. Interestingly BASIL performed best in the two cohorts with most severe Fontaine classification namely JUVENTAS and PADI. The PREVENT model showed the best performance in the AE. Moreover, we studied the effects of the different components used in the risk prediction models and show a similar rank in importance of risk factors. An important findings of this study is that by adding diabetes to the PREVENT model, classification significantly improved for both amputation and AFS 12 months after intervention.

Upon interpretation of these results it is essential to notice the differences in the study cohorts compared with the initial validation cohorts. The initial study cohorts are all comprised of patients undergoing surgical revascularization. Patients in the JUVENTAS-study however, were not eligible for either surgical or endovascular revascularization often due to severe comorbidities. Interestingly, the AUCs of the different models ranged from 62.2% to 70.9% in the JUVENTAS-cohort and were comparable to the AUCs of 63.0% to 65.0% of the initial validation cohorts. Moreover, patients in PADI were oldest and often had a diagnosis of diabetes (62.6%) but models showed similar performance compared with the initial cohorts.

When we critically review the performances of the three different models we can conclude that overall model performance can be classified as fair to poor. When we compare the AUCs with historical cohorts we can confirm that these are in line with previous publications.[17] There were some differences, however, between model performance of the three interventions studies compared to the initial validation cohorts. When looking at 12 month AFS the BASIL model performed better than PREVENT and FINNVASC. However, amputation at 12 months is best predicted by the FINNVASC model. Mortality after 12 months was best predicted by the BASIL model in the JUVENTAS cohort. The reason for limited model performance can partly be explained by the multifactorial complexity of CLTI which makes it difficult to give one clear explanation for the found differences. However, something that does stand out is the implementation of different risk factors. Considering that diabetes is an important risk factor and is known to influence the pathophysiology of CLTI, addition of this risk factor seemed as a first logical step. As demonstrated by our prediction model analyses we were able to improve the PREVENT-model by adding diabetes into the risk score and thereby improve risk classification.

One could argue that current risk prediction models simply do not cover all factors associated with CTLI disease progression and poor outcome. CLTI populations often suffer from a form of end-stage atherosclerotic disease. This systemic atherosclerotic burden is reflected by the large prevalence of other affected vascular beds such as the coronary and the cerebral circulation. However, despite the fact that CLTI is a subgroup of advanced disease by definition, it still represents a heterogeneous population. This end-stage phenotype with often multi-segment vascular pathology can make risk prediction by use of four to five risk factors as used in PREVENT and FINNVASC an unjustified simplification for a complex problem. Considering the large heterogeneity in CLTI cohorts a possible approach for a new risk classification could come from novel statistical techniques such as phenomapping.[18] This unbiased clustering analysis may help identify distinct phenotypes in CLTI cohorts and has shown promising results in other heterogeneous clinical syndromes such as for instance heart failure with preserved ejection fraction.[18] This mapping strategy can help distinguish homogenous patient subcategories that have comparable success and outcome rates to the different treatment strategies.

Starting point for improvements of PAD risk prediction can come from different angles. Simple improvements could come from implementing information on exercise capacity or medical therapy prescription and compliance.

While functional scores such as the ankle-brachial index (ABI) are used only in BASIL, other scoring methods such as transcutaneous oxygen measurement and toe pressure measurements also merit consideration. Especially when taking into account that ABI is notoriously unreliable among diabetics, which were highly prevalent in these populations. Moreover, there is strong evidence emerging that hematological parameters can be used for improvement of secondary risk prediction. Recent studies have shown that by using specific features of full blood analyses such as counts and percentages from red blood cells, platelets and leukocytes, risk prediction significantly improved.[19,20] In a cohort of coronary angiography patients a panel of hematological predictors even outperformed high-sensitivity troponin I (hsTnI) and N-terminal pro-brain natriuretic peptide (NT-pro-BNP) in secondary risk prediction.[20]

In a cross-sectional study of 6950 participants, the red cell distribution width, a marker of erythrocyte cell size heterogeneity, showed a strong graded increase in prevalence of PAD.[21] Moreover, this study showed that the predictive accuracy of the American College of Cardiology / American Heart Association (ACC/AHA)-defined PAD screening criteria improved from an AUC of 0.657 to 0.727 after addition of RDW.[22] These studies provide compelling evidence that future risk prediction models likely comprise a wide range of variables. Deep learning algorithms can be used to combine the broad array of phenotypic data ranging from patient characteristics, blood biomarkers, imaging results to outcomes of functional vascular tests all in order to improve risk prediction.

Strengths and limitations

An important strength of this study is that we were able to study the performance of three different prediction models in three different CTLI-cohorts. First, we were able to study model performance in the surgical AE-cohort that is conceivably best comparable to the initial cohorts that are also comprised of patients undergoing surgical interventions. Second, we were able to show that prediction models initially build using data derived from interventional cohorts could be extrapolated to a no-option for revascularization population receiving autologous bone marrow cell therapy alone. Lastly, we were able to improve the risk PREVENT-model by adding diabetes into the risk score which could help improve clinical decision making. A limitation of this study is that since these cohorts were different in design some data on covariates were missing such as a history of previous hemodialysis.

CONCLUSION

In general the three models perform poor to fair (AUC ROC 0.60-0.71) on predicting amputation and AFS. The best performing model in predicting AFS is the BASIL survival prediction model. We consider this model to be a useful addition to the CLTI decision-making process in clinical practice.

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PART

three

CIRCULATING BIOMARKERS IN CARDIOVASCULAR DISEASE

6

Impaired kidney function is associated with intraplaque hemorrhage in patients undergoing carotid endarterectomy

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ABSTRACT

Background and aim

Previously we showed that patients undergoing carotid endarterectomy have an increased risk for major atherosclerotic events in the presence of moderate or poor kidney function. Acceleration of vascular inflammatory responses is considered to be causally involved in progression of atherogenesis and poor outcome in chronic kidney disease patients. The association between kidney function and plaque composition has not been thoroughly investigated yet. The aim of this study was to investigate the association between kidney function and atherosclerotic plaque composition in patients undergoing carotid endarterectomy.

Methods

Atherosclerotic plaques harvested from 1796 patients who underwent carotid endarterectomy were immunohistochemically stained for, macrophages, smooth muscle cells, calcifications, collagen, microvessels, lipid core size and intraplaque hemorrhage. Cytokines were measured in plaque and plasma and associated with kidney function. Quantitative proteomics were performed on 40 carotid plaques and associated with kidney function.

Results

Decreased kidney function was associated with increased odds ratio of intraplaque hemorrhage, OR 1.15 (95%CI; 1.02-1.29 (p=0.024)) and increased odds ratio of fibrous-atheromatous plaques (plaques with lipid core presenting more than 10% of total plaque surface) OR 1.21 (95%CI; 1.07-1.38 (p=0.003)) per decrease of 20 points in eGFR. Proteomics revealed that decreased kidney function was associated with upregulation of the classical pathway of the complement system and the intrinsic pathway of the coagulation system.

Conclusion

Decreased kidney function was associated with plaque hemorrhage but not with inflammatory plaque characteristics. Our data suggests that other pathways than the inflammation-pathway are involved in plaque vulnerability and poor outcome in patients with decreased kidney function.

INTRODUCTION

Chronic kidney disease (CKD) and cardiovascular disease (CVD) are profound problems increasingly diagnosed in the elderly patient in Western society today.[1] Due to large interactions between the heart, blood vessels and kidney function these three are intrinsically bound and together form the cardio-renal-axis.[2]

The pivotal role of inflammation in development of atherosclerosis and kidney failure has been clearly established.[3,4] Beside inflammation various other plaque features are described that might be associated with MACE, such as neovascularization, calcium deposition, and vessel wall remodeling.[5] Autopsy studies showed that decreased kidney function associated with vascular calcifications and smooth muscle cell apoptosis. In patients with manifestations of cerebrovascular disease, CKD is an important risk factor for future major cardiovascular events (MACE).[6] In patients with end-stage renal disease mortality rate after myocardial infarction from cardiac causes is approximately 40 percent at one year, 51 percent at two years and 70 percent at five years.[7]

In hemodialysis patients, oxidative stress and a state of chronic inflammation have been described as a cause and a consequence of atherogenesis.[8,9] Recent ongoing clinical trials show promising results targeting chronic inflammation for improvement of vascular function.[10,11] Inflammation is an established contributor to atherosclerotic disease, however it is not clear to what extend decreased kidney function contributes to atherosclerotic inflammation, and whether it is associated with different plaque characteristics.[10–12]

Current evidence on associations of CKD with atherosclerotic plaque characteristics is scarce, obtained post-mortem or obtained with small sample sizes. We report associations of CKD with plaque characteristics in a large cohort that represent viable patients. This patient domain is rapidly growing in size due to improved survival rates following a cardiovascular event. We hypothesized that decreased kidney function is associated with increased inflammatory plaque characteristics which may explain the increased risk for adverse secondary manifestations in this patient group.

We present analyses of a cohort of 1796 patients with advanced atherosclerosis who underwent CEA with a normal distribution of glomerular filtration rate and completed three year follow-up. We studied histological characteristics of the harvested carotid plaques, together with pro- and anti-inflammatory plasma and plaque protein levels. In addition, a quantitative proteomic analysis was performed to examine the plaque proteome in relation to kidney function.

PATIENTS AND METHODS

Athero-Express Biobank Study

The Athero-Express Biobank is world's largest ongoing atherosclerotic plaque biobank and started on March 24th, 2002 in the University Medical Center Utrecht and the St. Antonius Hospital Nieuwegein in the Netherlands.[13] Study design has been reported before, but in short, atherosclerotic plaques of patients undergoing CEA are harvested and subjected to

histological staining and protein extraction. Between April 2002 and August 2015, 2281 patients undergoing CEA at one of our institutions were included in the Athero-Express. Patients were considered eligible for inclusion in the current study when both plaque histology and eGFR were available.118 patients were excluded for the current analyses due to missing eGFR and another 337 patients due to missing information on plaque histology. The first three years after surgery, all patients were contacted annually for their medical status. If any adverse events were reported, information was gathered at the respective hospitals or general practitioner.

Kidney function estimation

An estimation of the glomerular filtration rate (eGFR) is considered a reliable measure for kidney function.[14] For all patients, eGFR was calculated with the modification for diet (MDRD) formula and expressed in ml/min/1.73m^2. Serum creatinine was measured in peripheral blood, withdrawn before surgery. Patients were classified based on their kidney function according to the international guidelines of the National Kidney Foundation (KDOQI): eGFR ≥90 ml/min/1.73m^2 (CKD stage 1: normal kidney function), eGFR 60-89 ml/min/1.73m^2 (CKD stage 2: mildly impaired kidney function), eGFR 30-59 ml/min/1.73m^2 (CKD stage 3: moderately impaired kidney function).[15,16] In our cohort only 30 patients were identified with severely impaired kidney function and end stage kidney disease (CKD stage 4 and 5: eGFR <30 ml/min/1.73m^2). We considered this patient group too small to compare with the other groups and was therefore excluded from the current study.

Atherosclerotic plaque assessment

After CEA all atherosclerotic plaques were immediately processed. The culprit lesion was divided into segments of 5 mm thickness along the longitudinal axis. The segment with the largest plaque burden was chosen as culprit lesion and subjected to histological examination. A detailed description of atherosclerotic plaque assessment has been reported previously and has been added to the supplemental methods.[13,17–19]

Inflammatory plaque and plasma proteins

To investigate if plaque protein levels associate with kidney function, cytokine levels were measured in randomly selected subgroups with a normal distribution of eGFR. Cytokines, interleukins and matrix metalloproteinases (MMP) were selected based on known influence on plaque progression and stability. The selection of protein markers in plasma was based on the previously selected markers that were measured in plaque. Circulating inflammatory marker high-sensitive C-reactive protein (hsCRP) was added to this essay to gain knowledge on the general inflammatory status of the patients. All used kits and essays are added to the supplemental methods.

Quantitative Plaque Proteomics

Quantitative proteomics measurements were performed in carotid plaques from 40 patients, including 20 patients suffering from MACE during three years of follow-up and 20 age and sex matched controls without MACE during three years of follow-up. The methods of the quantitative proteomics experiment have been reported in detail previously.[20] In short, proteins were isolated from carotid atherosclerotic plaques and peptides were separated with high-performance liquid chromatography using a SCX column in 15 salt fractions. Bioworks 3.3 was used to generate a list of identified proteins per salt fraction. Proteins that were detectable in at least 32 of all 40 investigated plaques were included for analysis. Proteins detected with quantitative proteomics were correlated with eGFR using Pearson's correlation tests. Proteins that showed significant correlations ($p<0.05$) with eGFR were entered into Ingenuity Pathways Analysis (Ingenuity® Systems, "http://www.ingenuity.com/").

Clinical outcome

To confirm previously reported associations between kidney failure and adverse outcome during follow-up after CEA we analyzed the impact of CKD on outcome in the current study cohort.[6] Primary endpoint of interest consisted of all major manifestations of CVD including myocardial infarction, stroke and cardiovascular death. Secondary endpoint of interest was all-cause mortality. Definitions of endpoints were previously described.[6,13] Two members of the outcome assessment committee validated endpoints. If no consensus was reached, a third observer was consulted for final judgment of the endpoint.

Statistical analysis

Data were inspected for patterns of missing values. The proportion of randomly missing values for baseline characteristics varied between 0-5%. Missing values were imputed using multiple imputation to prevent limitation of incomplete case analyses in multivariable regression analyses.[21] Differences in binary characteristics between the three groups were analyzed with Pearson's Chi square. Differences in continuous parameters between the groups were calculated with One-Way ANOVA or Pearson correlation tests where appropriate. To investigate independent associations between histological plaque characteristics and kidney function, and to correct for potential confounders, we conducted multivariable linear and binary logistic regression models for continuous and binominal plaque characteristics respectively. Non-normally distributed quantitative histological parameters, including macrophages and SMCs, required logarithmic transformation, to enter them into linear regression models. Impact of CKD on clinical outcome was studied with Cox proportional hazard models. We corrected for all baseline characteristics that showed an association with kidney function (p-value <0.10) in all multivariable analyses. To assess associations between kidney function groups and various protein levels measured in plaque and plasma, UNIANOVA with correction for multiple confounders was used. Proteins detected with quantitative plaque proteomics were correlated to eGFR using Pearson correlation tests. To assess whether sex stratification was necessary, interaction terms were built into the model. SPSS 21.0 (SPSS Inc, Chicago, Illinois, USA) was used for all statistical analyses.

RESULTS

Patient characteristics

Baseline characteristics of the 1796 included patients before CEA are reported in Table 1. The percentage of patients with relevant comorbidities at baseline increased with every decrease in kidney function. Patients with moderately and mildly impaired kidney function were older, and were more frequently: female, treated for hypertension and had a higher prevalence of coronary artery disease as compared to patients with normal kidney function. The percentage of diabetic patients was highest in the moderately impaired kidney function group but surprisingly enough lowest in the mildly impaired kidney function group. Clinical presentation did not differ between kidney function groups. TIAs were most prevalent in all kidney function groups directly followed by stroke. Other clinical presentation was ocular symptoms and asymptomatic carotid stenosis. Prescription of anticoagulants, diuretics, beta-blockers and ACE-inhibitors significantly increased with decreasing kidney function. There were no differences observed in the percentage of statins and antiplatelet-drug-use across our cohort. Total cholesterol, HDL and LDL cholesterol significantly declined concomitantly with declining kidney function.

Plaque characteristics and kidney function

After correcting for possible confounders, IPH was more frequently observed in patients with decreased kidney function, OR 1.15 (95% CI; 1.02-1.29(p=0.024)) per decrease of 20 points in eGFR (Table 2). Fibrous-atheromatous plaques (plaques with lipid core presenting more than 10% of total plaque surface) were more often observed in patients with impaired kidney function, OR 1.21 (95% CI; 1.07-1.38 (p=0.003)) per decrease of 20 points in eGFR. For continuous measures of plaque characteristics an association with eGFR was found with the percentage of smooth muscle cell staining (p=0.001) in univariate analysis, however not in multivariate analysis. No significant association was found between eGFR and macrophage staining. In addition, large lipid core (plaques with lipid core covering more than 40% of total plaque surface), moderate/heavy calcifications, collagen staining and the number of micro vessels were not associated with eGFR. In Figure 1 a typical example of a carotid plaque with IPH is depicted characterized by heavy glycophorine staining central in the plaque specimen.

Inflammatory protein markers in plaque and kidney function

We examined the relation between various inflammatory protein markers in plaque and kidney function (Table 3, Supplemental Figure 1). Levels of chemokine RANTES (CCL5) increased in plaques from patients with impaired kidney function (p=0.030). The levels of pro-inflammatory chemokine monocyte chemoattractant protein-1 (MCP-1), Osteoprotegerin (OPG) and vascular endothelial growth factor A (VEGFA) were not associated with eGFR. The interleukin analysis in plaque varied between kidney function groups but did not consistently increase with decreasing kidney function (Table 3). No statistically significant associations were observed for plaque MMP2, MMP8 and MMP9 with eGFR.

Table 1. Baseline characteristics of patients with normal kidney function (eGFR ≥ 90 ml/min/1.73 m^2), mildly impaired kidney function (eGFR 60-89 ml/min/1.73 m^2) and moderately impaired kidney function (eGFR 30-59 ml/min/1.73 m^2).

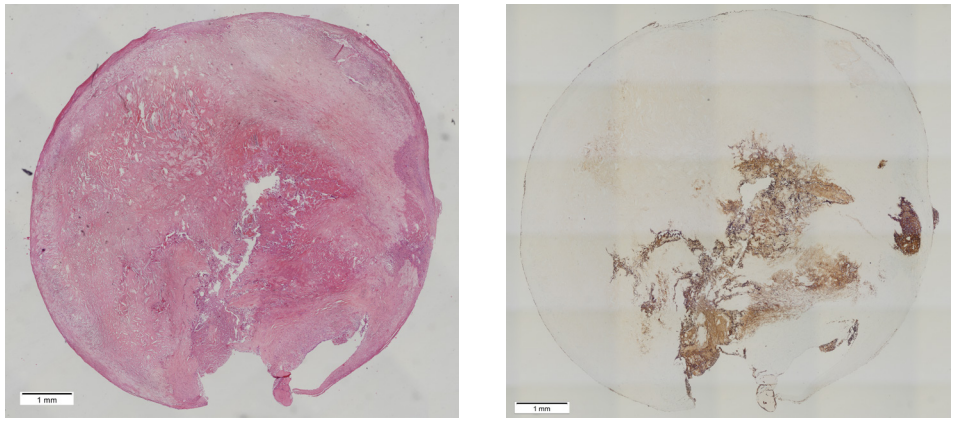
Patient characteristics	**eGFR ≥ 90 ml/ min/1.73 m^2**	**eGFR 60-89 ml/ min/1.73 m^2**	**eGFR 30-59 ml/ min/1.73 m^2**	**P-value**
	(*n*=340)	(*n*=988)	(*n*=468)	
eGFR in mL/min/1.73 m^2 [median; IQR]	98.4 [93.5-107.0]	74.1 [67.5-81.4]	50.7 [42.8-55.5]	<0.001
Sex, male n (%)	253(74.4)	685(69.3)	288(61.5)	<0.001
Age, years [median; IQR]	65 [57-71]	69 [62-75]	74 [68-79]	<0.001
BMI [median; IQR]	25.8 [23.5-28.1]	26.0 [24.1-28.4]	26.1 [24.1-28.7]	0.132
Current smoker, n (%)	159(48.5)	322(33.2)	122(26.8)	<0.001
Diabetes mellitus, n (%)	84(24.7)	201(20.3)	135(28.8)	0.001
Treated hypertension, n (%)	220(67.7)	693(71.8)	369(81.6)	<0.001
Treated hypercholesterolaemia, n (%)	209(66.6)	614(67.0)	284(68.4)	0.832
History of CAD, n (%)	80(23.6)	296(30.0)	195(41.8)	<0.001
Triglycerides in mmol/l [median; IQR]	1.40 [1.06-1.98]	1.40 [1.04-2.00]	1.40 [1.00-2.10]	0.942
Total cholesterol in mg/dL [median; IQR]	4.97 [4.00-5.70]	4.68 [3.90-5.60]	4.40 [3.50-5.23]	<0.001
HDL in mmol/l [median; IQR]	1.15 [0.94-1.39]	1.13 [0.92-1.41]	1.07 [0.87-1.34]	0.001
LDL in mmol/l [median; IQR]	2.93 [2.20-3.60]	2.71 [2.07-3.42]	2.50 [1.80-3.10]	<0.001
Clinical presentation				
Ø Asymptomatic, n (%)	41(12.1)	129(13.2)	76(16.3)	0.172
Ø Ocular, n (%)	59(17.5)	154(15.7)	67(14.3)	0.488
Ø TIA, n (%)	131(38.8)	447(45.7)	198(42.4)	0.075
Ø Stroke, n (%)	107(31.7)	249(25.4)	126(27.0)	0.085
Pre-operative medication use				
Statin use, n (%)	252(74.1)	753(76.4)	356(76.2)	0.692
Antiplatelet use, n (%)	312(91.8)	870(88.3)	410(88.0)	0.169
Anti-coagulant use, n (%)	24(7.1)	119(12.1)	74 (15.8)	0.001
Diuretic use, n (%)	89(26.2)	308(31.2)	236(50.5)	<0.001
RAAS medication use, n (%)	144(42.4)	4782(48.9)	284(60.8)	<0.001
Betablocker use, n (%)	119(35.0)	437(44.3)	248(53.1)	<0.001

Abbreviations: BMI, Body Mass Index; CAD, Coronary Artery Disease; PAOD, Peripheral Arerial Occlusive Disease; eGFR, estimated Glomerular Filtration Rate; TIA, Transient Ischemic Attack; RAAS, Renin Angiotensin Aldosteron System. Bold values were considered statistically significant with a P<0.05.

Inflammatory protein levels in plasma and kidney function

In plasma, the moderately impaired kidney function group had a higher osteoprotegerin level when (p<0.002) compared to the normal and mildly impaired kidney function groups (Table 4, Supplemental Figure 1). Lastly the hsCRP levels giving an overall impression of the inflammatory state of a patient were decreased in the mildly impaired kidney function group compared to the normal and moderately impaired kidney failure group (p=0.022) (Table 4, Supplemental Figure 1).

Figure 1. Typical example of an atherosclerotic plaque with intraplaque hemorrhage.



Left panel, typical example of an atherosclerotic plaque with intraplaque hemorrhage quantified using Hematoxylin and eosin staining. Bar, 1mm. Right panel showing same atherosclerotic plaque with glycophorine staining depicting intraplaque residues of erytrocytes. Bar, 1mm.

Plaque proteome pathway analysis and kidney function

Mean eGFR among the 40 patients included in the proteomic analysis was 65.8 (±22.8) ml/min/1.73m^2. Plaque proteomics resulted in detection of peptides that identified 3873 proteins. Overall, 565 of 3873 proteins (14.6%) were detectable in at least 32 of 40 plaques (≥80%). Of these 565 proteins, 105 (18.6%) correlated with eGFR with a p-value <0.05 (Supplemental Table 1). The (relative) quantitative values of these 105 associated proteins were uploaded into Ingenuity pathway analyses. Pathway analyses revealed that a decrease in eGFR was inversely associated with proteins involved in the intrinsic pathway of the coagulation system (Supplemental Table 2 + Figure 2). In addition, proteins of the classic complement pathway were also associated with decreased kidney function (Supplemental Figure 3).

Clinical outcome and kidney function

To confirm previously described observations that impaired kidney function is associated with adverse cardiovascular outcome after carotid endarterectomy, we performed survival analyses within the current cohort (Figure 2 + 3). In total 1573 patients completed three year follow-up or endured a major endpoint, 151 patients (8.8%) were lost to follow-up. In a cox proportional hazards analysis, patients with moderately impaired kidney function have a 1.75 HR (95%CI 1.08-2.85 p=0.024) for major adverse cardiovascular events (MACE) during 3-year follow-up compared to patients with normal kidney function. All-cause mortality associated with the moderately impaired kidney function group HR 1.85 (95%CI 1.05-3.26 p=0.035) in both univariate and multivariate analysis after correcting for possible confounders (age, sex, smoking status, diabetes, hypertension, history of coronary artery disease, symptom status and lipid levels.

Table 2. Histological atherosclerotic plaque characteristics of 1796 carotid plaques.

Binominal carotid plaque characteristics	**P-value Univariate**	**Odds ratio unadjusted**	**P-value Multivariate**	**Odds ratio adjusted**
Presence of lipid core ≥40%, % (n)	0.498	1.039 [0.931-1.159]	0.303	1.074 [0.937-1.231]
Presence of lipid core ≥10%, % (n)	**0.035**	1.117 [1.008-1.239]	**0.003**	1.213 [1.067-1.379]
Moderate/heavy calcifications, % (n)	0.106	1.082 [0.984-1.189]	0.867	0.990 [0.882-1.112]
Moderate/heavy collagen, % (n)	0.835	0.987 [0.876-1.113]	0.882	0.989 [0.854-1.145]
Presence of intraplaque hemorrhage, % (n)	0.097	1.085 [0.985-1.195]	**0.024**	1.145 [1.018-1.289]
Continuous carotid plaque characteristics	**P-value**	**Beta Unadjusted**	**P-value adjusted**	**Beta Adjusted**
Mean number of microvessels per hotspot	0.304	0.026 [-0.019-0.060]	0.419	0.024 [-0.028-0.067]
% of positive macrophage staining per plaque	0.217	-0.030 [0.036-0.008]	0.354	-0.026 [-0.040-0.014
% of positive SMC staining per plaque	**0.001**	-0.078 [-0.078- -0.019]	0.124	-0.043 [-0.062-0.008]

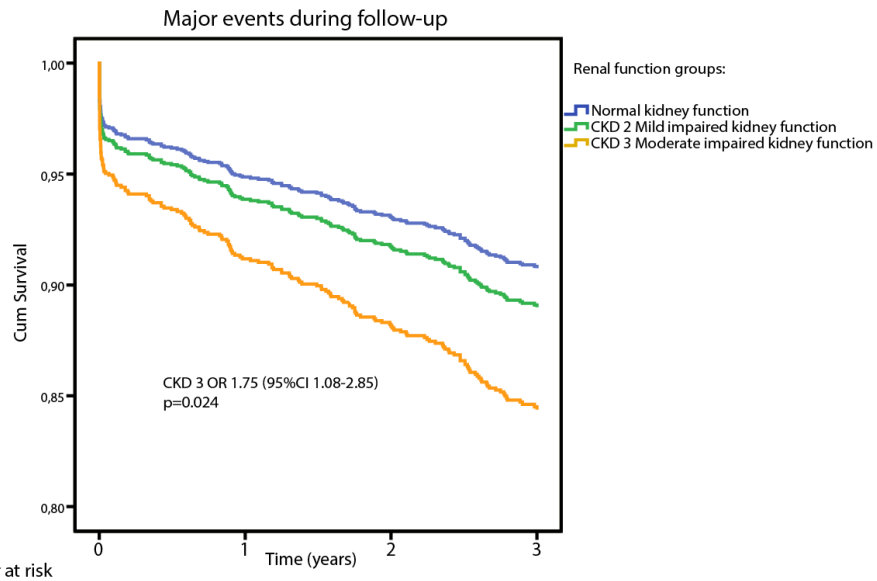
Odds ratios and regression coefficients represent the risk per decrease of 20 points eGFR. Multivariate analysis corrected for sex, age, smoking status, diabetes mellitus, hypertension, history of CAD, lipid levels, anticoagulant therapy, Beta blocker use, diuretics use, RAAS medications. Abbreviations: CI, confidence interval; SMC, Smooth Muscle Cell. Bold values were considered statistically significant with a P<0.05.

Table 3. Plaque protein levels in patients with normal kidney function (eGFR ≥ 90 ml/min/1.73 m^2), mildly impaired kidney function (eGFR 60-89 ml/min/1.73 m^2) and moderately impaired kidney function (eGFR 30-59 ml/min/1.73 m^2).

Plaque protein	**n**	**eGFR ≥ 90 ml /min/1.73 m^2**	**eGFR 60-89 ml/ min/1.73 m^2**	**eGFR 30-59 ml/ min/1.73 m^2**	**P-value**
RANTES (pg/ug)	597	1.19 [1.12-1.26]	1.30 [1.26-1.35]	1.28 [1.22-1.35]	**0.030**
OPG (pg/ml)	581	906.87 [778.40-1056.96]	818.93 [757.64-885.70]	759.00 [674.36-854.68]	0.207
MCP-1 (pg/ug)	1176	1.40 [1.34-1.45]	1.44 [1.41-1.47]	1.44 [1.40-1.49]	0.384
VEGFA (ng/ml)	564	555.57 [357.25-864.13]	471.07 [370.94-598.76]	564.53 [394.98-806.96]	0.642
Interleukin 4 (pg/ml)	552	35.30 [22.33-55.83]	67.90 [54.02-85.27]	42.31 [29.97-59.68]	**0.010**
Interleukin 5 (pg/ml)	552	50.96 [33.42-77.70]	81.37 [65.96-100.41]	51.99 [37.86-71.38]	**0.023**
Interleukin 6 (pg/ml)	552	26.21 [18.27-37.58]	36.67 [30.64-43.89]	25.92 [19.76-33.98]	0.054
Interleukin 8 (pg/ml)	552	48.04 [29.62-77.91]	42.14 [33.11-53.60]	42.56 [29.58-61.21]	0.890
Interleukin 10 (pg/ml)	552	9.55 [6.82-13.38]	14.00 [11.84-16.54]	10.99 [8.55-14.15]	0.069
Interleukin 12 (pg/ml)	552	24.93 [15.95-38.98]	46.43 [37.15-57.99]	34.09 [24.36-47.70]	**0.030**
MMP 2 activity*	587	5.10 [4.68-5.55]	5.35 [5.13-5.58]	5.17 [4.84-5.52]	0.483
MMP 8 activity*	587	5.72 [4.92-6.65]	5.98 [5.54-6.45]	6.69 [5.96-7.52]	0.196
MMP 9 activity*	587	1.79 [1.65-1.94]	1.92 [1.84-1.99]	1.96 [1.85-2.09]	0.193

Values presented as mean [95% CI]. Associations are corrected for sex, age and year of surgery. Abbreviations: OPG, Osteoprotegerin; MCP-1, monocyte chemoattractant protein-1; VEGFA, Vascular endothelial growth factor A; MMP, Matrix metalloproteinase. * Values represent arbitrary units corrected for total protein content. Bold values were considered statistically significant with a P<0.05.

Figure 2. Hazard curves for CKD and major cardiovascular events after carotid endarterectomy.



Number at risk				
eGFR: ≥ 90	325	305	292	261
eGFR: 60-89	947	870	822	729
eGFR: 30-59	452	385	340	305

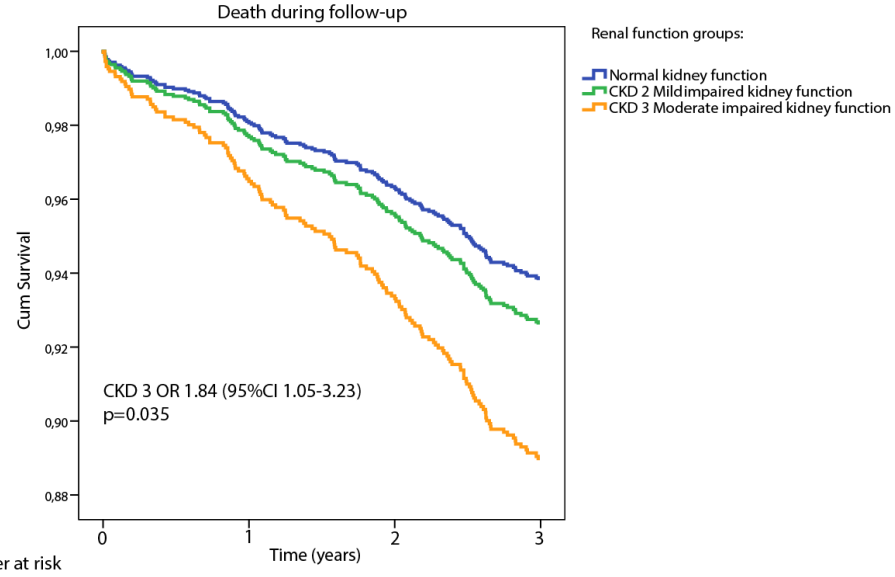
Major cardiovascular events during 3 year follow-up. CKD 3 OR 1.75 (95%CI 1.08-2.85 p=0.024) corrected for age, sex, smoking status, diabetes, hypertension, history of coronary artery disease, symptom status, and lipid levels.

Table 4. Plasma protein levels in patients with normal kidney function (eGFR ≥ 90 ml/min/1.73 m^2), mildly impaired kidney function (eGFR 60-89 ml/min/1.73 m^2) and moderately impaired kidney function (eGFR 30-59 ml/min/1.73 m^2).

Plasma protein	n	eGFR ≥ 90 ml/ min/1.73 m^2	eGFR 60-89 ml/ min/1.73 m^2	eGFR 30-59 ml/ min/1.73 m^2	P-value
RANTES (ng/ml)	1028	3.82 [3.57-4.09]	3.77 [3.63-3.92]	3.85 [3.63-4.07]	0.841
OPG (ng/ml)	1054	1.77 [1.67-1.87]	1.74 [1.68-1.80]	1.93 [1.84-2.01]	0.002
MCP-1 (pg/ml)	580	85.54 [69.93-104.61]	91.29 [82.25-101.26]	80.72 [69.05-94.40]	0.429
VEGFA (pg/ml)	902	440.54 [367.10-529.16]	416.13 [377.28-458.59]	410.76 [354.08-476.09]	0.830
hsCRP (ug/ml)	1055	4.21 [3.58-4.94]	3.66 [3.35-4.00]	4.53 [3.96-5.18]	0.022

Values presented as mean [95% CI]. Associations are corrected for sex, age and year of surgery. Abbreviations: OPG, Osteoprotegerin; MCP-1, monocyte chemoattractant protein-1; VEGFA, Vascular endothelial growth factor A. Bold values were considered statistically significant with a P<0.05.

Figure 3. Hazard curves for all-cause mortality after carotid endarterectomy.



Number at risk				
eGFR: ≥ 90	325	312	299	271
eGFR: 60-89	947	910	866	779
eGFR: 30-59	452	411	378	339

Death during 3 year follow-up. CKD 3 OR 1.84 (95%CI 1.05-3.23 p=0.035) corrected for age, sex, smoking status, diabetes, hypertension, history of coronary artery disease, symptom status, and lipid levels.

DISCUSSION

To our knowledge, this is the first large atherosclerotic plaque study reporting on associations between kidney function and atherosclerotic plaque composition. We observed a higher prevalence of fibrous-atheromatous lesions and intraplaque hemorrhage in patients with decreased kidney function. These histological plaque features are considered to characterize the vulnerability of atherosclerotic lesions. In our cohort of patients undergoing carotid endarterectomy, we confirmed that impaired kidney function associated with poor outcome during follow up. However, we could not find supportive evidence for a role of inflammation as neither inflammatory plaque characteristics nor inflammatory plaque proteins were associated with decreased kidney function.

We found that plaque hemorrhage was increasingly prevalent in patients with impaired kidney function .[19,22] Plaque hemorrhage is believed to result from disrupted micro vessels that are formed in response to plaque growth. In atherosclerotic plaques there is a relative shortage of support tissue such as collagen and extracellular components which results in leaky or disrupted endothelial linings of newly formed microvessels.[23,24] Earlier work

within our biobank revealed that plaque hemorrhage is the only histological plaque feature that independently predicts MACE during follow-up, yet this finding was restricted to males only.[25] We tested whether the increased risk for MACE in CKD-patients may be explained by the increased incidence of plaque hemorrhage, but found that both plaque hemorrhage and CKD are independent risk factors. This implicates that CKD is associated with MACE through alternative mechanisms.

We hypothesized that inflammation could be such an alternative mechanism, as disease progression in both atherosclerosis and CKD are strongly driven by inflammatory processes. However, associations of decreased kidney function with inflammatory histological plaque features were absent or inconsistent. Neither did we find an association between CKD and calcifications, a characteristic that is commonly used as reported as a strong prognostic marker of coronary atherosclerotic plaque burden. In Autopsy studies a high prevalence of calcifications of the vessel wall among patients with CKD has been reported which is considered a strong prognostic marker of coronary atherosclerotic plaque burden. In both univariate and multivariate regression models such association were not found suggesting that other underlying mechanisms such as plaque hemorrhage are responsible for accelerated progression of atherosclerotic plaques in patients with CKD.[7,26,27]

In addition, we investigated the plaque proteome of patients with and without CKD. These analyses revealed that patients with decreased kidney function have an up regulation of the classical complement pathway and the intrinsic coagulation pathway. The classical complement system has been suggested to have a stabilizing effect on plaques by promoting clean-up of apoptotic cells and cell debris, and hereby removing the inflammatory triggers within the arterial wall.[28,29] Although the complement pathway was upregulated in CKD patients, this was contradictory with our histology results which showed increased risk of plaque features that are considered to represent a vulnerable atherosclerotic plaque type in patients with CKD. Pathway analyses also revealed alterations in the coagulation pathway, which is in line with previous reports that showed patients with CKD have altered coagulation pathways.[30] Furthermore, alterations in coagulation are in line with our main finding that plaque hemorrhage was more prevalent in patients with CKD. These results will have to be interpreted with caution, since we observed differences in the prescription of anti-coagulants and aspirin between kidney function groups in the current cohort. To correct for differences in medication-use possibly driving our effect we added anticoagulant-use to the multivariate model. Moreover, when we tested the association of anticoagulant-use with presence of intraplaque hemorrhage, such association was not found (data not included).

Our results are of clinical relevance, as recent clinical trials show a possible therapeutic target in inhibition of inflammation. In a relatively small cohort of 42 CKD patients, IL-1 inhibition improved the brachial artery flow-mediated dilatation.[10] Targeting IL-1βin a high-risk cardiovascular patient cohort of more than 500 patients decreased levels of hsCRP and IL-6 after four months of follow-up.[11] Targeting inflammation in CKD patients has resulted in beneficial short-term results on vascular function however no long-term effects of anti-inflammatory drugs in CKD patients have been published until date. Moreover data

on decreased manifestations of CVD such as MI, stroke and cardiovascular death in patients treated with anti-inflammatory drugs are still lacking. We found inconsistent associations between decreased kidney function and systemic inflammatory proteins. This contradicts earlier studies that showed a significant increase in systemic inflammatory proteins.[31] Our data suggests that increased systemic inflammation is not the primary cause for increased risk of CVD. Highlighting the lack of robust data on the long-term effect of anti-inflammatory drugs in CKD patients combined with the absence of a role for inflammation in the present study, our findings suggest that targeting the inflammatory pathway in CKD patients would not result in a significant decrease in morbidity and mortality.

Since no strong consistent associations was found between eGFR and inflammatory plaque markers additional analyses is performed for all patients that had a major cardiovascular adverse event during three year follow-up. Since we hypothesized that inflammation is a key driver of increased morbidity and mortality during follow-up in patients with decreased kidney function, we aimed to investigate the associations between plaque histology and eGFR in this subset of our cohort. In total 235 patients (13.5%) developed a major cardiovascular adverse event during follow-up and associations of plaque characteristics and inflammatory plaque proteins with eGFR are reported in Supplemental Table 3 and 4. No associations of eGFR with binominal or continuous plaque characteristics were observed for patients that developed a major cardiovascular event during three year follow-up. No consistent increase in inflammatory plaque proteins was observed with decreasing kidney function. A statistically significant difference between Interleukin 4, 5 and 12 was observed between different kidney function groups however these differences did not show a gradual decline or increase over different kidney function groups.

This study suffers from several limitations. First, we describe a large cohort of patients that underwent CEA, with a wide variation in eGFR. However, because patients had to be fit for surgery, only few patients with end stage kidney failure or dialysis could be identified. Conclusions concerning atherosclerotic plaque composition and inflammatory status for this highly vulnerable patient group can therefore not be inferred from this study. However the distribution of kidney function in our cohort is a good reflection of elderly patients in the general population affected by atherosclerotic disease. Second, eGFR estimated by the MDRD equation has its limitations. Ideally eGFR would have been measured at multiple time points so patients are grouped based on multiple measurements narrowing the chance that patients are wrongfully classified. Last, no measures of urinary protein excretion were available which can potentially drive cardiovascular outcomes during follow-up in our cohort.

In conclusion, in patients suffering from carotid artery disease, decreased kidney function was associated with intraplaque hemorrhage and poor secondary outcome but not with inflammatory histological plaque characteristics after carotid endarterectomy. Furthermore, the current data suggests that plaque complement and coagulation pathways are involved in subsequent poor outcome in patients with decreased kidney function and severe atherosclerotic disease. Our data suggests that mechanisms other than inflammation explain the poor cardiovascular outcome in patients with impaired kidney function following

carotid endarterectomy. Future efforts on reducing cardiovascular disease burden in CKD patients may not merely address the reduction of inflammation driven disease progression. The current study shows that other effectors such as intraplaque hemorrhage offer potential therapeutic targets to reduce atherosclerotic disease progression in CKD patients.

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SUPPLEMENTAL MATERIAL

Supplemental Figure 1. Plaque and plasma protein levels in patients with normal kidney function (CKD1), mildly impaired kidney function (CKD2) and moderately impaired kidney function (CKD3)

Supplemental Figure 2. Pathway derived from Ingenuity Pathway analysis. Coagulation system and upregulation of intrinsic pathway with decreasing eGFR.

Supplemental Figure 3. Pathway derived from Ingenuity Pathway analysis. Complement system and upregulation of classical pathway with decreasing eGFR.

Supplemental Table 1. Alphabetically ordered list of plaque proteins correlated with eGFR in 40 patients.

Supplemental Table 2. Ingenuity pathway Analysis results reveals enrichment of the coagulation and complement system.

Supplemental Table 3. Histological atherosclerotic plaque characteristics of patients that did develop major adverse cardiovascular events during three year follow-up (n=235).

Supplemental Table 4. Plaque and plasma protein levels in patients with normal kidney function (eGFR ≥ 90 ml/min/1.73 m^2), mildly impaired kidney function (eGFR 60-89 ml/min/1.73 m^2) and moderately impaired kidney function (eGFR 30-59 ml/min/1.73 m^2) of patients with major adverse cardiovascular events during three year follow-up.

SUPPLEMENTAL METHODS

Supplemental material is omitted because of space limitations.

7

Testosterone to estradiol ratio reflects systemic and plaque inflammation and predicts future cardiovascular events in men with severe atherosclerosis

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ABSTRACT

Aims

The effects of testosterone on cardiovascular disease (CVD) as reported in literature have been ambiguous. Recently, the interplay between testosterone and estradiol as assessed by testosterone/estradiol (T/E2) ratio was suggested to be better informative on the normal physiological balance. Considering the role in CVD, we hypothesized that a low T/E2 ratio in men with CVD is associated with increased inflammation, a more unstable plaque and a worse cardiovascular outcome.

Methods and results

Testosterone and estradiol concentrations were determined in blood samples of 611 male carotid endarterectomy patients included in the Athero-Express Biobank Study. T/E2 ratio was associated with baseline characteristics, atherosclerotic plaque specimens, inflammatory biomarkers and three-year follow-up information. Patients with low T/E2 ratio had more unfavorable inflammatory profiles compared to patients with high T/E2 as observed by higher levels of C-reactive protein (CRP) (2.81 μg/mL vs. 1.22 μg/mL ($p<0.001$)) and higher leukocyte counts ($8.98*10^9$/L vs. $7.75*10^9$/L ($p=0.001$)) in blood. In atherosclerotic plaques, a negative association between T/E2 ratio and number of neutrophils (B=-0.366($p=0.012$), plaque calcifications (OR: 0.816($p=0.044$)), interleukin-6 (IL-6) (B=-0.15 $p=0.009$) and Il-6receptor (B=-0.13 $p=0.024$) was found. Furthermore, in multivariate Cox regression analysis, low T/E2 ratio was independently associated with an increased risk for major cardiovascular events (MACE) during three-year follow-up (HR 1.67(95%CI: 1.02-2.76) $p=0.043$). In men with elevated body mass index, these effects were strongest.

Conclusions

In male patients with manifest atherosclerotic disease, low T/E2 ratio was associated with increased systemic inflammation, increased inflammatory plaque proteins and an increased risk of future major cardiovascular events as compared to men with normal T/E2 ratio. These effects are strongest in men with elevated body mass index and are expected to be affected by aromatase activity in white fat tissues. Normalization of T/E2 ratio may be considered as target for the secondary prevention of CVD in men.

INTRODUCTION

The potential harmful and protective effects of sex hormones on cardiovascular disease (CVD) progression have been a matter of controversy over the last decades. The late onset of CVD in women, when compared to men, has been attributed to the protective effects of estrogen during reproductive years and is lost after menopause.[1] In males, low levels of circulating testosterone have been associated with an increased risk of atherosclerotic manifestations, but results have been inconsistent and contradictory.[2,3] Normalization of testosterone levels in a large cohort of elderly male veterans has shown convincing results with a significant reduction in all-cause mortality, myocardial infarction and stroke.[4] That said, the underlying pathophysiological phenomenon leading up to these health benefits remains poorly understood.

A possible shortcoming of previous studies reporting on the effects of sex hormones on CVD is that these studies often focused on the individual effects of either testosterone or estradiol. Synergistic effects or co-dependency of estradiol on testosterone are thereby missed. In male patients, for instance, most estradiol production occurs via conversion of testosterone to estradiol through aromatase in white adipose tissue, making estradiol dependent of both circulating free testosterone and aromatase.[5] The interplay between these two key players on vascular function can be assessed by calculating the ratio of testosterone to estradiol (T/E2) in males or the estradiol to testosterone ratio (E2/T) in females.

The first results of focusing on T/E2 ratio in males and E2/T ratio in females on CVD have been promising and show that a disturbance of the normal physiological balance could contribute to CVD progression.[6–8] A T/E2 imbalance rather than the absolute levels of androgens associated with the development of coronary artery disease (CAD) in a cohort of 115 male patients.[7] Furthermore, androgen replacement therapy with the aim to restore an adequate E2/T ratio suppressed the development of atherosclerosis by reducing lipid lesions, decreasing endothelial cell injury, modulating the coagulation system and reduction of inflammatory processes.[6] Another recent study focusing on soluble glycoprotein130 (SGP130), a natural antagonist of interleukin-6 (IL6) trans-signaling, showed a strong positive correlation with T/E2 ratio in 254 male CAD patients.[8] Although these results seem compelling they have been obtained in small studies or in experimental animal models only.

In order to investigate the interplay of sex hormones and CVD, we investigated the underlying culprit of CVD, namely the atherosclerotic plaque, and sex hormones in a cohort of 611 men undergoing carotid endarterectomy (CEA). We studied histological plaque characteristics, inflammatory plaque proteins and blood biomarkers together with three-year follow-up and their association with T/E2 ratio in these men.

MATERIALS AND METHODS

Study population

The Athero-Express Biobank Study (AE) is a prospective ongoing biobank, which includes blood and atherosclerotic plaque specimens as well as extensive baseline characteristics and three-year follow-up data from patients undergoing carotid endarterectomy. The study design is described previously in more detail.[9,10] In brief, since 2002 all patients scheduled for CEA in the St Antonius Hospital Nieuwegein or the University Medical Centre Utrecht (UMC Utrecht) were asked to participate in the AE. Indications for CEA were reviewed by a multidisciplinary vascular team and experienced surgeons performed the endarterectomy in accordance with local and international guidelines.[11–14] Patient characteristics are obtained through standardized preoperative questionnaires and from hospital medical records. Medical ethics committees of both participating centers approved the study protocol, and all of the included patients provided written informed consent. The study is conducted in accordance with the declaration of Helsinki. All males with sex-hormone level measurements above the detection limit and complete three year follow-up were included in the present study. Atherosclerotic plaques specimens, blood, and patient characteristics were collected between 2002 and 2016.

Tissue collection and plaque processing

Atherosclerotic plaque specimens collected during CEA were immediately processed after surgery according to a standardized protocol. Briefly, after removal, the carotid plaques were divided into cross-sectional segments of 5 mm thick. The segment with the greatest plaque burden was classified as a culprit lesion and subjected to histological examinations. The culprit lesion was routinely stained for assessment of calcification (hematoxylin-eosin), collagen (picrosirius red), lipid core (hematoxylin-eosin, picrosirius red), macrophages (CD68), smooth muscle cells (βactin), intraplaque haemorrhage (hematoxylin-eosin, Elastin von Gieson), vessel density (CD34) and neutrophils (CD66b)[10]. The amount of collagen and calcification were scored semi-quantitatively at a 40× microscopic magnification and grouped in no/minor and moderate/heavy staining. Computerized analyses were used to quantify macrophages and smooth muscle cells infiltration. The size of lipid core was assessed using polarized light and plaques with lipid-content >40% were classified as atheromatous. CD34-positive microvessels were scored in the three areas of the plaque with the highest microvessel density and expressed as an average number of vessels per hot spot.[15] The number of neutrophils was determined by counting the CD66b-positive cells per plaque, as described before.[10] Figure 1 shows one example of a single atherosclerotic plaque stained for histological assessment. All histologic slides were assessed and showed good inter- and intra-observer variability (β0.6-0.9).[16] Additional cytokine and chemokine protein levels were measured in a selection of atherosclerotic plaque specimens (n=333). Measurements were performed in isolates after Tripure (Roche) protein isolation. IL-6, interleukin-6receptor (IL-6R), interleukin-8 (IL-8), adiponectin, macrophage colony stimulating factor 1 (MCSF), and monocyte chemotactic protein 1

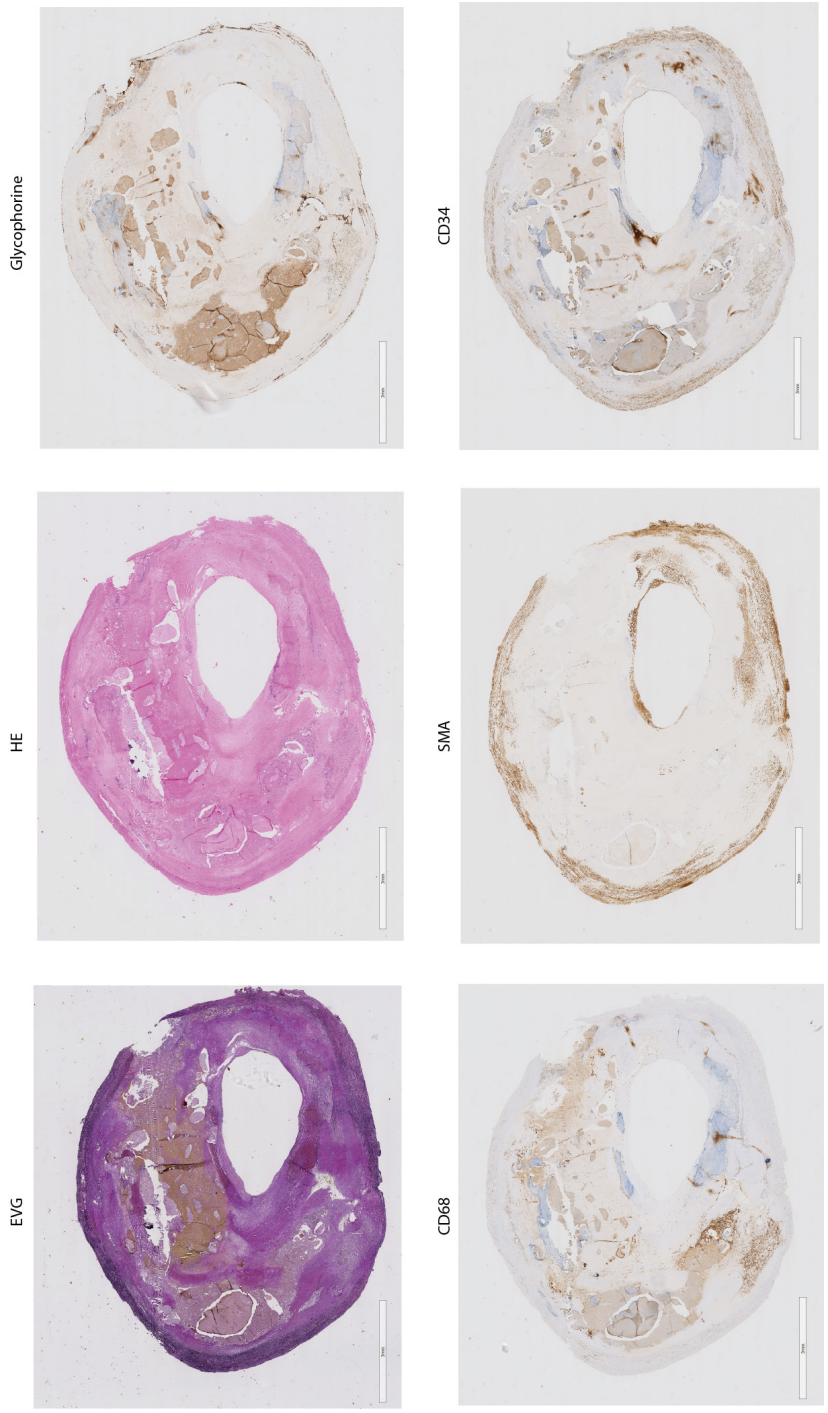


Figure 1. Example of one single atherosclerotic plaque stained for histological assessments. Panels showing an example of a culprit carotid lesion stained for the presence of Elastin von Gieson (EVG), hematoxylin-eosin (HE), glycophorine, macrophages (CD68), smooth muscle cells (SMA(α-actin)) and vessel density (CD34).

(MCP1) levels were measured at the in-house Luminex core facility (Whilhemina Children`s Hospital, UMC Utrecht, the Netherlands).

Blood collection and sex-hormone measurements

All blood samples were collected prior to surgical incision. In 611 male patients sufficient blood was available for measurements of both sex hormones. Preoperative lithium-heparin EDTA plasma, stored at -80 degrees Celsius, was used for the sex hormone measurements. Plasma testosterone and estradiol were measured by immunoassay on an ARCHITECT ci8200 system (Abbott Diagnostics, Abbott Laboratories, USA) at the Laboratory of Clinical Chemistry and Hematology of the University Medical Centre Utrecht. The testosterone to estradiol ratio was calculated using the following formula: Testosterone/(10*Estradiol).

Hematological measurements

For this study data from the Utrecht Patient-Oriented Database (UPOD) was used. UPOD is an infrastructure of relational databases comprising data on patient characteristics, hospital discharge diagnoses, medical procedures, medication orders and laboratory tests for all patients treated at the University Medical Center Utrecht since 2004. All hematological parameters were measured using the Abbott CELL-DYN Sapphire. The CELL-DYN is an automated hematology analyzer using laser light scattering, electrical impedance and spectrophotometry to characterize morphological traits of white blood cells, red blood cells, and platelets. Irrespectively of the clinician's request, the CELL-DYN will perform a complete blood count, only the requested results are reported to the physician, however, all results are stored in UPOD for research purposes. UPOD data acquisition and management is in accordance with current regulations concerning privacy and ethics. The structure and content of UPOD have been described in more detail previously.[17] For the current study hematological measurements were selected within a one-month preoperative range. Measurement closest to the moment of surgery was used for analyses. In 351 males a preoperative blood draw was available for analysis.

Follow-up

After the initial procedure, patients included in the AE undergo three-year follow-up. Three-year follow-up data were obtained through annual questionnaires addressing the occurrence of cardiovascular events and/or hospitalizations. If a questionnaire is answered positively, cardiovascular events were validated using hospital data systems or health records kept by the general practitioner. Furthermore, all events were assessed by two members of an outcome assessment committee. If patients did not respond, their general practitioner was contacted. A major adverse cardiovascular event (MACE) was defined as myocardial infarction (MI), stroke or cardiovascular death. Cardiovascular death was defined as one of the following: fatal MI, fatal stroke (either bleeding or ischemic), fatal ruptured abdominal aneurysm, fatal heart failure, or sudden death. In patients who reached multiple endpoints during follow-up, only the first manifestation of a cardiovascular event was used for analysis.

Statistical analyses

Both sex-hormone levels and clinical follow-up were available for 611 patients. Patients were stratified into quartiles based on their calculated T/E2 ratio; Q1: <0.9878 (n=152), Q2; 0.9878 – 1.4191 (n=153), Q3; 1.4192 – 1.8916 (n=153) and Q4; >1.8916 (n=153). A flowchart of patient stratification and studies performed was added to the supplemental (Supplemental Figure 1). Plaque characteristics were available for 500 patients included in this study. Chi-square tests were used to compare categorical baseline characteristics of patients across the different T/E2 ratio groups, one-way ANOVA for continuous, normally distributed variables and Kruskall-Wallis tests for continuous, non-normally distributed variables. To test the association between the calculated T/E2 ratio and plaque characteristics, multivariate logistic regression analyses with correction for possible confounders were performed. Baseline characteristics that differed in univariate analyses over the T/E2 ratio groups ($p<0.2$) and associated with the plaque characteristic of interest ($p<0.2$) were considered possible confounders. Furthermore, to examine the risk of future cardiovascular events in relation to the testosterone to estradiol ratio, a multivariable cox proportional hazard model was used. Due to relative small power in the survival analyses, Q1 + Q2 were grouped together to serve as the low T/E2 ratio group and Q3 + Q4 were grouped together to serve as the high T/E2 ratio group. Possible confounders were selected based on both a univariable analysis and empirical evidence. Variables that were added to the final model were: age, BMI, diabetes mellitus, kidney function, contralateral stenosis, HDL-cholesterol, and history of CAD. Measured levels of plaque cytokines and chemokines required logarithmic transformation in order to enter them into linear regression models. The effect of T/E2 ratio on the different outcomes is expected to be affected by aromatase in white adipose tissue. Favorably this aromatase activity would be quantified and added to the multivariate models. Unfortunately this metabolic activity is hard to measure and therefore interpretation of these analyses should be done with acknowledgement of this serious limitation. The BMI, however, is a good measure for body composition and is likely to be related with adipose tissue. In order to appreciate this interplay between T/E2 ratio, body composition and cardiovascular events, BMI stratification analyses were performed after BMI interaction was proven to be highly significant. Missing data was handled by multiple imputation in order to prevent incomplete case analyses in multivariate models.[18] All statistical tests were performed in SPSS version 21. A two-sided *P* value < 0.05 was considered significant.

RESULTS

Baseline characteristics

Baseline characteristics of the 611 male patients stratified by T/E2 quartiles are presented in Table 1. Moreover in Supplemental Figure 2 a scatterplot was added demonstrating the testosterone and estradiol concentrations across the different T/E2 quartiles. Mean testosterone and estradiol concentrations were 12.3 nmol/L (SD±5.56) and 92.8 pmol/L (SD±38.44), respectively. Testosterone concentrations in the study population were low

but normally distributed. The low concentrations of testosterone in the study population are in line with testosterone concentrations of men the same age in the general population [19]. Age and most other cardiovascular risk factors, such as smoking and hypertension, did not differ across the different T/E2 ratio groups. Patients with low T/E2 ratios had a high body mass index (BMI) (p<0.001) and more often had comorbidities associated with overweight such as diabetes mellitus. Conversely, patients with high T/E2 ratio had higher LDL, HDL and total cholesterol levels while triglyceride levels were comparable in all groups. Indication for surgery (i.e. asymptomatic, transient ischemic attacks (TIA), Amaurosis Fugax (AFX) or stroke) did not differ across the different T/E2 ratio groups.

Table 1. Baseline characteristics of 611 male patients stratified by T/E2 ratio quartiles.

Patient characteristics	**Q1: <0.9878** *n* = 152	**Q2: 0.9878 – 1.4191** *n* = 153	**Q3: 1.4192 – 1.8916** *n* = 153	**Q4: >1.8916** *n* = 153	**P-value**
Age (SD)(years)	69.0 (10.1)	69.8 (8.2)	69.4 (8.8)	69.6 (8.3)	0.853
BMI (SD)	27.3 (3.8)	26.9 (3.5)	26.0 (3.4)	25.4 (3.1)	**<0.001**
Glucose (SD) (mmol/L)	6.76 (1.92)	6.86 (2.22)	6.44 (1.66)	6.37 (1.45)	0.196
eGFR (SD) (mL/min/1.71m2)	71.96 (24.96)	72.48 (20.88)	76.04 (16.79)	75.92 (18.41)	0.191
Hypertension, yes(n)	106/145 (73.1)	105/147 (71.4)	101/150 (67.3)	114/151 (75.5)	0.425
Hypercholesterolemia, yes(n)	99/137 (72.3)	104/136 (76.5)	89/139 (64.0)	100/140 (71.4)	0.146
Diabetes mellitus, yes(n)	42/152 (27.6)	41/153 (26.8)	35/153 (22.9)	25/153 (16.3)	0.079
Current smoker, yes(n)	51/150 (34.0)	50/153 (32.7)	54/153 (35.3)	40/151 (26.5)	0.367
History of CAD, MI or coronary intervention, yes(n)	54/152 (35.5)	54/153 (35.3)	47/153 (30.7)	40/153 (26.1)	0.244
Total cholesterol (mmol/L) (SD)	3.71 (0.99)	3.91 (1.01)	4.10 (0.96)	4.36 (1.06)	**<0.001**
LDL cholesterol (mmol/L) (SD)	1.98 (0.73)	2.12 (0.73)	2.35 (0.76)	2.59 (0.85)	**<0.001**
HDL cholesterol (mmol/L) (SD)	0.91 (0.26)	0.97 (0.26)	1.03 (0.28)	1.08 (0.28)	**<0.001**
Triglycerides (mmol/L) (SD)	1.73 (1.16)	1.78 (0.99)	1.66 (0.87)	1.60 (0.79)	0.391
Hormone levels					
Testosterone (nmol/L) (SD)	7.56 (4.04)	11.60 (3.48)	13.58 (3.90)	16.26 (6.32)	-
Estradiol (pmol/L) (SD)	112.10 (49.1)	102.26 (30.36)	88.47 (25.20)	68.41 (30.04)	-
Clinical presentation					
Ø Asymptomatic, (n)	25/151 (16.6)	25/152 (16.4)	24/151 (15.9)	20/152 (13.2)	0.836
Ø Ocular symptoms, (n)	25/151 (16.6)	28/152 (18.4)	19/151 (12.6)	24/152 (15.8)	
Ø TIA, (n)	64/151 (42.4)	63/152 (41.4)	65/151 (43.0)	60/152 (39.5)	
Ø Stroke, (n)	37/151 (24.5)	36/152 (23.7)	43/151 (28.5)	48/152 (31.6)	
Pre-operative medication use					
Statin use, yes(n)	127/152 (83.6)	126/153 (82.4)	120/153 (78.4)	120/153 (78.4)	0.577
Antiplatelet use, yes(n)	132/152 (86.8)	132/153 (86.3)	135/152 (88.8)	133/152 (87.5)	0.920
Anticoagulant use, yes(n)	26/152 (17.1)	18/153 (11.8)	14/153 (9.2)	14/153 (9.2)	0.103

Abbreviations: BMI, Body Mass Index; CAD, Coronary Artery Disease; eGFR, estimated Glomerular Filtration Rate; HDL, High-Density Lipoprotein; LDL, Low-Density Lipoprotein; MI, Myocardial Infarction; TIA, Transient Ischemic Attack. Bold values were considered statistically significant with a p < 0.05.

Plasma biomarkers

Plasma inflammatory biomarkers stratified by T/E2 ratio groups are presented in Table 2. Patients within the lowest T/E2 ratio group had higher levels of C-reactive protein in comparison to the other T/E2 ratio groups (p<0.001). Moreover, the measurements showed a consistent direction of effect over all ratio groups. Patients with low T/E2 ratio had higher number of blood leukocytes when compared to patients with high T/E2 ratio (p=0.001). The higher leukocyte counts in the blood of patients in the lowest T/E2 ratio group can be attributed to the higher number of neutrophils (p=0.005) and monocytes (p=0.038). The neutrophil-to-lymphocyte ratio which is a strong predictor for future adverse events and reflects inflammatory status in CVD patients, was lowest in patients with high T/E2 ratio and showed an direction of effect over the four groups, however not reaching statistical significance (p=0.385) [20].

Plaque histology

Results from univariate and multivariate plaque analyses are presented in Table 3. In univariate analyses, T/E2 ratio was negatively associated with plaque calcifications, the percentage of macrophage staining, and the total number of plaque neutrophils. After multivariate correction T/E2 ratio was independently associated with moderate/heavy plaque calcifications and the total number of neutrophils per plaque. In addition, even though not reaching statistical significance, six out of eight plaque characteristics showed negative trends of effect with T/E2 ratio. Moreover, one important plaque characteristic showing a positive trend with T/E2 ratio was collagen staining, this is the plaque feature best characterizing stable atherosclerotic lesions. To test the overall direction of effect of T/E2 ratio and features associated with the vulnerability of the atherosclerotic plaque we performed binomial testing. This correlation showed a negative association of T/E2 with vulnerable plaque features (p=0.031).

Table 2. Inflammatory blood biomarkers of patients in the different T/E2 ratio quartiles.

	Q1: <0.9878	**Q2: 0.9878 – 1.4191**	**Q3: 1.4192 – 1.8916**	**Q4: >1.8916**	**P-value**
Plasma proteins	*n* = 121	*n* = 131	*n* = 126	*n* = 114	
CRP (µg/mL) median [IQR]	2.81 [1.37-6.26]	1.78 [0.78-3.67]	1.44 [0.72-2.84]	1.22 [0.57-2.86]	**<0.001**
Blood counts	*n* = 69	*n* = 86	*n* = 91	*n* = 105	
Number of leukocytes in blood (10^9/L)	8.98 (2.60)	7.75 (1.66)	8.24 (2.06)	7.75 (2.24)	**0.001**
Number of lymphocytes in blood (10^9/L)	2.18 (0.90)	1.94 (0.67)	2.03 (0.65)	2.00 (0.68)	0.216
Number of neutrophils in blood (10^9/L)	5.77 (2.24)	4.85 (1.29)	5.23 (1.74)	4.86 (2.03)	**0.005**
Number of monocytes in blood (10^9/L)	0.75 (0.27)	0.68 (0.22)	0.73 (0.22)	0.66 (0.18)	**0.038**
Neutrophil to lymphocyte ratio, median [IQR]	2.81 [1.37 – 6.26]	1.78 [0.78 – 3.67]	1.44 [0.72 – 2.84]	1.22 [0.57 – 2.86]	0.385

Abbreviations: CRP; C-Reactive Protein. Bold values were considered statistically significant with a p < 0.05.

Table 3. Histological atherosclerotic plaque characteristics of 500 male CEA patients.

Binominal carotid plaque characteristics	**P-value Univariate**	**Odds ratio unadjusted**	**[95%CI]**		**P-value Multivariate**	**Odds ratio adjusted**	**[95%CI]**	
Presence of lipid core ≥40%	0.165	0.885	0.744	1.052	0.184	0.876	0.720	1.065
Moderate/heavy calcifications	**0.004**	0.784	0.666	0.924	**0.044**	0.816	0.670	0.994
Moderate/heavy collagen	0.682	0.962	0.798	1.159	0.774	1.031	0.839	1.265
Presence of intraplaque hemorrhage	0.292	0.914	0.774	1.080	0.940	0.993	0.816	1.207
Continuous carotid plaque characteristics	**P-value Univariate**	**Beta Unadjusted**	**[95%CI]**		**P-value Multivariate**	**Beta adjusted**	**[95%CI]**	
Mean number of microvessels per hotspot	0.551	0.029	- 0.415	0.776	0.381	0.047	- 0.341	0.888
% of positive macrophage staining per plaque	**0.001**	- 0.158	- 0.099	- 0.026	0.140	- 0.071	- 0.065	0.009
% of positive SMC staining per plaque	0.206	- 0.060	- 0.085	0.018	0.189	- 0.065	- 0.091	0.018
Total number of neutrophils per plaque	**0.005**	- 0.339	- 1.047	- 0.198	**0.012**	- 0.366	- 1.191	- 0.151

Abbreviations: CI, Confidence Interval; SMC, Smooth Muscle Cell. Odds ratios and regression coefficients represent the difference per increase in T/E2 quartile. Multivariate analyses performed with correction for possible confounders. Bold values were considered statistically significant with a $p < 0.05$.

Table 4. Inflammatory plaque proteins of 333 patients in the different T/E2 ratio quartiles.

	Q1: <0.9878	**Q2: 0.9878 – 1.4191**	**Q3: 1.4192 – 1.8916**	**Q4: >1.8916**
Plaque protein concentrations	*n* = 88	*n* = 100	*n* = 75	*n* = 70
Interleukin-6 (pg/ug) (SD)	0.14 (0.37)	0.19 (0.68)	0.07 (0.07)	0.05 (0.06)
Interleukin-6 receptor (pg/ug) (SD)	0.21 (0.25)	0.20 (0.22)	0.15 (0.14)	0.14 (0.12)
Interleukin-8 (pg/ug) (SD)	0.32 (0.62)	0.60 (1.52)	0.29 (0.32)	0.38 (0.62)
Adiponectin (pg/ug) (SD)	65.69 (119.58)	70.74 (79.05)	71.33 (69.73)	88.98 (124.11)
MCSF (pg/ug) (SD)	0.08 (0.07)	0.06 (0.05)	0.06 (0.06)	0.07 (0.07)
MCP1 (pg/ug) (SD)	0.58 (0.78)	0.66 (0.98)	0.61 (0.54)	0.54 (0.43)

Abbreviations: IL-6; Interleukin 6, IL-6R; interleukin-6 receptor, IL-8; interleukin 8, MCSF; Macrophage colony stimulating factor 1, MCP1; Monocyte chemotactic protein 1.

Plaque proteins

Proteins were isolated from 333 atherosclerotic plaque specimens. Plaque protein content stratified by the four T/E2 groups is presented in Table 4. Correlation plots of the different cytokine and chemokine levels with T/E2 ratio are presented in Figure 2. T/E2 ratio showed a negative correlation with Il-6 (B=-0.149 (p=0.009)) and Il-6 receptor (B=-0.127(p=0.024)). IL-8, MCP1 and CSF1 did not show a statistically significant correlation with circulating T/E2 ratio. Lastly adiponectin showed a positive correlation with T/E2 ratio (B=0.166(p=0.003)). Both the negative correlations with IL-6 and IL-6receptor and the positive correlation with adiponectin point towards a more favorable plaque with less inflammation in patients with preserved T/E2 ratio.

Clinical outcomes

For the outcome analyses Q1 and Q2 were binned together into the low T/E2 ratio group and Q3 and Q4 in the high T/E2 ratio group. Clinical follow-up data was available for 611 male patients included in the Athero-Express. During a mean follow-up of 2.7 years, 72 patients (11.8%) reached a major cardiovascular endpoint. Forty patients (6.5%) suffered from stroke, 18 patients endured an MI (2.9%), and in total 24 patients (3.9%) died due to cardiovascular causes. All adverse outcomes and results of the multivariate analyses are presented in Table 5. After correction for possible confounders (age, BMI, diabetes mellitus, eGFR, HDL-cholesterol, contralateral stenosis and history of CAD) with the use of a multivariable Cox regression model, T/E2 ratio was an independent predictor for the occurrence of major cardiovascular events (hazard ratio low T/E2 ratio; 1.67 (95%CI: 1.02-2.76) p=0.043)(Figure 3). Moreover low T/E2 was associated with an increased incidence of stroke during three-year follow-up (HR 2.09 (95%CI: 1.06-4.14) p=0.034) Figure 4). All-cause mortality showed a trend towards poor survival in patients with low T/E2 ratio, which is in line with the increased incidence of major events however not reaching statistical significance (p=0.150)(Figure 5).

BMI interaction and stratified results

In Supplemental Table 1 data of the multivariate model with and without BMI is displayed. The hazard ratio of T/E2 ratio on major events changed from 1.67 to 1.58 with the addition of BMI. This suggests that BMI is not explaining the relation between T/E2 and outcome and is not a confounder. To identify whether the relation of T/E2 on outcome was dependent on high or low BMI, interaction was tested. After a significant interaction was found, the data was stratified for low and high BMI groups (BMI<25 and BMI≥25). The hazard ratio of T/E2 ratio for future MACE is largest in the elevated BMI cohort (hazard ratio low T/E2 ratio; 2.42 (95%CI 1.09 – 5.38 p=0.030) and no longer statistically significant in the non-elevated BMI group (Supplemental Table 2). This indicates that BMI acts as an important effect modifier of T/E2 ratio on future events. Additional stratified plaque protein analyses have been performed and results are shown in Supplemental Figure 3. These figures show that the inverse correlation between T/E2 ratio and pro-inflammatory markers (Il-6 + Il-6r) is highest in the elevated BMI-group while the linear correlation with the anti-inflammatory marker adiponectin is strongest in the normal BMI group. These interaction analyses show that the effects we found on secondary outcome and plaque proteins and strongest in men with elevated BMI.

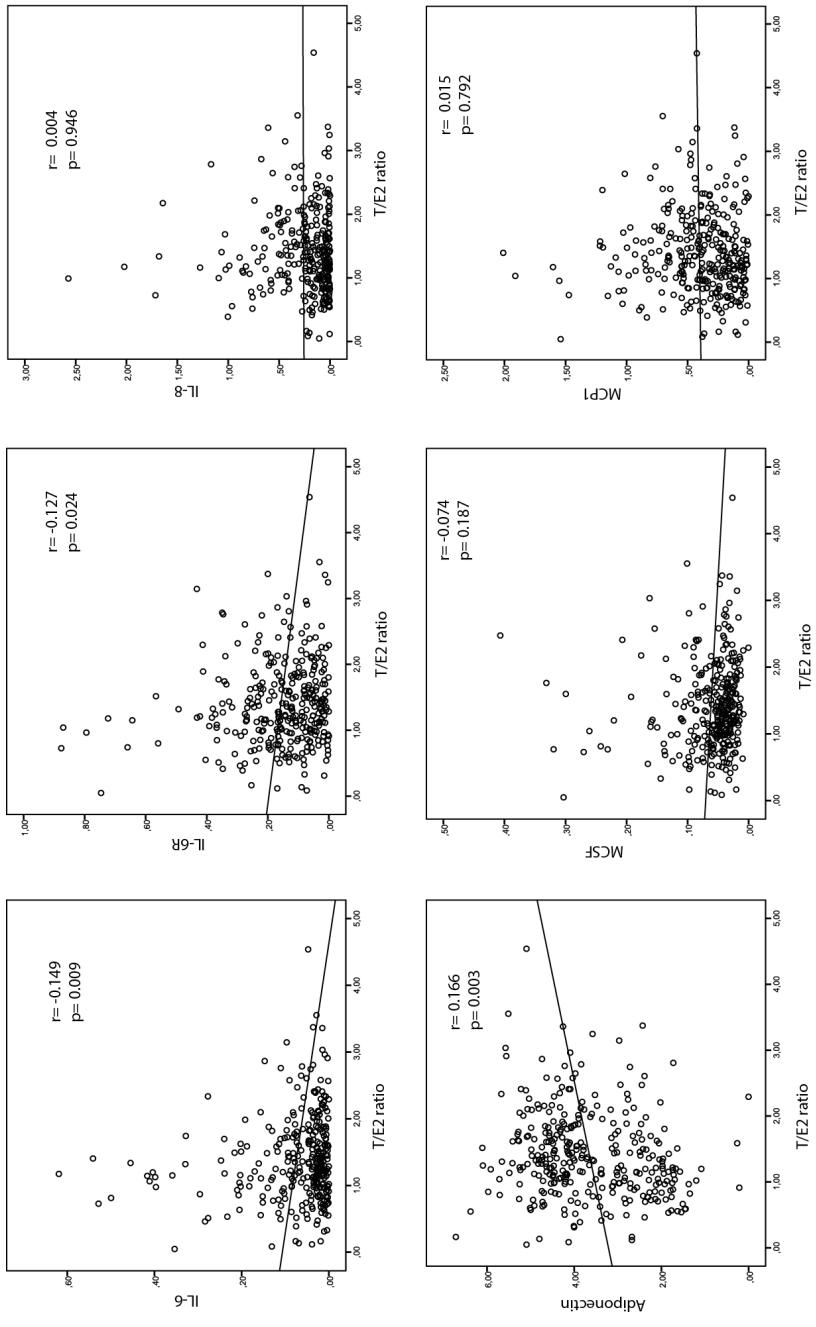


Figure 2. The correlation between T/E2 ratio and plaque proteins.

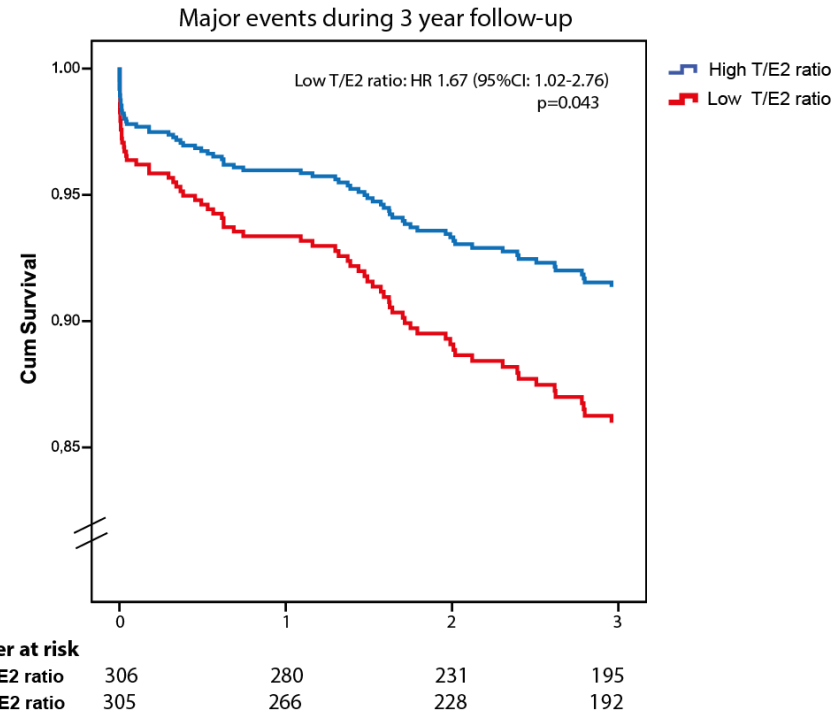
Scatter diagrams illustrate the correlation between circulating T/E2 ratio and cytokine and chemokines in plaques obtained from male patients after carotid endarterectomy (based on log transformed data). IL-6; Interleukin 6, IL-6R; interleukin-6 receptor, IL-8; interleukin 8, MCSF; Macrophage colony stimulating factor 1, MCP1; Monocyte chemotactic protein 1 .

Table 5. Three year clinical outcomes of 611 CEA-patients

	Low T/E2 Ratio (n=305)	High T/E2 ratio (n=306)	Multivariable analysis HR [95%CI]	P-value
EP Major, n(%)	46/305 (15.1%)	26/306 (8.5%)	1.67 (1.02-2.76)	**0.043**
Stroke, n(%)	27/305 (8.9%)	13/306 (4.2%)	2.09 (1.06-4.14)	**0.034**
MI, n(%)	10/305 (3.3%)	8/306 (2.6%)	1.13 (0.43-2.94)	0.804
CV death, n(%)	14/305 (4.6%)	10/306 (3.3%)	1.21 (0.51-2.84)	0.667
All-cause mortality, n(%)	40/305 (13.1%)	25/306 (8.2%)	1.48 (0.87-2.53)	0.150

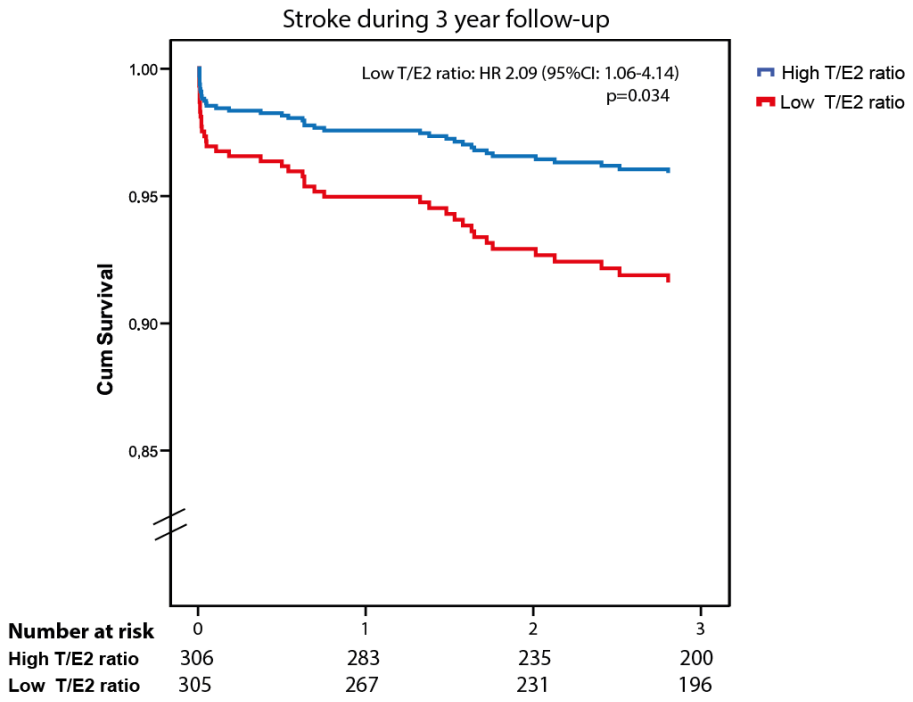
Abbreviations: CV death; cardiovascular death, MI; myocardial infarction. Bold values were considered statistically significant with a $p < 0.05$.

Figure 3. Adjusted event curves for T/E2 ratio and major cardiovascular events after carotid endarterectomy using Cox proportional-hazards model.



Major cardiovascular events during 3 year follow-up. Low T/E2 ratio HR 1.67 (95%CI 1.02 - 2.76 p = 0.043) corrected for age, BMI, eGFR, diabetes mellitus, history of CAD, HDL-cholesterol and contralateral stenosis.

Figure 4. Adjusted event curves for T/E2 ratio and stroke after carotid endarterectomy using Cox proportional-hazards model.

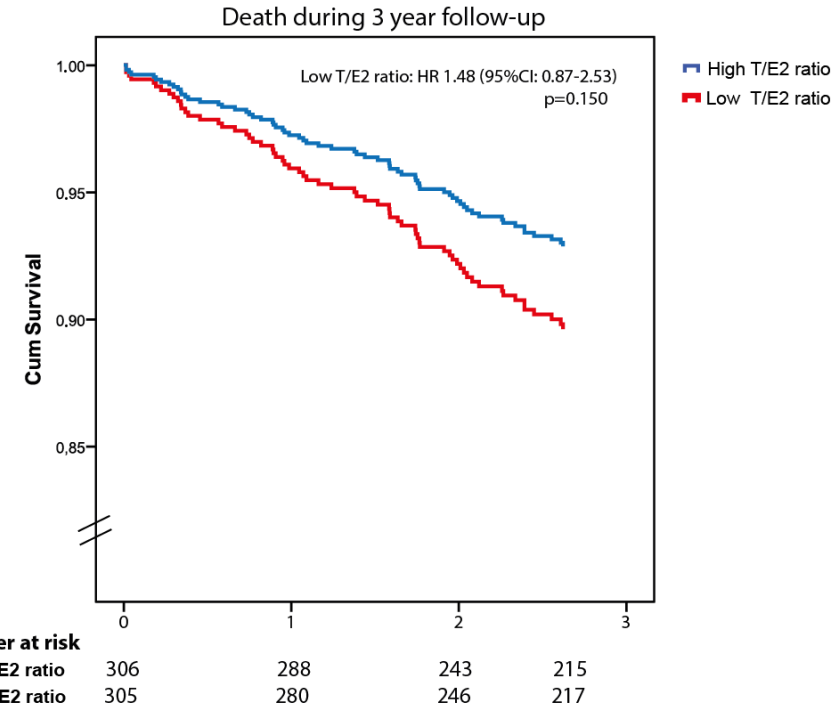


Stroke during 3 year follow-up. Low T/E2 ratio HR 2.09 (95%CI 1.06 – 4.14 p = 0.034) corrected for age, BMI, eGFR, diabetes mellitus, history of CAD, HDL-cholesterol and contralateral stenosis.

Individual sex-hormone analyses

To exhibit the clear added value in using T/E2 ratio over individual hormone measurements additional analyses have been performed for the individual testosterone and estradiol levels and these results were added to the supplemental. As illustrated by Supplemental Tables 3-5, testosterone levels alone were not associated with plaque features and a modest negative correlation with inflammatory markers in the blood was seen. For the individual estradiol measurements as illustrated in Supplemental Tables 6-8, no correlation with inflammatory blood biomarkers was seen but a positive correlation with percentage of smooth muscle cells (B=0.068(p=0.008). In univariate survival analyses, low levels of testosterone were associated with secondary stroke, however, this association lost statistical significance after correction for possible confounders (Supplemental Table 9).

Figure 5. Adjusted event curves for T/E2 ratio and death after carotid endarterectomy using Cox proportional-hazards model.



Death during 3 year follow-up. Low T/E2 ratio HR 1.48 (95%CI: 0.87 - 2.53 p = 0.150) corrected for age, BMI, eGFR, diabetes mellitus, history of CAD, HDL-cholesterol and contralateral stenosis.

DISCUSSION

To our best knowledge, this is the first study reporting on the testosterone/estradiol ratio in a large cardiovascular cohort in which atherosclerotic plaques, patient characteristics, and three-year follow-up were available. The current study shows that men with low T/E2 ratio have an increased risk for development of MACE during three-year follow-up independent of known cardiovascular risk factors. Low T/E2 ratio showed an overall trend towards histological features, which represent the vulnerability of atherosclerotic plaques. Moreover, we show that the T/E2 ratio reflects both systemic and plaque inflammations, as assessed by levels of CRP and leukocyte counts in blood and Il-6 in the plaque, possibly explaining the poor outcome after carotid revascularization. Considering that interaction analyses showed that these effects were predominantly found in men with elevated BMI, our results are expected to be affected by aromatase activity in white fat tissue.

In the last decades, cardiovascular research focusing on the individual effects of testosterone and estradiol has provided inconsistent and contradictory evidence. Both positive and negative associations between the two sex hormones and mortality have been found in men.[21,22] Nevertheless, more recent findings suggest that testosterone and estradiol may have opposing effects on cardiovascular risk factors and the progression of CVD.[23] For instance, low levels of testosterone but high levels of estradiol have been associated with increased intima-media thickness, a widely recognized marker for atherosclerosis.[23] In addition, several studies have demonstrated that testosterone has anti-inflammatory effects, whereas estradiol has pro-inflammatory effects.[24–27] These findings all show that a disrupted hormonal balance is associated with poor vascular function and increased systemic inflammation. It can of course be postulated that these associations are indirect in nature and the T/E2 ratio is merely reflective of het inflammatory processes and aromatase activity in white adipose tissue.

To further expand on our understanding of the interaction between BMI, T/E2 ratio and future MACE we have performed interaction and stratified analyses. We show that BMI is an important effect modifier of T/E2 ratio on future events. Men with elevated BMI and decreased T/E2 ratio had the highest risk of developing future MACE, while in men without elevated BMI this risk was no longer statistically significant. These different effects in patients with different body composition might explain different traits of adipose tissue apart from total volume. Adipose tissue is distributed throughout the human body in two main compartments namely the subcutaneous compartment and the visceral compartment.[28] In males this distribution is typically apple-shaped and represents visceral/abdominal adipose tissue (VAT) while in females fat distribution is more pear-shaped and represents subcutaneous adipose tissue (SAT).[29] The mechanisms by which sex hormones regulate energy balance and adiposity is not fully understood but it has been shown that VAT express higher concentrations of androgen receptors (ARs) while SAT expresses higher concentrations of estrogen receptors (ERs).[30] More importantly, SAT exerts protective effects, while VAT induces harmful metabolic alterations and increases the risk of cardiovascular disease.[31,32] The effects on the cardiovascular system are partially exerted by the endocrine effects of VAT since it expresses key inflammatory players such as TNFβ Il-6 and PAI-1 which strongly affects inflammatory processes throughout the body. SAT, however, is less metabolically active and produces protective effects such as adiponectin and leptin.[33] As presented by the BMI-stratified results it appears that the effects we find are largely driven by T/E2 abnormalities in patients with BMI ≥25. Since all patients are males it is likely that a contributing factor to poor prognosis and increased inflammation is indeed induced by the endocrine effects of VAT. And it is possible that T/E2 ratio reflects the aromatase activity and might therefore be a marker of total VAT volume and increased cardiovascular risk. In overweight men, T/E2 ratio may thus serve as risk marker of VAT and identify high-risk patients that may require more intensive monitoring after cardiovascular interventions.

Recent findings state that T/E2 ratio is associated with the anti-inflammatory protein SGP130, moreover this the same study showed the synergistic effect of testosterone and estradiol on SGP130 levels in culture supernatant of human umbilical vein endothelial cells (HUVECs).[8] The anti-inflammatory properties of SGP130 have mainly been attributed to endogenous inhibition of IL-6 trans-signaling; a decrease in SGP130 would result in a decrease of these anti-inflammatory capacities. To further expand on these mechanisms leading up to diminished vascular function we measured a range of inflammatory cytokines and chemokines in the atherosclerotic plaque. Within the present study we show that T/E2 ratio was inversely associated with IL-6 and IL-6receptor concentrations in the plaque. The increased Il-6 and IL-6receptor concentrations in the atherosclerotic plaque of patients with decreased T/E2 ratio suggests an inflammatory pathway affected by hormonal misbalance. Our results are in line with these studies showing lowered T/E2 ratio associated with decreased vascular function and increased incidence of future CVD onset, however, if these effects are direct through the actions of T/E2 ratio or an indirect effect through the added risk of VAT remains uncertain.[6–8,34,35]

An important consideration when studying T/E2 ratio is the enzyme aromatase, which is responsible for the transformation of androgens into estrogens. This aromatization step is responsible for most production of estradiol in men and occurs predominantly in white adipose tissue.[5] Male patients with a mutation in the gene coding for aromatase and the corresponding knockout mouse model showed similar features of a disturbed T/E2 balance as the current CEA-population.[36] These patients suffered from early onset of atherosclerosis, increased VAT (present in the current cohort as a higher BMI in patients with a lower T/E2 ratio) and a disturbed glucose homeostasis (present in the current cohort as an increased incidence of diabetes mellitus in patients with a lower T/E2 ratio). Patients with a mutation in the gene coding for aromatase logically have a high T/E2 ratio, which is in contrast to the patients with poor outcome after CEA in the present analysis that have a lowered T/E2 ratio. Not to mention, Zheng *et al.* showed a higher T/E2 ratio in coronary artery disease (CHD) patients in comparison to age-matched healthy controls.[7] Nevertheless, the mean T/E2 ratio in the above-mentioned control groups was similar to the mean T/E2 ratio of patients in the higher quartiles for the T/E2 ratio in the current cohort. These studies suggest that in CVD the optimal concentrations curves of testosterone and estradiol could be U-shaped. Either concentrations that are on the high or low range of normal could impair vascular function and promote clot formation. Hence, disequilibrium between testosterone and estradiol, resulting in either too high or too low T/E2 ratio, might contribute to CVD progression. This said, when assessing sex hormones in CVD-patients the T/E2 ratio should always be combined with the individual levels of testosterone and estradiol for appropriate understanding of hormonal status. The T/E2 ratio can then serve as a practical tool to determine CVD-risk, especially in overweight men.

Future implications

Future efforts in restoring hormonal balance in order to further reduce cardiovascular risk and preserve vascular function should not merely try to improve just one of both sex hormones. The present study provides important insights in how hormonal dysregulation is associated with increased systemic inflammation, inflammation within the atherosclerotic plaque and poor outcome. Moreover, we provide essential insights through which pathophysiological mechanisms obesity influences cardiovascular function and outcomes. Considering the unknown role of aromatase activity in this interaction which is suggested by the different outcomes in the BMI strata we can only speculate on the effects of clinical aromatase inhibition (AI). In a recent small (n=29) proof-of-concept trial no clear cardio metabolic benefits were seen in men receiving AI.[37] A small reduction in levels of CRP was visible; however, possibly due to small sample size these differences were not statistically significant. In females there is more evidence on the cardiovascular effects of AI, since these agents are used in the treatment of estrogen receptor positive breast cancer. A large observational study (n=9350) showed an increases risk for myocardial infarction (HR 2.02 (95%CI: 1.16-3.53)) in women receiving AI compared to women receiving Tamoxifen.[38] Considering these results we expect AI to have no beneficial cardiovascular effects in women and in men these effects remain largely unknown.

Strengths and limitations

The current study suffers from some limitations, first, the production of estradiol via aromatase occurs predominantly in white adipose tissue. As a consequence, males with higher BMI will show decreased T/E2 ratios. This effect was also present in our cohort ($p<0.001$) and it could be suggested that the poor outcome after CEA is caused by obesity and not T/E2 ratio. In multivariate analyses, however, we corrected for this possible confounder and show that decreased T/E2 ratio was a risk factor for major events during follow-up, independent of BMI or obesity. Moreover, in univariate logistic regression analysis BMI was not associated with the occurrence of future major cardiovascular events (HR 0.96 (95% CI 0.89 – 1.04 p=0.321) while T/E2 strongly predicts future events. Implicating that T/E2 ratio had prognostic value for MACE independent of BMI. One could argue that BMI does not accurately represent the degree of abdominal adipose tissue and could advocate the use of waist circumference. Nonetheless, it has been shown that BMI and waist circumference similarly estimate the degree of abdominal adipose tissue in males.[39] Lastly the activity of aromatase activity in human subjects is difficult to quantify. However, T/E2 proved to be predictive for MACE, independent of BMI. BMI alone had no predictive value in our cohort.

Concluding, in male patients with manifest atherosclerotic disease, low T/E2 ratio was associated with increased systemic inflammation, increased inflammatory plaque proteins and an increased risk of future major cardiovascular events as compared to men with normal T/E2 ratio. These effects are strongest in men with elevated body mass index and are expected to be affected by aromatase activity in white fat tissues. Normalization of T/E2 ratio may be a useful tool for the secondary prevention of CVD in men.

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SUPPLEMENTAL

Supplemental Table 1. BMI multivariate models.

	Multivariable analysis HR [95%CI]	P-value
HR of T/E2 ratio for future MACE in multivariate cox regression analysis with correction for BMI	1.67 (1.02-2.76)	**0.043**
HR of T/E2 ratio for future MACE in multivariate cox regression analysis without correction for BMI	1.58 (0.96-2.59)	0.070
Fold change %	5.39%	

Abbreviations: MACE; Major adverse cardiovascular events includes all stroke, myocardial infarctions and cardiovascular death. Bold values were considered statistically significant with a $p < 0.05$.

Supplemental Table 2. BMI-group stratified interaction analyses.

		Multivariable analysis HR [95%CI]	P-value
Patients with BMI over 25 (n=375)	HR of T/E2 ratio for future MACE	2.42 (1.09-5.38)	**0.030**
Patients with BMI below 25 (n=236)	HR of T/E2 ratio for future MACE	1.32 (0.66-2.64)	0.434

Abbreviations: MACE; Major adverse cardiovascular events includes all stroke, myocardial infarctions and cardiovascular death. Multivariate model corrected for age, BMI, eGFR, diabetes mellitus, history of CAD, contralateral stenosis and lipid levels. Bold values were considered statistically significant with a $p<0.05$.

Supplemental Table 3. Baseline characteristics of patients in the different testosterone (nmol/L) quartiles.

	≤8.770	8.780-11.730	11.740-15.140	≥15.150	
Patient characteristics	*n* = 153	*n* = 153	*n* = 153	*n* = 152	**P-value**
Age (SD) (years)	69.5 (9.5)	69.2 (8.8)	69.3 (9.1)	69.8 (7.9)	0.934
BMI (SD)	27.0 (3.7)	26.6 (3.7)	26.2 (3.5)	25.8 (3.3)	**0.025**
Glucose (SD) (mmol/L)	6.84 (2.01)	6.76 (1.80)	6.61 (2.06)	6.17 (1.28)	0.068
eGFR (SD) (mL/min/1.71m2)	73.65 (25.12)	71.11 (19.45)	76.12 (18.46)	75.36 (18.45)	0.172
Hypertension, yes(n)	108/146 (74.0)	109/148 (73.6)	102/151 (67.5)	107/148 (72.3)	0.581
Hypercholesterolemia, yes(n)	89/137 (65.0)	107/139 (77.0)	96/137 (70.1)	100/139 (71.9)	0.175
Diabetes mellitus, yes(n)	49/153 (32.0)	37/153 (24.2)	32/153 (20.9)	25/152 (16.4)	**0.012**
Current smoker, yes(n)	49/153 (32.0)	53/151 (35.1)	49/152 (32.2)	44/151 (29.1)	0.745
History of CAD, MI or coronary intervention, yes(n)	49/153 (32.0)	51/153 (33.3)	47/153 (30.7)	48/152 (31.6)	0.969
Total cholesterol (mmol/L) (SD)	3.75 (0.98)	4.00 (1.07)	4.08 (1.01)	4.24 (1.01)	**<0.001**
LDL cholesterol (mmol/L) (SD)	2.06 (0.70)	2.24 (0.81)	2.34 (0.83)	2.39 (0.82)	**0.001**
HDL cholesterol (mmol/L) (SD)	0.91 (0.25)	0.98 (0.30)	1.00 (0.23)	1.09 (0.29)	**<0.001**
Triglycerides (mmol/L) (SD)	1.92 (1.26)	1.74 (0.90)	1.53 (0.78)	1.57 (0.78)	**0.001**
Hormone levels					
Estradiol (pmol/L) (SD)	74.9 (39.2)	88.6 (32.8)	97.9 (35.6)	109.8 (37.5)	**<0.001**
Clinical presentation					
Ø Asymptomatic, (n)	26/151 (17.2)	26/153 (17.0)	23/152 (15.1)	19/150 (12.7)	0.348
Ø Ocular symptoms, (n)	25/151 (16.6)	22/153 (14.4)	27/152 (17.8)	22/150 (14.7)	
Ø TIA, (n)	60/151 (39.7)	74/153 (48.4)	53/152 (34.9)	65/150 (43.3)	
Ø Stroke, (n)	40/151 (26.5)	31/153 (20.3)	49/152 (17.8)	44/150 (29.3)	
Pre-operative medication use					
Statin use, yes(n)	123/153 (80.4)	120/153 (78.4)	120/153 (78.4)	130/152 (85.5)	0.349
Antiplatelet use, yes(n)	130/153 (85.0)	137/152 (90.1)	133/153 (86.9)	132/151 (87.4)	0.598
Anticoagulant use, yes(n)	22/153 (14.4)	12/153 (7.8)	15/153 (9.8)	23/152 (15.1)	0.139

Abbreviations: BMI, Body Mass Index; CAD, Coronary Artery Disease; eGFR, estimated Glomerular Filtration Rate; HDL, High-Density Lipoprotein; LDL, Low-Density Lipoprotein; MI, Myocardial Infarction; TIA, Transient Ischemic Attack. Bold values were considered statistically significant with a $p < 0.05$.

Supplemental Table 4. Inflammatory blood biomarkers of patients in the different testosterone (nmol/L) quartiles.

	≤8.770	8.780-11.730	11.740-15.140	≥15.150	
Plasma proteins	*n* = 119	*n* = 133	*n* = 122	n = 118	**P-value**
CRP (µg/mL)	2.10 [1.07 – 5.24]	1.93 [0.93 – 3.86]	1.38 [0.75 – 3.35]	1.37 [0.60 – 2.46]	**0.022**
Blood counts	*n* = 88	*n* = 83	*n* = 89	*n* = 91	
Number of leukocytes in blood (10^9/L)	8.69 (2.63)	8.10 (2.02)	8.04 (2.22)	7.65 (1.70)	**0.015**
Number of lymphocytes in blood (10^9/L)	2.16 (0.90)	2.03 (0.70)	1.96 (0.62)	1.95 (0.63)	0.190
Number of neutrophils in blood (10^9/L)	5.51 (2.21)	5.09 (1.60)	5.13 (2.14)	4.78 (1.38)	0.076
Number of monocytes in blood (10^9/L)	0.72 (0.26)	0.70 (0.23)	0.70 (0.21)	0.67 (0.17)	0.557
Neutrophil to lymphocyte ratio	2.52 [1.76 – 3.57]	2.42 [2.00 – 3.34]	2.57 [1.93 – 3.42]	2.44 [1.86 – 3.30]	0.409

Abbreviations: CRP, C-Reactive Protein. Bold values were considered statistically significant with a $p < 0.05$.

Supplemental Table 5. Association of the different testosterone quartiles with histological atherosclerotic plaque characteristics.

Binominal carotid plaque characteristics	**P-value Univariate**	**Odds ratio unadjusted**	**[95%CI]**	**P-value Multivariate**	**Odds ratio adjusted**	**[95%CI]**
Presence of lipid core ≥40%	0.885	1.013	0.853 1.202	0.799	0.977	0.814 1.171
Moderate/heavy calcifications	0.842	0.983	0.833 1.161	0.111	0.862	0.718 1.035
Moderate/heavy collagen	0.195	1.140	0.935 1.389	0.371	1.093	0.900 1.328
Presence of intraplaque hemorrhage	0.888	1.012	0.858 1.194	0.487	0.939	0.786 1.121
Continuous carotid plaque characteristics	**P-value Univariate**	**Beta Unadjusted**	**[95%CI]**	**P-value Mulitvariate**	**Beta adjusted**	**[95%CI]**
Mean number of microvessels per hotspot	0.370	0.268	-0.320 0.857	0.428	0.239	-0.353 0.830
% of positive macrophage staining per plaque	0.883	0.003	-0.034 0.039	0.939	0.001	-0.033 0.036
% of positive SMC staining per plaque	0.939	-0.002	-0.053 0.049	0.928	-0.002	-0.057 0.052
Total number of neutrophils per plaque	**0.041**	**-0.409**	**-0.801 -0.017**	0.081	-0.359	-0.763 0.046

Abbreviations: CI, Confidence Interval; SMC, Smooth Muscle Cell. Odds ratios and regression coefficients represent the difference per increase in testosterone quartile. Multivariate analyses performed with correction for possible confounders. Bold values were considered statistically significant with a $p < 0.05$.

Supplemental Table 6. Baseline characteristics of patients in the different estradiol (pmol/L) quartiles.

	≤67.0	**68.0-89.0**	**90.0-112.0**	**≥113.0**	
Patient characteristics	*n* = 153	*n* = 157	*n* = 150	*n* = 151	**P-value**
Age (SD) (years)	70.2 (8.9)	69.9 (8.3)	69.5 (9.2)	68.3 (9.0)	0.256
BMI (SD)	25.7 (3.4)	26.5 (3.6)	26.7 (3.4)	26.8 (3.7)	**0.026**
Glucose (SD) (mmol/L)	6.57 (1.80)	6.71 (1.86)	6.80 (2.11)	6.29 (1.44)	0.279
eGFR (SD) (mL/min/1.71m2)	75.41 (21.58)	73.48 (21.01)	73.58 (19.74)	73.82 (20.13)	0.849
Hypertension, yes(n)	104/147 (70.7)	119/155 (76.8)	105/147 (71.4)	98/144 (68.1)	0.394
Hypercholesterolemia, yes(n)	87/134 (64.9)	108/144 (75.0)	95/137 (69.3)	102/137 (74.5)	0.213
Diabetes mellitus, yes(n)	38/153 (24.8)	41/157 (26.1)	37/150 (24.7)	27/151 (17.9)	0.318
Current smoker, yes(n)	54/151 (35.8)	49/157 (31.2)	40/150 (26.7)	52/149 (34.9)	0.314
History of CAD, MI or coronary intervention, yes(n)	43/153 (28.1)	47/157 (29.9)	52/150 (34.7)	53/151 (35.1)	0.469
Total cholesterol (mmol/L) (SD)	4.18 (1.04)	4.08 (1.06)	3.96 (0.99)	3.84 (1.01)	**0.025**
LDL cholesterol (mmol/L) (SD)	2.46 (0.79)	2.38 (0.83)	2.19 (0.75)	2.00 (0.76)	**<0.001**
HDL cholesterol (mmol/L) (SD)	1.00 (0.27)	1.00 (0.30)	1.01 (0.26)	0.97 (0.26)	0.512
Triglycerides (mmol/L) (SD)	1.80 (0.96)	1.70 (0.79)	1.76 (1.17)	1.50 (0.88)	**0.036**
Hormone levels					
Testosterone (nmol/L) (SD)	9.3 (4.8)	11.8 (4.3)	13.7 (5.2)	14.4 (6.3)	**<0.001**
Clinical presentation					
Ø Asymptomatic, (n)	26/152 (17.1)	21/154 (13.6)	21/150 (14.0)	26/150 (17.3)	0.527
Ø Ocular symptoms, (n)	22/152 (14.5)	18/154 (11.7)	31/150 (20.7)	25/150 (16.7)	
Ø TIA, (n)	64/152 (42.1)	72/154 (46.8)	54/150 (36.0)	62/150 (41.3)	
Ø Stroke, (n)	40/152 (26.3)	43/154 (27.9)	44/150 (29.3)	37/150 (24.7)	
Pre-operative medication use					
Statin use, yes(n)	119/153 (77.8)	125/157 (79.6)	120/150 (80.0)	129/151 (85.4)	0.366
Antiplatelet use, yes(n)	138/152 (90.8)	131/156 (84.0)	135/150 (90.0)	128/151 (84.8)	0.164
Anticoagulant use, yes(n)	9/153 (5.9)	17/157 (10.8)	16/150 (10.7)	30/151 (19.9)	**0.002**

Abbreviations: BMI, Body Mass Index; CAD, Coronary Artery Disease; eGFR, estimated Glomerular Filtration Rate; HDL, High-Density Lipoprotein; LDL, Low-Density Lipoprotein; MI, Myocardial Infarction; TIA, Transient Ischemic Attack. Bold values were considered statistically significant with a $p < 0.05$.

Supplemental Table 7. Inflammatory blood biomarkers of patients in the different estradiol (pmol/L) quartiles.

	<70.00	**70.00 – 90.00**	**90.01 – 111.50**	**>111.50**	
Plasma proteins	*n* = 126	*n* = 159	*n* = 133	*n* = 149	**P-value**
CRP (µg/mL)	1.41 [0.65 – 3.43]	1.60 [0.76 – 3.54]	1.46 [0.69 – 3.08]	2.30 [1.20 – 4.46]	0.815
Blood counts	*n* = 114	*n* = 128	*n* = 104	*n* = 71	
Number of leukocytes in blood (10^9/L)	8.23 (2.55)	8.24 (2.26)	8.19 (1.97)	7.61 (1.61)	0.277
Number of lymphocytes in blood (10^9/L)	2.09 (0.81)	2.06 (0.69)	2.03 (0.72)	1.85 (0.60)	0.229
Number of neutrophils in blood (10^9/L)	5.19 (2.25)	5.23 (1.95)	5.17 (1.61)	4.78 (1.33)	0.485
Number of monocytes in blood (10^9/L)	0.67 (0.23)	0.71 (0.21)	0.71 (0.22)	0.69 (0.23)	0.725
Neutrophil to lymphocyte ratio	2.43 [1.79 – 3.36]	2.52 [1.92 – 3.39]	2.52 [1.88 – 3.28]	2.63 [2.05 – 3.44]	0.688

Abbreviations: CRP, C-Reactive Protein. Bold values were considered statistically significant with a $p < 0.05$.

Supplemental Table 8. Association of the different estradiol quartiles with histological atherosclerotic plaque characteristics.

Binominal carotid plaque characteristics	**P-value Univariate**	**Odds ratio unadjusted**	**[95%CI]**		**P-value Multivariate**	**Odds ratio adjusted**	**[95%CI]**	
Presence of lipid core ≥40%	0.220	1.113	0.938	1.320	0.587	1.058	0.864	1.294
Moderate/heavy calcifications	**0.001**	1.344	1.135	1.592	0.748	1.033	0.848	1.259
Moderate/heavy collagen	0.304	1.107	0.912	1.345	0.637	1.052	0.853	1.430
Presence of intraplaque hemorrhage	**0.021**	1.214	1.029	1.432	0.922	0.990	0.815	1.203
Continuous carotid plaque characteristics	**P-value Univariate**	**Beta Unadjusted**	**[95%CI]**		**P-value Mulitvariate**	**Beta adjusted**	**[95%CI]**	
Mean number of microvessels per hotspot	0.834	-0.062	-0.642	0.518	0.292	-0.338	-0.967	0.291
% of positive macrophage staining per plaque	**<0.001**	0.077	0.042	0.112	0.171	0.026	-0.011	0.064
% of positive SMC staining per plaque	**0.008**	0.068	0.018	0.118	**0.008**	0.068	0.018	0.118
Total number of neutrophils per plaque	0.224	0.245	-0.153	0.643	0.792	0.060	-0.392	0.511

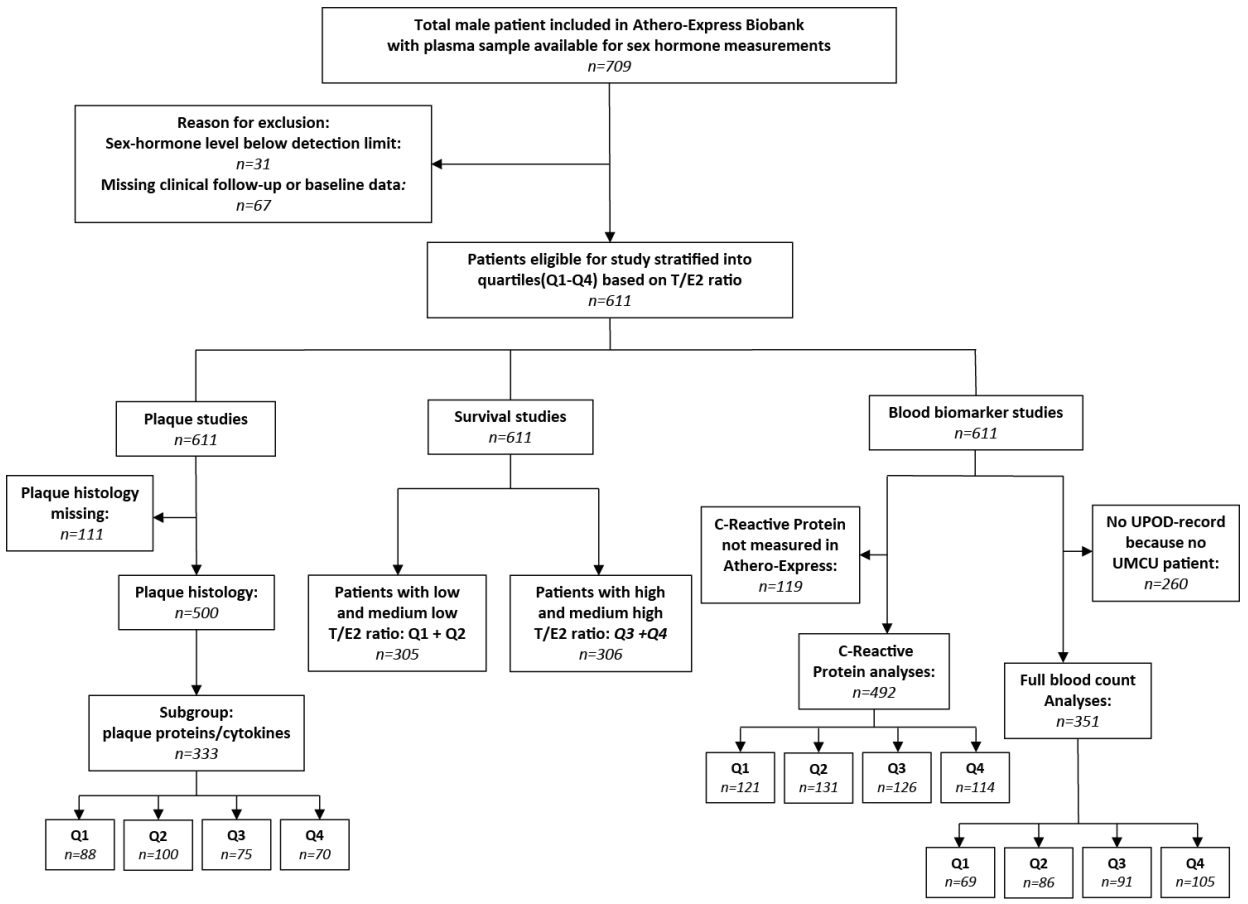
Abbreviations: CI, Confidence Interval; SMC, Smooth Muscle Cell. Odds ratios and regression coefficients represent the difference per increase in estradiol quartile. Multivariate analyses performed with correction for possible confounders. Bold values were considered statistically significant with a $p < 0.05$.

Supplemental Table 9. Hazard ratios for testosterone, estradiol and major endpoints, stroke & death during three-year follow-up after carotid endarterectomy.

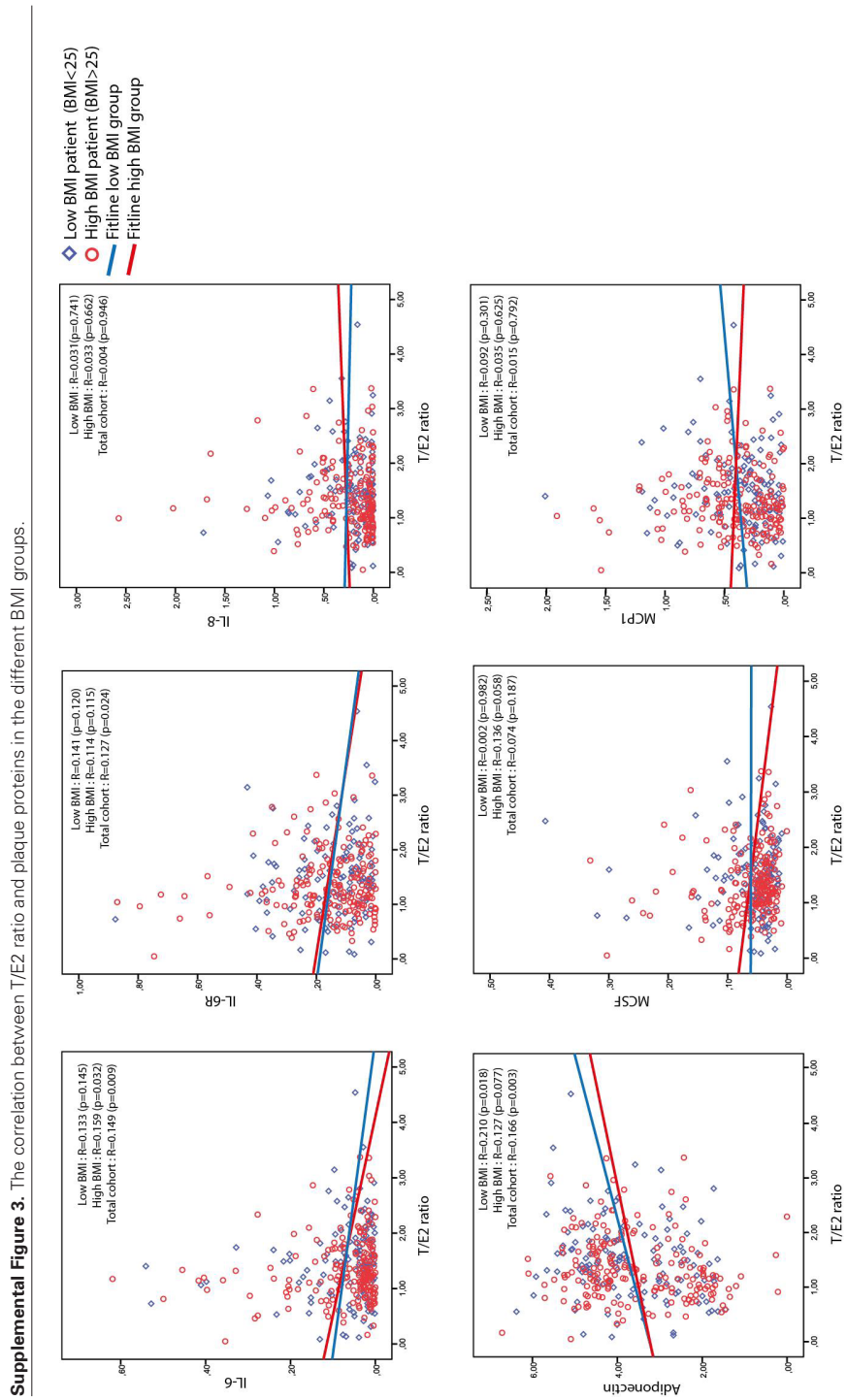
Major endpoints	**P-value Univariate**	**Odds ratio unadjusted**	**[95%CI]**		**P-value Multivariate**	**Odds ratio adjusted**	**[95%CI]**	
Testosterone (nmol/L)	0.081	1.521	0.950	2.436	0.272	1.314	0.807	2.138
Estradiol (pmol/L)	0.316	0.788	0.495	1.255	0.141	0.700	0.436	1.125
Stroke	**P-value Univariate**	**Odds ratio unadjusted**	**[95%CI]**		**P-value Multivariate**	**Odds ratio adjusted**	**[95%CI]**	
Testosterone (nmol/L)	**0.024**	2.139	1.104	4.146	0.052	1.966	0.996	3.880
Estradiol (pmol/L)	0.472	0.796	0.427	1.484	0.234	0.681	0.362	1.282
Death	**P-value Univariate**	**Odds ratio unadjusted**	**[95%CI]**		**P-value Multivariate**	**Odds ratio adjusted**	**[95%CI]**	
Testosterone (nmol/L)	0.252	1.333	0.816	2.177	0.755	1.085	0.651	1.808
Estradiol (pmol/L)	0.663	0.898	0.552	1.460	0.408	0.806	0.483	1.344

Abbreviations: CI, Confidence Interval. Multivariable cox regression analyses corrected for age, BMI, eGFR, diabetes mellitus, history of CAD, contralateral stenosis and lipid levels. Bold values were considered statistically significant with a $p < 0.05$.

Supplemental Figure 1. Flowchart of patient stratification and main studies performed.



Supplemental Figure 2. Testostorene to Estradiol ratio groups.



Supplemental Figure 3. The correlation between T/E2 ratio and plaque proteins in the different BMI groups.

Scatter diagrams illustrate the correlation between circulating T/E2 ratio and cytokine and chemokines in plaques obtained from male patients after carotid endarterectomy in the different BMI groups(based on log transformed data). Abbreviations: IL-6; Interleukin 6, IL-6R; interleukin-6 receptor, IL-8; interleukin 8, MCSF; Macrophage colony stimulating factor 1, MCP1; Monocyte chemotactic protein 1

8

A single preoperative blood test predicts postoperative sepsis and pneumonia after coronary bypass or open aneurysm surgery

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ABSTRACT

Background

Major surgery comes with a high risk for postoperative inflammatory complications. Preoperative risk scores predict mortality risk but fail to identify patients at risk for complications following cardiovascular surgery. We therefore assessed the value of preoperative red cell distribution width (RDW) as a predictor for pneumonia and sepsis after cardiovascular surgery.

Methods

RDW is an easily accessible, yet seldomly used parameter from routine hematology measurements. RDW was extracted from the Utrecht Patient Orientated Database (UPOD) for preoperative measurements in patients undergoing open abdominal-aortic-aneurysm-repair (AAA)(N=136) or coronary artery bypass grafting (CABG)(N=2193). Logistic regression analyses with correction for possible confounding was used to determine associations between RDW and postoperative inflammatory complications. Additional Standard Uptake Values (SUV) of hematopoietic tissues in FDG-PET scans were scored and associated with RDW using linear regression models.

Results

In total, 43(31.6%) and 73 patients(3.3%) suffered from inflammatory complications after AAA-repair or CABG, respectively; the majority being pneumonia in both cohorts. Preoperative elevated RDW was independently associated with postoperative inflammatory outcomes in the AAA-cohort (OR:1.80 (95%CI:1.23-2.61)p=0.002) and the CABG-cohort (OR:1.19 (95%CI:1.07-1.32)p=0.001). Postoperative inflammatory outcome incidence increased from 19.6% in the lowest to 48.9% in the highest RDW-tertile with a corresponding OR of 3.97 ((95%CI:1.47-10.72)p=0.022) in AAA-patients. In the CABG-cohort, the incidence of postoperative inflammatory outcomes increased from 1.8% to 5.3% with an adjusted OR of 2.12 ((95%CI:1.10-4.10)p=0.038) for the highest RDW-tertile compared with the lowest RDW-tertile. FDG-PET scans showed association of RDW with tissue activity in the spleen (B=0.517 (p=0.001)) and the lumbar bone marrow (B=0.480 (p=0.004)).

Conclusion

Elevated RDW associates with increased risk for postoperative inflammatory complications and hematopoietic tissue activity. RDW likely reflects chronic low-grade inflammation and should be considered to identify patients at risk for postoperative inflammatory complications following cardiovascular surgery.

INTRODUCTION

Postoperative pneumonia remains a frequent complication after major surgery with incidences ranging between 2 and 40% depending on clinical standards and surgical procedure.[1,2] Prophylactic antibiotics are mostly aiming at wound infections, but do not cover typical pneumonia pathogens. The estimated attributable health care costs associated with one case of postoperative pneumonia are approximately 46.400 U.S. dollar.[3] Prevention programs have proven to be effective in decreasing postoperative pneumonia incidence and reduce overall health care costs.[3] Unfortunately, ready-to-use implementable biomarkers to detect high-risk patients for postoperative inflammatory complications are not available.

Previous studies have shown that easily available parameters from automated hematology analyzers can predict outcome in different patient populations. In particular Red Blood Cell Distribution Width (RDW) associates with unfavorable outcome in numerous reports. The RDW is derived from the ratio of the erythrocyte`s mean corpuscular volume (MCV) and its variation and thus reflects greater cell size heterogeneity or, in morphologically terms, anisocytosis. It has been proposed that RDW increases upon a low-grade chronic inflammation, which is evident in both, cardiovascular disease and progressive cancer.[4] Postoperative inflammatory complications are often characterized by an accelerated innate immune response. Therefore, we hypothesized that the RDW, assessed prior to major CV surgery, can be used to identify patients at risk for postoperative adverse immunological events and mortality.

To test our hypothesis, we studied the predictive value of RDW for postoperative inflammatory complications in an open abdominal aortic aneurysm (AAA) repair cohort. AAA-surgery comes with a relatively high risk for postoperative inflammatory complications rendering it a useful discovery cohort despite its relatively small sample size.[5,6] We then replicated and validated these findings in a larger cohort of patients undergoing coronary bypass surgery (CABG).

We subsequently attempted to elucidate the mechanisms underlying increased RDW. Therefore, we compiled a cohort of patients undergoing fluor-18(^{18}F) deoxyglucose positron-emission-tomography (FDG-PET) scanning for screening purposes. This imaging modality uses a radiotracer which accumulates in tissue with increased metabolism and energy demand and thus serves as a marker for tissue activity. In the present study, we scored activity of several hematopoietic organs and tested its association with RDW.

METHODS

This study was conducted in accordance with the declaration of Helsinki. The institutional review board reviewed and approved the current study.

Patient cohorts

The clinical outcome populations consisted of 243 consecutive patients who underwent open AAA-repair (AAA-express Biobank study/discovery cohort) and 2508 consecutive patients undergoing isolated CABG (replication cohort) between 2007 and 2013 in the University Medical Center Utrecht (UMCU).[7] Indication for surgery was based on international guidelines for AAA-repair or cardiothoracic surgery.[8,9] Hematologic measurements were extracted from the Utrecht Patient Oriented Database (UPOD).[10] This unique research database was initiated in 2003 and continuously collects electronic patient information on demographics, hospital admissions, medication, diagnostics (e.g laboratory results) and surgical interventions. The closest preoperative hematological measurement available within a one-month preoperative range was selected. Patients without preoperative hematology measurements, missing complication registration or with ruptured AAA were excluded from the analyses.

Clinical adverse outcomes

Postoperative inflammatory complications (i.e. pneumonia, renal failure or sepsis) served as primary endpoint. Pneumonia was defined as: all patients with clinical symptoms of pneumonia (cough/fever/typical signs on chest X-ray/positive bronchoscopy/ positive sputum culture) which required oral or intravenous antibiotics. Renal failure was defined as: all patients requiring de-novo renal replacement therapy (hemodialysis or continuous venovenous hemodialysis) or patients with signs of acute kidney injury (doubling of serum creatinine levels) combined with a statement of the medical specialist on new onset of renal failure. Sepsis was defined as: all patients with clinical signs of sepsis (anomalous body temperature, increased heart rate, increased respiratory rate) combined with a positive blood culture. A composite endpoint including all postoperative inflammatory outcomes (i.e. pneumonia, renal failure or sepsis) was composed. Three year mortality and duration of postoperative intensive care unit-stay >24hour served as secondary endpoints. Information on survival after surgery was collected using an annual questionnaire or the in hospital data registry system.

Hematology measurements

Modern automated hemocytometers often measure a whole blood cell count irrespective of the clinician's request. Not all hematological parameters are reported to the physician; however these results can be stored. In the University Medical Center Utrecht (UMCU) all results are stored in the Utrecht Patient Oriented Database (UPOD). All hematology measurements used in this study were measured with the CELL-DYN Sapphire (Abbott Diagnostics, Santa-Clara, CA, USA), which is an automated multi-parameter hematology analyzer for clinical purposes.[11,12] The CELL-DYN Sapphire uses laser light scattering, spectrophotometry and electrical impedance to describe distinct morphological traits (cell size, shape, and granularity) of red blood cells, platelets, and white blood cells. An example of an overlay of two erythrocyte volume distribution curves is presented in Figure 1; both patients demonstrated comparable MCV but different RDW.

FDG-PET imaging

FDG-PET scans were performed using standardized in-house protocols. Patients fasted overnight and pre-scan glucose levels were obtained. FDG was administered intravenously. The total dose was calculated based on body weight (2 MBq/kg) and imaging was performed one hour after intravenous FDG injection. In the present study, reports from all FDG-PET-scans performed in 2016 in the UMCU were screened for eligibility. Patients were deemed eligible if they were >18 years of age and without signs of active infections (i.e. elevated C-reactive protein, fever or leukocytosis). Patients with a history of hematological malignancies, current active malignancies, major surgery, trauma, chemo or radiotherapy in the year before the scan were excluded. Furthermore, patients with granulomatous or autoimmune disorders and patients on anti-inflammatory drugs (i.e. methotrexate and prednisone) were excluded from the current analysis. Patients without RDW measurement within a one month range of FDG-PET scan were also excluded from analysis.

FDG-PET scoring

The EARL FDG-PET/CT image reconstruction was used in all analyses for replication purposes.[13] Scans were scored using the semi-automated image analysis software package ROVER.[14] Spleen, bone marrow (BM) of the first three lumbar vertebrae and bone marrow in the iliac crests were scored for hematopoietic activity. Spleen FDG-uptake was measured in the maximal axial, coronal and sagittal planes. FDG uptake of BM in the lumbar vertebrae was measured in axial slides of the first three lumbar vertebrae. FDG-uptake of BM in the iliac crests was measured in axial slides of the left and right iliac crests. SUV max and mean was determined in all regions of interest. Splenic activity was calculated as the mean of SUVmax in all three planes. SUVmax of the BM in the lumbar spine was calculated as the mean of the FDG-max of the three lumbar vertebrae, SUVmax of the BM in the iliac crest was calculated as the mean of the left and right iliac crest. Subcutaneous adipose fat tissue was scored as a reference. Similar methods for calculating the SUV mean of all tissues was used. The used scoring method has been validated in previous studies [15–17].

Statistical analysis

The association of RDW with postoperative inflammatory complications was tested both continuously (RDW was normally distributed) and categorical by use of tertiles. In the categorical analyses the median RDW-level was assigned to the patients in each tertile and then this variable was evaluated continuously. Since the clinical outcome measures were scored binary we used logistic regression models with correction for possible confounders. The cut-off point to determine possible confounders for postoperative inflammatory complications was chosen at $p < 0.1$. Student's t-test and Mann-Whitney U tests were used where appropriate to compare continuous baseline characteristics between patients with or without inflammatory complications after AAA-repair or CABG respectively. Chi-square tests were used to test categorical baseline characteristics between groups. Furthermore, age and renal function as known determinants of outcome were added to

the multivariate models in AAA-patients. Potential confounders added to the multivariable model of RDW and inflammatory outcomes were age, eGFR, current smoking and presence of symptoms for AAA patients and age, preoperative unstable angina, current smoking, eGFR, hemoglobin and white blood cell count for CABG patients. We reported both crude and adjusted odds ratios (OR) and their corresponding 95% confidence intervals (CI). Moreover, since RDW values might be difficult to interpret due to different hemocytometers used in different hospitals we have performed a stratified analysis based on the normal cut-off reference used in this hospital. Values greater than 13.5% were flagged as abnormal and for insight in the general usability of this cut-off, stratified analyses have been added. For comparison of the clinical prediction model compared to the prediction model with hematological parameters added, area under the curve (AUC) was compared using receiver operating characteristics (ROC) analysis.

In the FDG-PET study tissue organ activity was assessed by standard uptake values and the association with RDW was therefore analysed continuously by linear regression models with correction for possible confounders.

Due to a relative small sample size cut-off point to determine possible confounders for the FDG-PET study was chosen at $p < 0.5$. Possible confounders added to the multivariate model in the FDG-cohort were age, gender, WBC and eGFR. To avoid the limitation of complete case analyses, single imputation was used to calculate missing values (R computing platform version 3.0.2 (R Project for Statistical Computing, Vienna, Austria). SPSS version 21.0 (SPSS Inc, Chicago, IL) was used for statistical analyses. A p-value <0.05 was considered statistically significant.

RESULTS

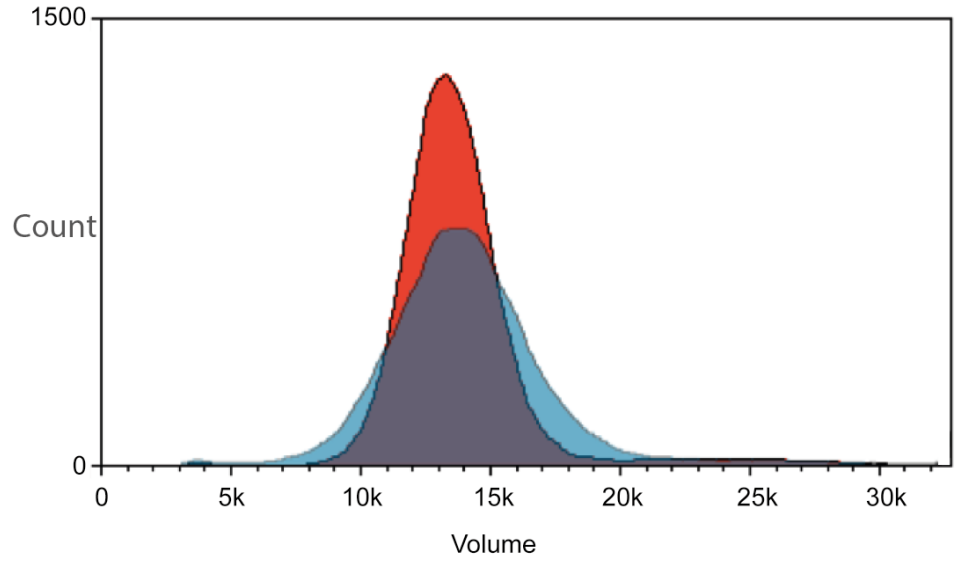
Patient cohorts

Inclusion flow charts are presented in supplemental Figures 1 and 2. In the AAA cohort, 77 patients (31.7%) were excluded due to missing preoperative hematological measurements; another 30 patients (12.4%) were excluded due to a diagnosis of ruptured AAA. In the CABG cohort, 260 patients (10.4%) were excluded due to missing preoperative hematological measurements, reoperation (46 patients; 1.8%) or missing complication registration (9 patients; 0.3%). In total, 136 AAA-patients (56.0%) and 2193 CABG (87.4%) were eligible and included for analysis.

Patient characteristics of the AAA- and the CABG-cohort were stratified by postoperative inflammatory complications and are presented in Table 1. In both cohorts, cardiovascular risk factors such as hypertension, diabetes, smoking status and elevated body mass index were highly prevalent. AAA-patients with postoperative inflammatory complications were more often symptomatic and current smokers compared to patients without postoperative inflammatory complications.

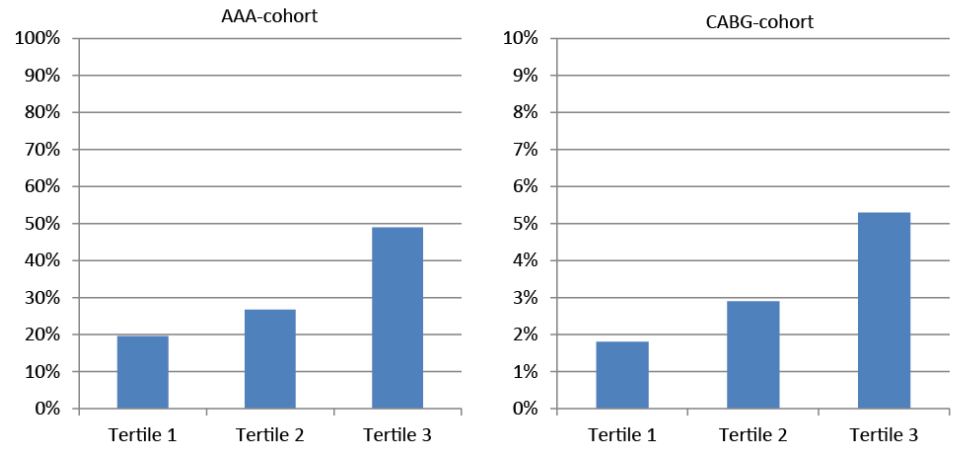
CABG-patients with postoperative inflammatory complications were on average 6 years older and more often reported unstable angina pectoris. They also smoked more often,

Figure 1. Overlay of two red cell size distribution curves.



Overlay of two red cell size distribution (RDW) curves, in red curve of patient with normal red cell size distribution (RDW = 11.64) characterized by steep slope and narrow curve. In light-blue curve of patient with abnormal red cell size distribution characterized by broad and flattened curve (RDW=17.59). Mean corpuscular volumes of both patients were similar (87.48 and 88.85).

Figure 2. Incidence of inflammatory outcome per RDW tertile.



Graphic presentation of the incidence of composite inflammatory events (renal failure, pneumonia, and sepsis) per RDW-tertile in the AAA-cohort and in the CABG-cohort. AAA, abdominal aortic aneurysm; CABG, coronary artery bypass grafting.

Table 1. Baseline characteristics of AAA- and CABG-patients stratified by inflammatory outcome.

	AAA-patients (Discovery cohort) *n*=136			CABG-patients (Replication cohort) *n*=2193		
Baseline characteristics	**No inflammatory complications; n= 93 (68.4%)**	**Inflammatory complications; n= 43 (31.6%)**	**p-value**	**No inflammatory complications; n= 2120 (96.7%)**	**Inflammatory complications; n=73 (3.3%)**	**p-value**
Gender (male) n(%)	73 (78.5)	31 (72.1)	0.413	1640 (77.4)	61 (83.6)	0.211
Age in years ± SD	66.5 ± 10.1	68.7 ± 9.0	0.211	66.1 ± 9.8	70.6 ± 9.1	**<0.001**
BMI ± SD	25.5 ± 3.9	25.0 ± 3.8	0.523	27.8 ± 4.2	27.0 ± 3.9	0.568
Hypertension, n(%)	73 (81.1)	34 (85.0)	0.592	1317 (62.1)	50 (68.5)	0.269
Diabetes, n(%)	22 (23.7)	11 (25.6)	0.808	511 (24.1)	21 (28.8)	0.361
Symptomatic AAA, n(%)	12 (12.9)	12 (27.9)	**0.033**	-	-	-
Preoperative unstable angina, n(%)	-	-	-	153 (7.2)	11 (15.1)	**0.012**
Preoperative recent MI, n(%)	-	-	-	495 (23.3)	20 (27.4)	0.422
Current smokers, n(%)	24 (25.8)	19 (44.1)	**0.032**	272 (14.1)	18 (26.9)	**0.004**
eGFR in mL/min/1.73 m2 ± SD	69.5 ± 22.5	63.5 ± 22.9	0.145	83.3 ± 31.3	62.8 ± 32.9	**<0.001**
Hemoglobin (Hb) mmol/L ± SD	8.5 ± 1.1	8.6 ± 1.4	0.569	8.6 ± 0.9	8.2 ± 1.0	**<0.001**
White blood cell count x10^9/L ± SD	8.6 ± 2.5	8.9 ± 3.0	0.547	8.2 ± 2.5	9.6 ± 4.9	**0.017**
HDL in mg/dL ± SD	1.0 ± 0.3	1.1 ± 0.5	0.684	1.06 ± 0.29	1.01 ± 0.34	0.125
RDW, % ± SD	12.5 ± 0.94	13.2 ± 1.44	**0.005**	12.2 ± 1.26	13.1 ± 1.90	**<0.001**

Values in parentheses are percentages unless indicated otherwise; values are median (i.q.r.). AAA, abdominal aortic aneurysm; BMI, Body Mass Index; CABG, coronary artery bypass grafting; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; MI, Myocardial infarction; SD, standard deviation.

had poorer renal function, higher white blood cell counts (WBC), and lower levels of circulating hemoglobin compared to CABG-patients without postoperative inflammatory complications.

Clinical cohort outcomes

The results from logistic regression analysis for prediction of adverse outcomes after AAA and CABG are shown in supplemental Tables 1 + 2 for univariate and multivariate analyses. In the AAA-cohort, 43 patients (31.6%) and 73 patients (3.3%) of the CABG-cohort reached the primary endpoint. The majority of adverse outcomes consisted of respiratory complications in both cohorts. Thirty-five (26%) patients of the AAA cohort and sixty (3%) patients of the CABG cohort developed respiratory inflammatory complications. Postoperative renal complications were experienced by 11 (8.1%) and 7 patients (0.3%) in the AAA and in the CABG-cohort respectively.

Logistic regression analyses in AAA-patients showed that postoperative sepsis, renal failure, and the composite inflammatory endpoint all associated with RDW in both univariate and multivariate models. In the CABG-cohort, RDW was associated in both univariate and multivariate analyses with postoperative respiratory complications and the composite inflammatory endpoint. Postoperative sepsis in the CABG-cohort only associated with RDW in univariate analyses. All reported odds ratios were calculated per 1% RDW increment. Adding CRP and WBC did not significantly alter these results indicating that RDW is a potent predictor of adverse events independent of known inflammation markers.

Secondary outcome measures were postoperative hospital mortality in 8 patients (5.8%) after AAA-repair and in 25 patients (1.1%) after CABG. Postoperative ICU-stay longer than 24 hours occurred in 48 (37.2%) AAA-patients and in 186 (8.5%) CABG-patients. In the AAA cohort, RDW was associated with in-hospital mortality in univariate but not in multivariate analysis. Furthermore, in AAA-patients no association of ICU-stay over 24hours with RDW was found. In CABG-patients, RDW was associated with in-hospital mortality in both univariate and multivariate analysis; an association of RDW and ICU-stay over 24hours was only statistically significant in univariate analysis.

The cohorts were stratified and analyzed by use of RDW-tertiles to assess for a continuous effect over the different RDW-groups (Table 2 + 3 and Figure 2). In AAA-patients, the incidence of pneumonia increased from 15.2% in the lowest tertile to 37.8% in the highest tertile with an unadjusted odds ratio (OR) of 3.38 (95%CI: 1.24-9.24) p=0.015) which remained statistically significant after correction for possible confounders (OR 3.19 (95%CI: 1.14-8.95) p=0.023). The composite inflammatory endpoint increased from 19.6% in the lowest RDW-tertile to 48.9% in the highest RDW-tertile. Both the unadjusted and adjusted ORs showed high predictive values for the development of postoperative inflammatory events.

In CABG-patients, the incidence of pneumonia increased from 1.5% in the lowest RDW-tertile to 4.1% in the highest RDW-tertile with an unadjusted OR of 2.80 (95%CI: 1.40-5.63) p=0.003) which did not remain statistically significant after multivariate correction (OR 1.80 (95%CI: 0.87-3.74) p=0.147). The composite inflammatory endpoint increased from 1.8%

Table 2. Risk of the primary endpoints (inflammatory event) and secondary composite endpoint (ICU-stay over 24hours and 3 year mortality) according to baseline RDW tertiles.

	AAA-cohort					CABG-cohort				
	All (*n*=136)	**T1** (*n*=46)	**T2** (*n*=45)	**T3** (*n*=45)	**p-value for trend***	**All** (*n*=2193)	**T1** (*n*=731)	**T2** (*n*=731)	**T3** (*n*=731)	**p-value for trend***
Pneumonia										
N (%)	35 (25.7%)	7 (15.2%)	11 (24.4%)	17 (37.8%)		60 (2.7%)	11 (1.5%)	19 (2.6%)	30 (4.1%)	
Unadjusted OR (95% CI)		-ref-	1.80 (0.63-5.17)	3.38 (1.24-9.24)	**0.015**	Unadjusted OR (95% CI)	-ref-	1.75 (0.83-3.70)	2.80 (1.40-5.63)	**0.003**
Adjusted OR (95% CI)†		-ref-	1.70 (0.59-4.91)	3.44 (1.25-9.45)	**0.014**	Adjusted OR (95% CI)§	-ref-	1.66 (0.78-3.53)	2.15 (1.06-4.39)	**0.038**
Adjusted OR (95% CI)‡		-ref-	1.62 (0.55-4.93)	3.19 (1.14-8.95)	**0.023**	Adjusted OR (95% CI)‖	-ref-	1.62 (0.76-3.45)	1.80 (0.87-3.74)	0.147
Composite inflammatory event										
N (%)	43 (31.6%)	9 (19.6%)	12 (26.7%)	22 (48.9%)		73 (3.3%)	13 (1.8%)	21 (2.9%)	39 (5.3%)	
Unadjusted OR (95% CI)		-ref-	1.50 (0.56-4.00)	3.93 (1.55-10.01)	**0.003**	Unadjusted OR (95% CI)	-ref-	1.63 (0.81-3.29)	3.11 (1.65-5.88)	**<0.001**
Adjusted OR (95% CI)†		-ref-	1.44 (0.53-3.90)	4.02 (1.56-10.37)	**0.014**	Adjusted OR (95% CI)§	-ref-	1.58 (0.78-3.19)	2.59 (1.36-4.93)	**0.005**
Adjusted OR (95% CI)‡		-ref-	1.50 (0.53-4.30)	3.97 (1.47-10.72)	**0.022**	Adjusted OR (95% CI)‖	-ref-	1.53 (0.76-3.11)	2.12 (1.10-4.10)	**0.038**
ICU-stay over 24 hours										
N (%)	84 (61.8%)	24 (52.2%)	30 (66.7%)	30 (66.7%)		186 (8.5%)	47 (6.4%)	58 (7.9%)	81 (11.1%)	
Unadjusted OR (95% CI)		-ref-	1.83 (0.79-4.28)	1.83 (0.79-4.28)	0.189	Unadjusted OR (95% CI)	-ref-	1.25 (0.84-1.87)	1.81 (1.25-2.64)	**0.001**
Adjusted OR (95% CI)†		-ref-	1.78 (0.76-4.18)	1.85 (0.79-4.33)	0.211	Adjusted OR (95% CI)§	-ref-	1.25 (0.84-1.88)	1.62 (1.10-2.37)	**0.017**
Adjusted OR (95% CI)‡		-ref-	1.74 (0.72-4.22)	1.65 (0.68-3.99)	0.362	Adjusted OR (95% CI)‖	-ref-	1.22 (0.82-1.83)	1.36 (0.92-2.02)	0.154
3-year mortality										
N (%)	20 (14.7%)	6 (13.0%)	4 (8.9%)	10 (22.2%)		81 (3.7%)	11 (1.5%)	17 (2.3%)	53 (7.3%)	
Unadjusted OR (95% CI)		-ref-	0.65 (0.17-2.48)	1.91 (0.63-5.78)	0.172	Unadjusted OR (95% CI)	-ref-	1.56 (0.73-3.35)	5.11 (2.65-9.88)	**<0.001**
Adjusted OR (95% CI)†		-ref-	0.58 (0.15-2.31)	1.81 (0.57-5.75)	0.355	Adjusted OR (95% CI)§	-ref-	1.50 (0.70-3.23)	4.46 (2.30-8.65)	**<0.001**
Adjusted OR (95% CI)‡		-ref-	0.50 (0.10-2.47)	1.69 (0.44-6.50)	0.382	Adjusted OR (95% CI)‖	-ref-	1.48 (0.68-3.18)	3.86 (1.97-7.58)	**<0.001**

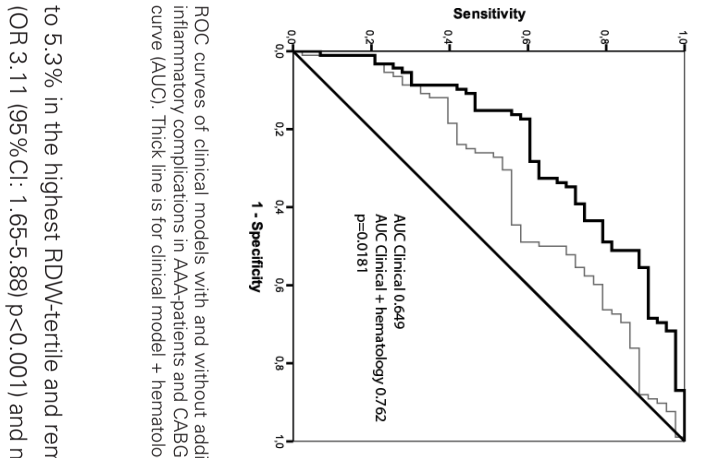
* The median RDW-level per tertile was assigned to subjects in the group and the grouping variable was analyzed continuously, † age, preoperative unstable angina, current smoking, eGFR; ; estimated glomerular filtration rate,‡ age, preoperative unstable angina, current smoking, eGFR, hemoglobin and white blood cell count, § age, eGFR, ‖ age, eGFR, current smoking and symptomatic status.

Table 3. Detailed outcome information stratified by RDW-tertiles.

	AAA-cohort					CABG-cohort			
	All	**T1**	**T2**	**T3**		**All**	**T1**	**T2**	**T3**
	(n=136)	**(n=46)**	**(n=45)**	**(n=45)**		**(n=2193)**	**(n=731)**	**(n=731)**	**(n=731)**
Detailed outcome information N (%)					Detailed outcome information N (%)				
Pneumonia	35	7	11	17	Pneumonia	60	11	19	30
Sepsis	8	0	2	6	Sepsis	7	1	2	4
Renal failure	11	2	2	7	Renal failure	7	1	1	5
Composite inflammatory event	43	9	12	22	Composite inflammatory event	73	13	21	39
ICU-stay over 24hours	84	24	30	30	ICU-stay over 24hours	186	47	58	81
In-hospital mortality	8	1	1	6	In-hospital mortality	25	4	4	17
3-year mortality	20	6	4	10	3-year mortality	81	11	17	53

AAA, abdominal aortic aneurysm; CABG, coronary artery bypass grafting; ICU, intensive care unit.

Figure 3. ROC curves of prediction models with and without hematological markers.



ROC curves of clinical models with and without adding hematological markers for prediction of postoperative inflammatory complications in AAA-patients and CABG-patients. P-value calculated for difference in area under the curve (AUC). Thick line is for clinical model + hematological markers, thin line is for the clinical model only.

to 5.3% in the highest RDW-tertile and remained statistically significant in both univariate (OR 3.11 (95%CI: 1.65-5.88) p<0.001) and multivariate models (OR 2.12 (95%CI: 1.10-4.10) p=0.038).
RDW-tertiles also showed high predictive value for three-year mortality in the CABG-cohort (adjusted OR 3.86 (95%CI: 1.97-7.58) p<0.001) but not in the AAA-cohort.

Clinical cutoff

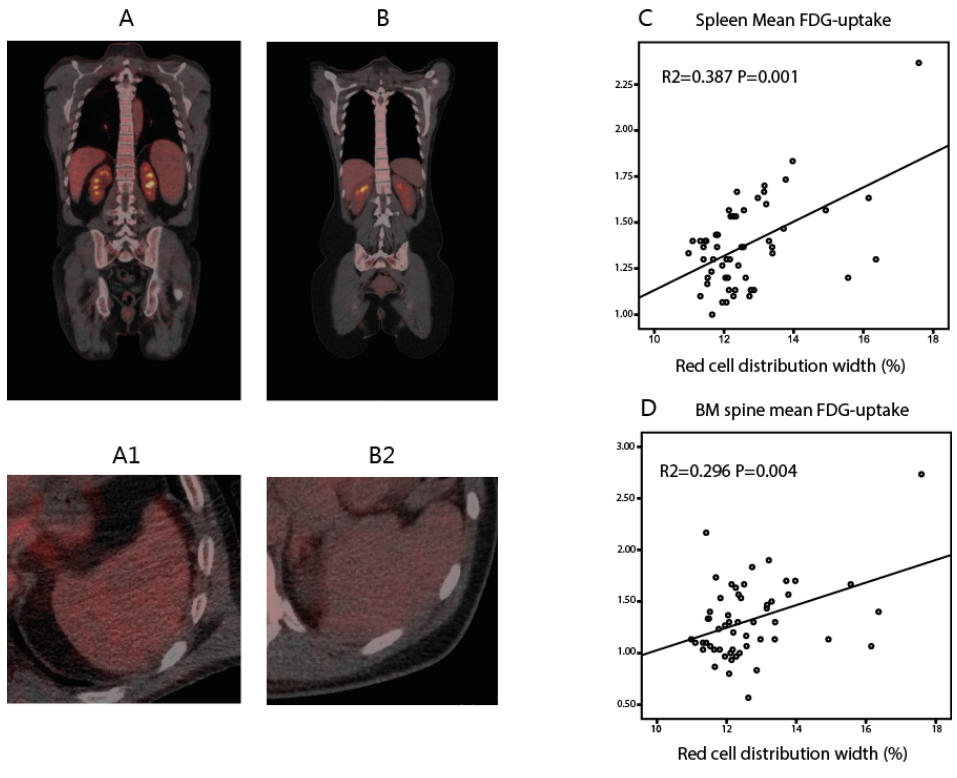
Since RDW values might differ depending on the technique (optic or impedance) that is used for the measurement, local cutoff values might vary. To assess the clinical usefulness of RDW we analyzed the cohort based on the 13.5% cutoff which is the upper reference value for the CELL-DYN hemocytometer. The results of the clinical cutoff analyses are shown in Table 4. In the AAA-cohort, 26 patients (19.1%) had RDW values ≥13.5% and in the CABG-cohort 197 patients (9.0%). The 13.5% cutoff value showed no statistically significant prediction in AAA-patients for pneumonia and the composite inflammatory endpoint. The predictive value for 3 year mortality was OR 3.63 ((95%CI: 1.30-10.13) p=0.014) and remained statistically significant after correction for possible confounders. In the CABG-cohort, the 13.5% cutoff showed high predictive value for pneumonia (adjusted OR 2.37(95%CI: 1.30-4.34) p=0.005) and the composite endpoint (adjusted OR 2.03 (95%CI: 1.02-4.04) p=0.043). Secondary endpoints in the CABG-cohort were statistically significant only in univariate analyses for ICU-stay over 24 hours but again highly significant for 3 year mortality in both unadjusted and adjusted analyses.

Table 4. Clinical threshold (cutoff 13.5%)

	AAA-cohort				CABG-cohort			
	All (*n*=136)	**RDW <13.5%** (*n*=110)	**RDW ≥13.5%** (*n*=26)	**p-value**	**All** (*n*=2193)	**RDW <13.5%** (*n*=1996)	**RDW ≥13.5%** (*n*=197)	**p-value**
Pneumonia								
No.Cases	35 (25.7%)	26 (23.6%)	9 (34.6%)		60 (2.7%)	46 (2.3%)	14 (7.1%)	
Unadjusted OR (95%CI)		-ref-	1.71 (0.68-4.29)	0.253	Unadjusted OR (95% CI)	-ref-	3.84 (2.23-6.62)	**<0.001**
Adjusted OR (95% CI)*		-ref-	1.89 (0.74-4.86)	0.185	Adjusted OR (95% CI)‡	-ref-	2.95 (1.68-5.19)	**<0.001**
Adjusted OR (95% CI)†		-ref-	1.76 (0.68-4.59)	0.248	Adjusted OR (95% CI)§	-ref-	2.37 (1.30-4.34)	**0.005**
Composite inflammatory event								
No.Cases	43 (31.6%)	31 (28.2%)	12 (46.2%)		73 (3.3%)	54 (2.7%)	19 (9.6%)	
Unadjusted OR (95% CI)		-ref-	2.18 (0.91-5.24)	0.080	Unadjusted OR (95% CI)	-ref-	3.24 (1.75-6.01)	**<0.001**
Adjusted OR (95% CI)*		-ref-	2.32 (0.94-5.75)	0.069	Adjusted OR (95% CI)‡	-ref-	2.46 (1.30-4.67)	**0.006**
Adjusted OR (95% CI)†		-ref-	2.12 (0.84-5.37)	0.112	Adjusted OR (95% CI)§	-ref-	2.03 (1.02-4.04)	**0.043**
ICU-stay over 24 hours								
No.Cases	84(61.8%)	68 (61.8%)	16 (61.5%)		186 (8.5%)	158 (7.9%)	28 (14.2%)	
Unadjusted OR (95% CI)		-ref-	0.98 (0.41-2.38)	0.979	Unadjusted OR (95% CI)	-ref-	1.93 (1.25-2.97)	**0.003**
Adjusted OR (95% CI)*		-ref-	1.05 (0.43-2.55)	0.923	Adjusted OR (95% CI)‡	-ref-	1.63 (1.05-2.55)	**0.031**
Adjusted OR (95% CI)†		-ref-	0.90 (0.36-2.26)	0.822	Adjusted OR (95% CI)§	-ref-	1.28 (0.79-2.06)	0.313
3-year mortality								
No.Cases	20 (14.7%)	12 (10.9%)	8 (30.8%)		81 (3.7%)	60 (3.0%)	21 (10.7%)	
Unadjusted OR (95% CI)		-ref-	3.63 (1.30-10.13)	**0.014**	Unadjusted OR (95% CI)	-ref-	3.85 (2.29-6.48)	**<0.001**
Adjusted OR (95% CI)*		-ref-	3.78 (1.25-11.42)	**0.019**	Adjusted OR (95% CI)‡	-ref-	3.40 (2.00-5.78)	**<0.001**
Adjusted OR (95% CI)†		-ref-	4.48 (1.32-15.22)	**0.016**	Adjusted OR (95% CI)§	-ref-	2.64 (1.49-4.65)	**0.001**

AAA, abdominal aortic aneurysm; CABG, coronary artery bypass grafting; ICU, intensive care unit. Multivariate logistic regression analyses adjusted for: * age, preoperative unstable angina, current smoking, eGFR; ; estimated glomerular filtration rate, † age, preoperative unstable angina, current smoking, eGFR, hemoglobin and white blood cell count, ‡ age, eGFR, § age, eGFR, current smoking and symptomatic status.

Figure 4. Hematopoietic tissue activity.



Examples showing FDG-PET with overlay CT-images of patient with high hematopoietic tissue activity (A) and low hematopoietic tissue activity (B). Detail image of the spleen (A1) shows increased FDG-uptake compared to splenic activity in B2. Splenic SUV-max scored in patient A was 2.4 and 1.3 in patient B. Relationship between red cell distribution width and splenic activity (C) and spinal bone marrow activity (D). The correlation of the expression of mean FDG-activity was analyzed and associated with RDW. FDG-PET, 8F-fluorodeoxyglucose positron emission tomographic; CT, computed tomography; SUV, standardized uptake values.

Risk prediction

Clinical models including predictors for postoperative inflammatory complications in the AAA and CABG cohort improved after adding hematological markers. Figure 3 shows traditional ROC analyses for the composite inflammatory outcome with and without the addition of hematological markers. In AAA-patients, the AUC improved from 0.649 in the clinical model to 0.762 in the combined model (p=0.0181). AUC in the CABG-cohort prediction improved from 0.696 in the clinical model to 0.741 in the combined model (p=0.0056). The interaction of RDW for sex did not reveal significant differences and therefore, no sex stratified analysis was included.

Table 5. FDG-uptakes of hematopoietic tissues and associations with RDW in univariate and multivariate linear regression analyses.

FDG-scores	p-value	Beta Unadjusted	[95%CI]	p-value adjusted	Beta Adjusted	[95%CI]
Spleen SUV-Max	**<0.001**	**0.478**	**(0.060-0.185)**	**0.004**	**0.443**	**(0.038-0.189)**
Spleen SUV-Mean	**<0.001**	**0.524**	**(0.051-0.135)**	**0.001**	**0.517**	**(0.039-0.141)**
BM lumbar Suv-Max	**0.003**	**0.396**	**(0.056-0.258)**	**0.012**	**0.422**	**(0.038-0.283)**
BM lumbar Suv-Mean	**0.003**	**0.399**	**(0.039-0.179)**	**0.004**	**0.480**	**(0.044-0.213)**
BM iliac SUV-Max	0.255	0.062	(-0.004-0.146)	0.098	0.254	(-0.014-0.155)
BM iliac SUV-Mean	0.374	0.123	(-0.036-0.095)	0.191	0.212	(-0.026-0.126)
SC fat SUV-Max	0.271	0.152	(-0.011-0.039)	0.414	0.150	(-0.019-0.046)
SC fat SUV-Mean	0.390	0.119	(-0.012-0.030)	0.189	0.242	(-0.009-0.046)

BM; Bone marrow. eGFR; estimated glomerular filtration rate; SUV, standard uptake value; SC fat, Subcutaneous fat tissue. Bold values are considered significant P-value <0.05 Multivariate models corrected for age, gender, white blood cell count and eGFR.

FDG-PET cohort

Next, we hypothesized that elevated RDW is a consequence of chronic inflammation which induces high turnover of red blood cells thereby increasing hematopoietic tissue activity. Chronic low-grade inflammation can continuously trigger the immune system inducing a state of hyporesponsiveness that makes it vulnerable to internal and external pathogens.

A total of 1771 FDG-PET scan reports were screened and the inclusion flow chart is presented in supplemental Figure 3. One-hundred forty-eight (148) patients met scan inclusion criteria. Another 19 patients were excluded due to immunosuppressive drug use and in 75 patients hematological measurements within 30 days before or after the FDG-PET scan were missing. The indications for FDG-PET scanning included screening for malignancy in 23 patients (42.6%), follow-up scan after resection of primary malignancy in 17 patients (31.5%) or screening for granulomatous/infectious disease in 14 patients (25.9%). All scans used for the current study were negative for these indications.

Baseline characteristics of the 54 eligible patients stratified by median RDW-values are shown in supplemental Table 3. RDW was robustly associated with mean and maximal FDG-uptake of the spleen and bone marrow tissues scored at the lumbar vertebrae in both univariate and multivariate linear regressions models (Table 5 + Figure 4). FDG-uptake in the iliac crest bone marrow was not associated with levels of RDW. RDW was not associated with subcutaneous fat tissue activity, which served as a negative control. Figure 4 shows an example of FDG-PET scans from two patients, one with high and one with low hematopoietic tissue activity. To investigate whether the increased bone marrow activity was due to acute inflammatory reactions, CRP was included in the analyses when available within a two-week period around the scan. Again, RDW showed to be independent of known markers of inflammation such as CRP and WBC (data not shown).

DISCUSSION

This is the first study to show that a single preoperative blood measurement can be used to identify patients in need for preemptive measures to prevent postoperative pneumonia or sepsis. A preoperative elevated RDW associated with a higher incidence of postoperative inflammatory outcomes in two cardiovascular surgery patient cohorts. These findings were irrespective of other established markers of inflammation, i.e. CRP and white blood cell count. Moreover, our data shows that elevated RDW can reflect increased hematopoietic activity as assessed by FDG-PET scanning of the spleen and lumbar bone marrow, possibly reflecting a state of low-grade inflammation.

Preventive measures such as preoperative optimization of pulmonary status or use of prophylactic antibiotics are most effective in patients who are most prone to develop these conditions. Since major surgery provokes a wide range of inflammatory responses, simple means for detection of inflammation prone individuals may serve as valuable tools. These high-risk patients need to be more closely monitored, might be eligible to receive prophylactic antibiotics and strict fluid resuscitation in order to prevent complications such as pneumonia and sepsis, thus shortening hospital-stay and preventing ICU-admissions.

Elevated RDW has been reported to associate with mortality and adverse events in cardiovascular disease (CVD) and cancer.[4,18–22] In non-cardiovascular diseases, RDW has been associated with poor outcome in patients with diabetes, kidney disease, respiratory disorders, and rheumatoid arthritis.[23–28] In secondary cardiovascular risk prediction, RDW outperforms traditional biomarkers such as high-sensitive troponin and NT-proBNP.[29,30] However, the underlying pathophysiological phenomenon of elevated RDW is poorly understood. Although CRP and WBC are commonly used for diagnosis and monitoring of acute inflammation, we hypothesized that an increase in erythrocyte cell size variability better reflects chronic inflammation considering their high numbers and relatively long life span (~110days).[31] Low-grade inflammation induces a state of increased hematopoiesis as reflected by increased activity of the spleen and bone marrow on FDG-PET scanning. Maturation of erythrocytes is impaired by inflammation and immature erythrocytes (reticulocytes) are actively shed into the peripheral blood circulation under inflammatory conditions.[32] Both, reticulocytes and erythrocytes, become more rigid and less deformable by Reactive Oxygen Species (ROS) and nitric oxide, contributing to elevated RDW.[32,33] Red cell viscoelastic properties and deformability are crucial in the microcirculation to pass the narrow capillary lumen and prevent microthrombus formation and obstruction. Loss of these functions contributes to hypoxia, tissue damage and impaired organ function, together contributing to a higher RDW.

Numerous studies have linked RDW with increased morbidity and mortality in cardiovascular patients. However, most lack compelling evidence on the underlying pathophysiologic phenomenon of how RDW interacts with the presented outcome measures. To increase our understanding of the interplay between anisocytosis and inflammatory responses, we focused on organs with key roles in red blood cell life cycle and immune responses by FDG-PET. In humans, the spleen is the largest lymphatic organ which acts both as a quality

control for red blood cells and as an important effector in the active immune response through humoral and cell-mediated pathways.[34,35] Scoring hematopoietic tissues on FDG-PET scans provided insights into hematopoietic tissue activity and their associations with RDW. Our data shows that RDW associates with splenic and lumbar bone marrow activity and thereby likely reflects low grade atherosclerotic inflammation. We did not find a correlation between RDW and bone marrow in the iliac crest; this can most likely be attributed to the confined small space and the resulting variation in intensity.

Our findings are in line with recent imaging studies showing that hematopoietic tissue activity is increased in patients with atherosclerosis compared to normal controls.[17] This increase in FDG-uptake was attributed to a chronically distressed hematopoietic system induced by a state of low-grade inflammation.[15] A larger follow-up study showed that splenic activity was increased after acute coronary syndrome and splenic metabolic activity serves an independent risk factor for recurrent cardiovascular events.[16] Taken together, these studies plea for a cardio-splenic axis triggered by atherosclerosis and acute coronary events explaining the high incidence of recurrent events in CVD patients. With the current study we show that an elevated RDW can reflect this chronically distressed hematopoietic system.

Future perspectives

Abnormal hematological parameters such as elevated RDW are often observed but rarely lead to clinical consequences as the clinical relevance of such deviations remains uncertain. We here provide evidence for a strong prognostic value of RDW, which may help improve clinical decision making. With the increasing possibilities of machine learning, great opportunities arise in using computational power to create algorithms for full blood counts. Capitalizing on routine patient generated data which now remains unused without the need for new measurements or new biomarkers will not only improve patient outcomes but also offer the opportunity to significantly reduce healthcare costs. Combining advanced technology to extract relevant information from readily available measurements will further help evolve current medical practice into twenty-first century personalized medicine.

Strengths and limitations

Due to the retrospective design of our study, we were not able to associate clinical outcome with FDG-PET in the same individuals. Nonetheless, our results are strengthened by validating and replicating our finding that a single preoperative RDW measurement can predict clinical outcome after cardiovascular surgery in two separate cohorts. For clinical application, it is also important to keep in mind that RDW values can depend on the applied analyzer technique. Hence, local cut-off values may differ. Finally, RDW can be used for the differential diagnosis of anemia together with MCV to differentiate between macrocytic and microcytic anemias. Most common type of anemias are acquired and caused by iron (micro) or vitamin B12 (macro) deficiencies, both of which we could not correct for. However, the vast majority of patients with elevated RDW have no iron or vitamin B12 deficiencies, show no MCV alterations, and were not anemic. To correct for the relatively

frequently occurring microcytic anemia, hemoglobin was added to the multivariate models to ensure that the effect of RDW was independent of anemic disorders.

CONCLUSION

Elevated RDW associates with increased risk for postoperative inflammatory complications and hematopoietic tissue activity, which likely reflects a state of low-grade chronic inflammation. This study suggests that a routinely accessible preoperative parameter, RDW, should be considered to identify patients at risk for postoperative inflammatory complications following cardiovascular surgery.

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SUPPLEMENTAL MATERIAL

Supplemental Table 1. Results from univariate logistic regression analysis of RDW as predictor for adverse outcomes after open AAA-repair (=136) or CABG (n=2193). Odds ratios presented are calculated for every increment of 1 percent in RDW.

Supplemental Table 2. Results from multivariate logistic regression analysis of RDW as predictor for adverse outcomes after open AAA-repair (=136) or CABG (n=2193). Odds ratios presented are calculated for every increment of 1 percent in RDW.

Supplemental Table 3. Baseline characteristics of FDG-PET cohort stratified by median RDW value.

Supplemental Figure 1. Flowchart of inclusion for AAA-patients in current analysis.

Supplemental Figure 2. Flowchart of inclusion for CABG-patients in current analysis.

Supplemental Figure 3. Flowchart of inclusion for FDG-PET-patients in current analysis.

Supplemental material is omitted because of space limitations

PART

four

STUDIES OF TIME-DEPENDENT EFFECTS ON ATHEROSCLEROTIC PLAQUE COMPOSITION

9

The impact of diabetes and time-dependence on the atherosclerotic plaque and cardiovascular outcome in patients undergoing iliofemoral endarterectomy

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ABSTRACT

Aim

The incidence of diabetes is rapidly increasing and diabetes is associated with an increased risk of peripheral artery disease. Recent studies have shown a time-dependent decline in vulnerable plaque features and secondary cardiovascular events in iliofemoral endarterectomy (IFE) patients. IFE patients with diabetes have a high risk for cardiovascular events. We do not know, however, if vulnerable plaque features and cardiovascular events reduced over time in IFE patients with diabetes.

Methods

Between 2003 and 2014, 691 atherosclerotic plaques were obtained by IFE, from 212 patients with and 479 patients without diabetes. Plaques were immunohistochemically stained and analysed for the presence of intraplaque hemorrhage, lipid core, calcifications, collagen, smooth muscle cells and macrophages. Patients were stratified according to their diabetic status and year of inclusion. All patients had a follow-up of three years in which cardiovascular adverse events were recorded.

Results

We observed a time-dependent decrease in intraplaque haemorrhage, plaque lipid core and percentage of macrophages in IFE patients with diabetes. After multivariate correction for changes in risk factors over time, intraplaque haemorrhage (64.2% (2002-2005) vs. 39.6% (2012-2014) (P = 0.01)) became significantly less prevalent. Interestingly, the percentage of severely calcified plaques remained high over time. The number of secondary events decreased over time in patients without diabetes (hazard ratio 1·80, 95%CI: 1·15 to 2·81; (P = 0·010) for 2002-2005 versus 2012-2014) but remained high and unchanged in patients with diabetes.

Conclusion

In patients with diabetes undergoing IFE, we found a time-dependent stabilization of atherosclerotic plaque features in line with previous observations in patients with severe atherosclerosis. The presence of severely calcified lesions remained high and unchanged. Secondary event rate remained high in patients with diabetes in contrast to a significant decrease in patients without diabetes. These findings stress the need for improvement of care in IFE patients with diabetes.

INTRODUCTION

The number of individuals diagnosed with type 2 diabetes is rapidly increasing.[1–3] Since diabetes is associated with an increased risk of peripheral artery disease (PAD), it is expected that vascular specialists will have to treat more limbs from diabetic patients in the near future.[4]

Although the incidence of diabetes is increasing, recent findings showed a time-dependent stabilization of both carotid and iliofemoral endarterectomy (IFE) atherosclerotic plaques. Histological features of plaque instability (large lipid core, high lipid content, plaque macrophages and intraplaque hemorrhage) were less prevalent within plaques obtained during most recent years when compared with plaques obtained in more early years. Furthermore a reduced incidence of cardiovascular events during three years of follow-up in IFE patients was reported.[5,6]

A possible explanation for the observed plaque stabilization and reduced incidence of secondary cardiovascular events are the dramatic changes made in cardiovascular risk management of patients with atherosclerotic disease.[7,8] Until the beginning of 2000, separate guidelines existed for each risk factor and patients were treated in case their risk factor exceeded a certain threshold. Nowadays, all cardiovascular risk factors are treated aggressively despite these previously determined thresholds. This is especially true for non-diabetic patients since most diabetic patients have already been subjected towards years of aggressive primary prevention regimes since their year of diagnosed diabetes.[9]

Furthermore It is currently unknown, however, if this reduction in vulnerable plaque features and secondary cardiovascular events also occurred in IFE-patients with diabetes who are known to have more severely calcified plaques.[10] Moreover, in the Netherlands, patients with claudication are nowadays first treated conservatively with supervised walking therapy before invasive therapy is started. It is unclear if these treatment strategies have influenced outcomes for diabetic patients.

Considering all the changes occurring in the diabetic PAD population, insight in the underlying culprit of PAD and clinical follow-up over the last decade is essential for understanding how disease course changed over time. For this study, we therefore investigated atherosclerotic plaques and three-year cardiovascular follow-up of IFE patients with and without diabetes included over a twelve year period.

METHODS

Athero-Express Biobank Study

For this study, data from the Athero-Express Biobank Study (AE) was used. The AE is an ongoing prospective study continuously collecting atherosclerotic plaques from patients undergoing IFE or carotid endarterectomy. The AE started at 2002 in the University Medical Centre Utrecht and the St. Antonius Hospital Nieuwegein in the Netherlands and has since collected over 2800 plaques making it the largest plaque biobank in the world.[11]

The University Medical Centre Utrecht is an academic centre and the St. Antonius Hospital Nieuwegein is a community centre and both hospitals serve as tertiary care centres. The study design has been reported previously but in short, atherosclerotic plaques of patients undergoing IFE are harvested during surgical revascularization and are directly processed for histological staining.[12,13] Between July 2002 and October 2014, 691 patients underwent IFE at one of the two institutions. Patients considered eligible for the current analysis did not have missing data on diabetic status or plaque histology. Furthermore, when patients had multiple entries in the AE (e.g. patients operated on both left and right iliofemoral arteries), only the first entry was used for analyses. All included patients provided written informed consent and medical ethics committees from both participating hospitals approved the study. Included patients filled-out an extensive questionnaire regarding medical history, cardiovascular history, medication use, family history of cardiovascular disease, functional status, quality of life and presence of general cardiovascular risk factors. Missing information was supplemented with information provided by the general practitioner or hospital data systems. The cohort was stratified based on diabetic status, and time-dependent plaque changes were investigated separately for diabetic and non-diabetic patients.

Definition of diabetes

Diabetic disease status was defined as follows: patients receiving insulin or oral glucose inhibitors as stated in the questionnaire of extracted from the hospital patient file or diabetes in medical history obtained from the patient file or self-reported diabetes mellitus in the questionnaire. Moreover, the blood glucose measurements presented, was the last preoperative measurement with a one-month range.

Follow-up

All patients were contacted annually during a three-year follow-up for the occurrence of adverse cardiovascular events or hospitalizations for cardiovascular disease. When a patient indicated an event or hospitalization had occurred hospital data records were checked for validation of the event. When no hospital records were present for the indicated event the general practitioner was contacted directly for validation of the event. In the case of no-response, the general practitioner was contacted for the occurrence of adverse cardiovascular events during three-year follow-up. For the current analyses we used a composite endpoint which consisted of: stroke, myocardial infarction, cardiovascular death, coronary interventions (percutaneous coronary intervention or coronary artery bypass grafting), peripheral intervention (percutaneous transluminal angioplasty or surgical endarterectomy) and leg amputation.

Atherosclerotic plaque processing

Plaque processing protocols have been reported previously, but in short, after IFE atherosclerotic plaques were immediately processed.[11] The culprit lesion was determined on location with the most severe atherosclerotic plaque burden. This culprit lesion was cut into 5 millimeter segments along the longitudinal axis. Hereafter the culprit lesion was then ~~be~~ further processed for immunohistochemical staining.[14] Plaques were stained with hematoxylin eosin (HE) for a general overview including fat content and calcifications, alpha-actin for smooth muscle cells (SMC), CD68 for macrophages picro-sirius Red (PSR) for collagen and HE and fibrin for intraplaque hemorrhage. Presence of calcifications, collagen content and intraplaque hemorrhage were scored semiquantitatively either as no/minor (0) or moderate/heavy staining (1). Furthermore, plaques were scored for lipid content in which a cut-off of more or less than 10% fat was used stratifying between fibrous (less than 10% Fat) and fibrous-atheromatous plaques. Macrophage content and SMC content were scored quantitatively using computerized analyses software AnalySIS 3.2 (Soft Imaging Systems GmbH, Münster, Germany), and reported as percentage positive staining per plaque area. Intraobserver and interobserver variability were examined previously and showed good reproducibility (β0.6-0.9).[13]

Statistical analyses

To study time-dependent differences in diabetic patients all diabetic patients were selected based on the previously determined diabetes definition. After this the cohort was stratified in quartiles based on date of inclusion. Differences in baseline characteristics over time were tested using Pearson Chi-Square for dichotomous variables and One-way ANOVA was used for normally distributed continuous variables. For parameters showing a nonparametric distribution the Kruskal Wallis test was applied to assess differences over time. The first quartile was then compared with the other quartiles using multivariate logistic regression analyses. To test the association between operation-year and binary plaque characteristics we performed multivariate logistic regression analyses with correction for possible confounders. Confounders for time-dependent differences were determined as follow: baseline characteristics which changed over time ($p<0.2$) and associated in univariate analyses with the plaque characteristic of interest ($p<0.2$). Possible confounders added to the multivariate models are operation type, serum cholesterol and HDL cholesterol for lipid core and surgery type for collagen. For smooth muscle cell the type of surgery and operated artery was added, for macrophages surgery type was added to the multivariate model. Cox regression was used for survival analyses. Baseline characteristics that changed over time and associated with the endpoint of interest ($p<0.2$) were determined as possible confounders and were added to the multivariable model. In non-diabetic patients eGFR and surgery type were added to the multivariate model, in diabetic patients operated artery, surgery type and HDL-cholesterol were added to the multivariate model. Values with a $p < 0.05$ were considered statistically significant. Missing data were imputed using single imputation with R (R computing platform version 3.0.2), all statistical analyses were performed using SPSS version 21.0 (SPSS Inc, Chicago, IL).

RESULTS

From 2002 until 2014, a total of 691 unique IFE plaques were collected in the AE. These plaques were obtained from 212 IFE patients with and 479 without diabetes. A table showing the baseline characteristics of patients with diabetes and without diabetes has been added to the supplemental file (Supplemental Table 1). Patients were first stratified on diabetic status and then further stratified into four groups of three years based on their date of inclusion.

In IFE patients with diabetes, no large differences in baseline characteristics for diabetic patients were observed (Table 1) within the 12 years of the study period. The number of patients with a previous history of coronary artery disease, stroke or peripheral intervention remained unchanged. Furthermore, the indication for surgery did not alter over time. The only significant differences that could be observed were lower levels of total and high-density lipoprotein (HDL) cholesterol within the last three time periods, while levels of low-density lipoprotein (LDL) remained stable over time.

In IFE patients without diabetes more differences were observed (Table 2). First of all, there was a non-significant trend with a higher Fontaine classification over time. Furthermore, patients who had surgery within the last time periods compared to the first time period were older, more often treated for hypertension and levels of both total as well as LDL cholesterol were significantly lower. For both patients with and without diabetes the percentage of remote endarterectomy decreased over time.

Plaque histology patients with diabetes

Over time, plaque morphology changed in the IFE population with diabetes. IFE patients with diabetes demonstrated a more stable plaque phenotype within the 2009-2012 and 2012-2014 time periods when compared to the 2002-2005 and 2006-2008 time periods (Table 3). The percentage of plaques with a lipid core, presence of intraplaque hemorrhage, and severe calcification decreased over time (Table 3). After correction for potential confounders, the presence of intraplaque hemorrhage was the only plaque feature that remained statistically significant (Table 4). The amount of quantitatively determined SMCs and macrophages did not show a gradual decline over time.

Figure 1 demonstrates the binary plaque features over time for both diabetic and non-diabetic IFE patients. Interestingly IFE patients with diabetes and IFE patients without diabetes demonstrate the same pattern in time for the percentage of plaques with a lipid core, intraplaque hemorrhage or moderate or heavy collagen staining. However, a different pattern was observed for the percentage of severely calcified lesions. Non-diabetic patients demonstrated a lower *percentage of plaque calcifications in the 2009-2011 and 2012-2014 periods. In patients with diabetes, the high percentage of severely calcified lesions persisted throughout the first three periods (2002-2011) and only showed a decrease in the 2012-2014 period.*

Table 1. Baseline characteristics diabetic IFE patients over time.

Year	2002-2005	2006-2008	2009-2011	2012-2014	p-value*
Number of patients	53	53	53	53	
Male gender, n(%)	43(81.1)	40(75.5)	37(69.8)	43(81.1)	0.453
Age in years, (mean)(±SD	67.3±9.6	67.6±9.0	68.7±9.1	69.6±7.6	0.520
BMI (mean)(±SD)	27.3±3.5	27.5±4.8	27.9±4.4	27.4±4.5	0.918
Current smoker, yes n(%)	21(40.4)	17(32.1)	14(26.4)	16(30.2)	0.474
Hypertension/drug use, yes n(%)	41(80.4)	39(75.0)	42(80.8)	43(86.0)	0.579
Hypercholesterolaemia, yes n(%)	38(74.5)	39(79.6)	34(81.0)	32(76.2)	0.870
History of CAD, yes n(%)	25(47.2)	30(56.6)	23(43.4)	26(49.1)	0.580
History of stroke, yes n(%)	4(7.8)	1(1.9)	3(6.0)	4(8.3)	0.497
History of peripheral intervention, yes n(%)	30(56.6)	22(41.5)	26(50.0)	32(60.4)	0.225
History of Amputation, yes n(%)	5(9.6)	6(11.5)	4(9.1)	4(8.2)	0.950
Fontaine Classification					
Ø Fontaine IIb	23(47.9)	21(42.0)	20(50.0)	14(35.0)	0.823
Ø Fontaine III	10(20.8)	12(24.0)	7(17.5)	12(30.0)	
Ø Fontaine IV	15(31.3)	17(34.0)	13(32.5)	14(35.0)	
Operated Artery					
Ø Femoral	46(86.8)	49(92.5)	53(100.0)	50(94.3)	0.054
Ø Iliac	7(13.2)	4(7.5)	-	3(5.7)	
Operation Type					
Ø REA	17(32.1)	17(32.1)	8(15.1)	7(13.2)	**0.022**
Ø TEA	36(67.9)	36(67.9)	45(84.9)	46(86.8)	
Systolic blood pressure (mm/Hg)(±SD)	148±29	144±22	149±18	143±25	0.599
Diastolic blood pressure (mm/Hg)(±SD)	79±13	75±11	77±12	74±16	0.339
Glucose in Mmol/) fasting (SD)(±SD)	8.7±4.6	7.8±2.5	8.6±2.7	7.2±1.8	0.269
eGFR in mL/min/1.73 m2 (mean)(±SD)	73.4±23.3	74.3±32.9	74.0±32.8	77.1±28.3	0.932
Triglycerides in mmol/L (mean)(±SD)	2.4±1.5	2.0±1.3	2.1±0.9	2.0±1.5	0.738
Total cholesterol in mmol/L (mean)(±SD)	5.0±1.3	4.1±1.3	4.1±1.1	4.2±1.1	**0.023**
HDL in mmol/L (mean)(±SD)	1.4±0.5	1.0±0.2	1.0±0.3	1.0±0.4	**<0.001**
LDL in mmol/L (mean)(±SD)	2.2±0.7	2.2±1.0	2.1±0.7	2.3±1.0	0.879
Statin use, yes n(%)	37(69.8)	42(79.2)	43(81.1)	43(81.1)	0.439
Antiplatelet use, yes n(%)	44(83.0)	38(71.7)	41(77.4)	45(84.9)	0.328
Insulin use, yes n(%)	18 (34.0)	21(39.6)	14(26.4)	17(32.1)	0.545
Oral glucose inhibitor, yes n(%)	36(67.9)	34(64.2)	41(77.4)	38(71.8)	0.491
Anti-coagulant use, yes n(%)	13(24.5)	15(28.3)	8(15.1)	8(15.1)	0.225

Abbreviations: BMI, Body Mass Index; CAD, Coronary Artery Disease; eGFR, estimated Glomerular Filtration Rate; REA, remote endarterectomy; TEA, thromboendarterectomy. *Comparison of the four groups by univariable analysis.

Table 2. Baseline characteristics non-diabetic IFE patients over time.

	2002-2005	2006-2008	2009-2011	2012-2014	p-value*
Number of patients	120	120	120	119	
Male gender, n(%)	86(72)	83(69)	86(72)	88(74)	0.880
Age in years, (mean)(±SD)	66.5±9.55	66.7±9.46	67.9±9.39	69.3±8.94	0.080
BMI (mean)(±SD)	25.4±3.7	25.2±4.2	25.5±3.3	25.6±4.0	0.864
Current smoker, yes	58(48)	50(43)	49(42)	45(38)	0.459
Hypertension/drug use, yes	70(58)	78(65)	91(78)	90(80)	**0.001**
Hypercholesterolaemia, yes	74(62)	74(65)	72(72)	77(73)	0.252
History of CAD, yes	37(31)	51(43)	49(41)	50(42)	0.203
History of stroke, yes	8(7)	4(3)	4(3)	9(8)	0.258
History of peripheral intervention, yes	53(44)	58(48)	53(45)	56(47)	0.901
History of Amputation, yes	2(2)	4(3)	4(4)	2(2)	0.669
Fontaine Classification					0.262
Ø Fontaine IIb	78(66)	65(57)	46(51)	54(55)	
Ø Fontaine III	28(24)	33(29)	30(33)	24(24)	
Ø Fontaine IV	13(11)	16(14)	15(16)	20(20)	
Operated Artery					
Ø Femoral	102(85)	102(85)	114(95)	116(97)	**<0.001**
Ø Iliac	18(15)	18(15)	6(5)	3(3)	
Operation Type					
Ø REA	55(46)	29(24)	17(14)	22(18)	**<0.001**
Ø TEA	65(54)	91(76)	103(86)	97(82)	
Systolic blood pressure (mm/Hg)(±SD)	153±25	147±22	146±25	150±26	0.158
Diastolic blood pressure (mm/Hg)(±SD)	80±12	77±11	77±12	77±12	0.120
Glucose Mmol/L fasting (SD)	5.8±1.0	6.0±2.0	5.9±1.3	5.8±0.8	0.948
eGFR in mL/min/1.73 m2 (mean)(±SD)	73.4±23.6	80.9±25.8	77.1±26.2	83.3±29.3	**0.027**
Triglycerides in mmol/L (mean)(±SD)	2.0±1.3	1.7±0.9	1.9±0.8	2.1±1.2	0.252
Total cholesterol in mmol/L (mean)(±SD)	5.1±1.2	4.4±0.9	4.5±1.0	4.4±1.0	**<0.001**
HDL in mmol/L (mean)(±SD)	1.2±0.3	1.1±0.3	1.1±0.3	1.2±0.5	0.397
LDL in mmol/L (mean)(±SD)	3.0±1.1	2.5±0.7	2.5±0.8	2.3±0.8	**<0.001**
Statin use, yes	79(66)	89(74)	91(76)	84(71)	0.325
Antiplatelet use, yes n(%)	102(85)	98(82)	102(86)	103(87)	0.736
Anti-coagulant use, yes n(%)	21(18)	24(20)	18(15)	12(10)	**<0.001**

Abbreviations: BMI, Body Mass Index; CAD, Coronary Artery Disease; eGFR, estimated Glomerular Filtration Rate; REA, remote endarterectomy; TEA, thromboendarterectomy. *Comparison of the four groups by univariable analysis.

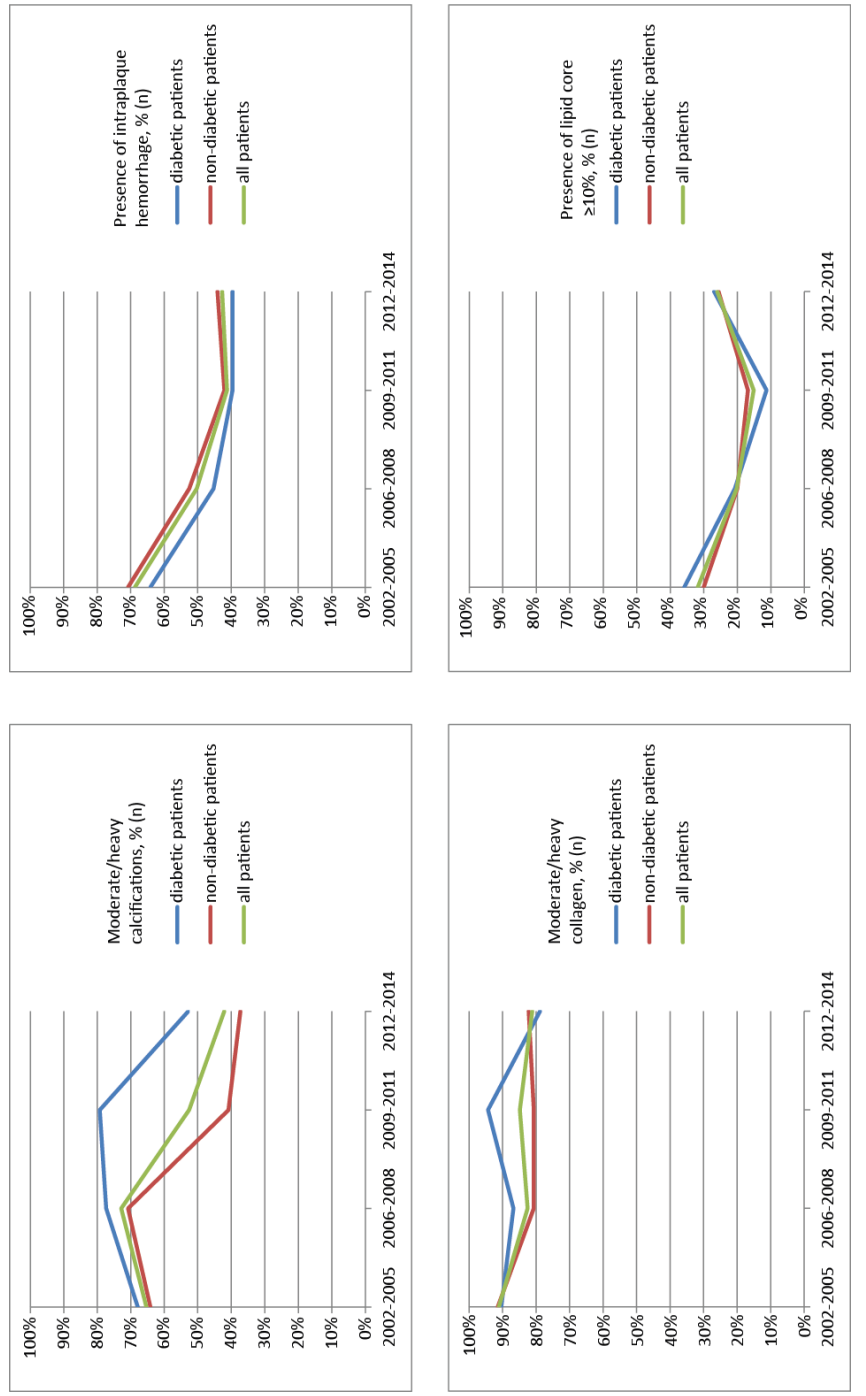


Figure 1. Percentage of plaque characteristics per two-year cohort stratified by diabetic status. Overview of semi-quantitatively scored ilio-femoral plaque characteristics over time. Blue line: diabetic patients; red line: non-diabetic patients; green line: all patients.

Table 3. Histological atherosclerotic plaque characteristics of diabetic IFE-patients.

Year	2002-2005	2006-2008	2009-2011	2012-2014	p-value*
Semi-quantitative plaque features	53	53	53	53	
Presence of lipid core ≥10%, % (n)	19 (35.8)	11(20.8)	6(11.3)	14(26.9)	**0.03**
Moderate/heavy calcifications, % (n)	36(67.9)	41(77.4)	42(79.2)	28(52.8)	**0.01**
Moderate/heavy collagen, % (n)	47(90.4)	46(86.8)	50(94.3)	41(78.8)	0.10
Presence of intraplaque hemorrhage, % (n)	34(64.2)	24(45.4)	21(39.6)	21(39.6)	**0.04**
Continuous plaque features					**p-value**
% of positive macrophage staining per plaque (median IQR)	0.036 (0.010 -0.235)	0.197 (0.039 -0.898)	0.029 (0.008 -0.113)	0.027 (0.007-0.130)	**<0.001**
% of positive SMC staining per plaque (median IQR)	1.317 (0.426-3.385)	2.377 (1.015-4.492)	1.597 (0.496-4.238)	1.543 (0.310-2.547)	0.10

Abbreviations: IQR:interquartile range, SMC, Smooth Muscle Cell. *Comparison of the four groups by univariable analysis.

Cardiovascular outcomes

Overall, patients with diabetes had a higher incidence of secondary cardiovascular endpoints (any cardiovascular endpoint) compared with non-diabetic patients (Table 5). Although it was not our primary goal to directly compare endpoints between IFE-patients with and without diabetes, the average incidence of all separate components of the composite endpoint was higher in patients with diabetes. The occurrence of endpoints in IFE patients with diabetes did not change and remained high over time (Table 6). However, for patients without diabetes the percentage of patients with a composite endpoint was significantly lower during the 2002-2005 and 2006-2008 periods when compared with the 2012-2014 period. This was mainly caused by a significant decrease in peripheral interventions during the 2009-2011 and 2012-2014 periods. These differences remained significant after correction for confounders (Table 6 and Figure 2). Considering that peripheral interventions were an important part of the secondary interventions, insight into the specific target vessel revascularization (TVR) is crucial. As presented in Table 5, TVR remained unchanged in IFE patients with diabetes but decreased over time in IFE-patients without diabetes (p=0.002). Considering the relative small number of events and small groups no multivariate analyses was performed on these secondary events.

Sex can be an important factor influencing the number of secondary events in patients with atherosclerotic disease. This interaction for sex was tested by adding an interaction term to the multivariate analyses. Within IFE patients with diabetes no statistical significant interaction between male and female patients on cardiovascular outcomes was seen and this is likely due to an insufficient power. However, female patients are known to have a worse outcome after surgical revascularization and sex-stratified outcomes have therefore been added to the supplemental (Supplemental Table 2 and 3).[15]

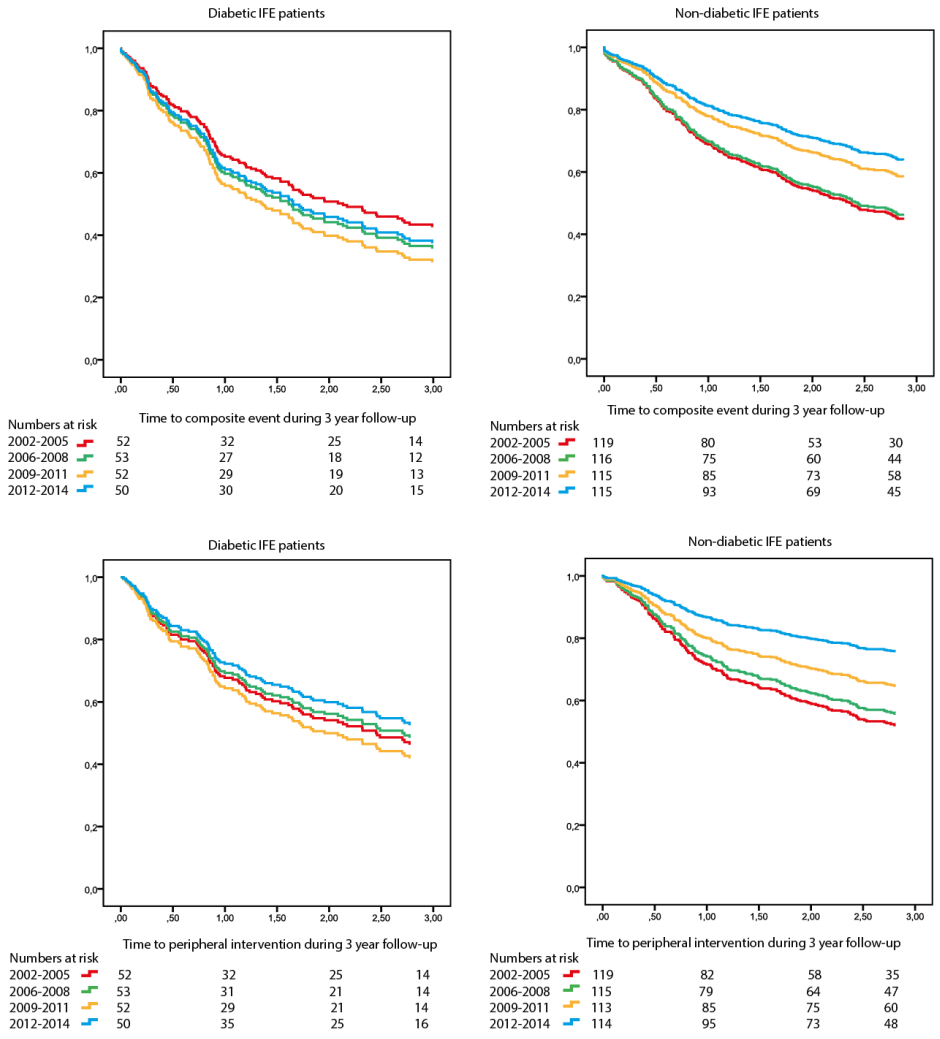
Table 4. Multivariable plaque analysis of diabetic IFE-patients.

Binary plaque characteristics	OR of 2009–2011 vs. 2002–2005 (95% CI)	p-value	OR of 2012–2014 vs. 2002–2005 (95% CI)	p-value
Presence of lipid core ≥10%	0.22 (0.08-0.65)	**0.01**	0.67 (0.28-1.65)	0.39
Moderate/heavy calcifications	1.93 (0.75-4.98)	0.17	0.62 (0.26-1.42)	0.25
Moderate/heavy collagen	2.03 (0.45-9.01)	0.36	0.45 (0.14-1.44)	0.18
Presence of intraplaque hemorrhage	0.37 (0.17-0.81)	**0.01**	0.37 (0.17-0.81)	**0.01**
Continuous plaque characteristics	**Beta of 2009–2011 vs. 2002–2005 (95% CI)**	**p-value**	**Beta of 2012–2014 vs. 2002–2005 (95% CI)**	**p-value**
% of positive macrophage staining per plaque	-0.08 (-0.19-0.06)	0.31	-0.04 (-0.20-0.12)	0.59
% of positive SMC staining per plaque	0.11 (-0.09-0.43)	0.19	-0.02 (-0.36-0.30)	0.84

Binary plaque characteristics	OR of 2006–2008 vs. 2002–2005 (95% CI)	p-value
Presence of lipid core ≥10%	0.51 (0.21-1.24)	0.14
Moderate/heavy calcifications	2.06 (0.78-5.46)	0.15
Moderate/heavy collagen	0.70 (0.21-2.338)	0.57
Presence of intraplaque hemorrhage	0.46 (0.21-1.01)	**0.05**
Continuous plaque characteristics	**Beta of 2006–2008 vs. 2002–2005 (95% CI)**	**p-value**
% of positive macrophage staining per plaque	0.31 (0.11-0.35)	**<0.001**
% of positive SMC staining per plaque	0.16 (-0.06-0.50)	0.06

Odds ratios (OR) and betas are given for depicted cohorts compared with the cohort 2002–2005. CI, confidence interval.

Figure 2. Hazard curves for composite events after iliofemoral endarterectomy in diabetic and non-diabetic patients.



Multivariate cox regression survival analyses for both composite cardiovascular endpoints and peripheral events during 3 year follow-up. Multivariate model corrected for age, eGFR, hypertension and Fontaine classification in non-diabetic patients and operated artery and type of surgery in diabetic patients.

DISCUSSION

With an increasing prevalence of type 2 diabetes, knowledge of the interaction of diabetes on atherosclerotic plaque morphology is of great importance. Furthermore, primary and secondary prevention of cardiovascular disease has changed within the last decades, especially for non-diabetic patients. Analysis of a large number of atherosclerotic plaques

Table 5. 3-year cohort clinical outcomes diabetic and non-diabetic IFE patients during 3-year follow-up.

Diabetic patients	**Cohort 2002-2005**	**Cohort 2006-2008**	**Cohort 2009-2011**	**Cohort 2012-2014**	**Total cohort**	**p-value***
	n=52	*n*=53	*n*=52	*n*=50	*n*=207	
EP composite, n(%)	30(58)	29(55)	33(63)	29(58)	121(58)	0.835
EP major, n(%)	3(6)	7(13)	13(25)	8(16)	31(15)	0.052
Death, n(%)	9(17)	15(28)	17(33)	5(10)	46(22)	**0.024**
CV death, n(%)	3(6)	4(8)	10(19)	3(6)	20(10)	0.060
Peripheral interventions, n(%)	28(54)	23(43)	26(50)	21(42)	98(47)	0.586
TVR, n (%)	10(19)	8(15)	7(14)	10(20)	35(17)	0.776
PTA, n(%)	25(48)	18(34)	22(42)	21(42)	86(42)	0.535
Leg amputations, n(%)	6(12)	9(17)	8(15)	2(4)	25(12)	0.185
Non-diabetic patients	*n*=119	*n*=116	*n*=115	*n*=116	*n*=466	p-value
EP composite, n(%)	64(54)	59(51)	47(41)	38(33)	208(45)	**0.004**
EP major, n(%)	13(11)	12(10)	10(9)	11(9)	46(10)	0.945
Death, n(%)	20(17)	20(17)	16(14)	17(15)	73(16)	0.875
CV death, n(%)	9(8)	8(7)	8(7)	6(5)	31(7)	0.897
Peripheral interventions, n(%)	55(46)	48(41)	39(34)	25(22)	167(36)	**0.001**
TVR, n (%)	26(22)	15(13)	7(6)	11(10)	59(13)	**0.002**
PTA, n(%)	53(45)	44(38)	37(32)	25(22)	159(34)	**0.002**
Leg amputations, n(%)	6(5)	7(6)	4(3)	2(2)	19(4)	0.367

EP composite: composite endpoint includes: cardiovascular death (CV), stroke, myocardial infarction (MI), coronary interventions (CI) and peripheral interventions. EP major: major endpoint includes: all cardiovascular death, and all cerebral and myocardial infarctions. TVR; target vessel revascularization, PTA: percutaneous transluminal angioplasty. *Comparison of the four groups by univariable analysis.

Table 6. Multivariable survival analysis diabetic and non-diabetic cohort ➔ outcome.

	Diabetic patients		**Non-diabetic patients**	
Operation year	**HR composite endpoints (95% CI)**	**p-value**	**HR composite endpoints (95% CI)**	**p-value**
2002-2005	0.87 [0.52-1.46]	0.595	**1.80 [1.15-2.81]**	**0.010**
2006-2008	1.05 [0.62-1.77]	0.973	**1.69 [1.08-2.64]**	**0.022**
2009-2011	1.18 [0.71-1.97]	0.524	1.10 [0.68-1.78]	0.669
2012-2014	- ref-	- ref-	- ref-	- ref-

Multivariate model corrected for age, eGFR, hypertension and Fontaine classification in non-diabetic patients and operated artery, HDL-cholesterol and type of surgery in diabetic patients.

over a large period enabled us to investigate trends in plaque development. We analysed a cohort of 691 IFE-patients in which 212 patients with diabetes were identified. Our study showed a time-dependent decrease in intra-plaque haemorrhage in patients with diabetes. Other plaque features such as lipid content, collagen, calcifications, macrophage content and SMC did not show a gradual decline over time in patients with diabetes. Moreover, our follow-up data showed that the occurrence of adverse cardiovascular events decreased

in IFE-patients without diabetes but not in patients with diabetes. This decrease in adverse events in follow-up was attributable to a decrease in peripheral interventions in IFE patients without diabetes; in patients with diabetes, the percentage of peripheral interventions remained high during follow-up. These findings stress the need for improvement of outcomes in IFE patients with diabetes.

The decline in intra-plaque hemorrhage as found in previous work was also observed in patients with diabetes.[6] Diabetic patients are known to have a high percentage of calcified plaques, but we could not detect a decline in time in this diabetes-specific plaque feature.[10] One of the possible explanations is that the persistent high prevalence of severely calcified lesions within patients with diabetes might be an effect of statin treatment. Statin-use has been associated with increased calcifications of coronary arteries. Within a large post hoc analysis of more than 3400 patients with coronary artery disease undergoing serial coronary IVUS, statin therapy was significantly associated with an increase in calcification of the coronary atheroma. Moreover, high intensity statin treatment was compared with low intensity and no statin treatment and associated with the largest increase in coronary atheroma calcification.[16] Patients with diabetes demonstrated a consistently high percentage of statin use throughout the years of inclusion. As a consequence, no significant differences in LDL levels could be observed in patients with diabetes. In non-diabetic patients, however, an increase in statin use and subsequently lowering of LDL-levels was observed. Another explanation might be the fact that patients undergoing IFE have a longer duration of symptoms before the plaque is removed and plaques stabilize after an initial event. In a sample of 1455 CEA patients, no clear differences in calcifications were observed between the diabetic and non-diabetic patients.[17] This might be explained by the fact that the time between event and removal of the plaque is much lower compared with IFE patients in which surgical revascularization are often postponed as long as possible, since more severely calcified lesions were found in the diabetic IFE group. This suggests that timing of surgical intervention could have had an effect on the plaque calcification and suggesting that plaque remodeling in patients with diabetes is different from plaque remodeling in patients without diabetes.

Recent guideline changes favouring conservative therapy for patients with claudication as a first treatment and an increase in endovascular interventions results in a delay between first symptoms and plaque harvesting within the more recent years. Furthermore the number of remote endarterectomy procedures significantly declined over time, which is probably caused by an increase of endovascular revascularization procedures. Since patients only enter the AE biobank in case of an endarterectomy procedure the exact number of patients treated endovascular are unknown. In addition, the superficial femoral artery is currently increasingly more often treated by endovascular procedures as well. As a consequence relatively more plaques derived from the common femoral artery were collected in most recent years. These effects were assessed and did not influence the plaque histology analyses.

The numbers of secondary peripheral interventions over time are declining probably due to guideline changes which have increasingly advocated conservative therapy in most recent years.[18] This can explain the decline in peripheral interventions we found in IFE patients without diabetes. This conservative therapy, however, might be less effective in diabetic patients. It is conceivable that therapies which strongly depend on functional status are less effective in diabetic patients due to higher body mass index (BMI) and a higher incidence of foot ulcers. Both of these features are increasingly present in the diabetic cohort with an overall BMI of 27.5 compared to 25.4 in non-diabetic patients ($p<0.001$) and a Fontaine classification grade 4 in 33.1% of all diabetic patients compared to 15.2% in non-diabetic patients ($p<0.001$). Overall, the higher Fontaine classifications in IFE-patients with diabetes likely contributed to the overall higher incidence of secondary peripheral interventions. Moreover, in diabetic patients disease progression is often characterized by neuro-ischemic components such as diabetic neuropathy which warrant more aggressive re-intervention strategies as stated by the current guidelines.[19] In interaction models focussing on the combined effects of diabetic status and blood glucose levels on secondary events we only found a small effect on outcome. These models showed that elevated blood glucose levels did not impose an additional effect on future events over diabetic disease status. This indicates that better glucose control might not provide better outcomes in this severely affected atherosclerotic population.

Clinical (treatment) perspectives

An important finding of the current study is that the percentage of plaque calcifications did not decrease over time in patients with diabetes undergoing IFE. Moreover, within the more recent years, patients with diabetes had a higher prevalence of severely calcified plaques, compared with non-diabetic patients. The remaining high prevalence of severely calcified plaques is important in light of different treatment modalities applied to treat iliofemoral stenosis. It has been previously established that following angioplasty, severe calcifications are associated with poor outcome.[20–22] Calcifications are an important risk factor for stent fracture and consecutively vessel patency.[23] Furthermore, a negative relationship between circumferential calcification, assessed at Computed Tomography Angiography (CTA), and vessel patency following paclitaxel coated balloon angioplasty was previously described.[24] These calcifications are believed to prevent formation of a reservoir of paclitaxel and affect drug penetration into the adventitial layer thereby influencing outcome negatively.[24] Therefore (novel) debulking endovascular strategies are being developed in which severely calcified plaques are being removed after which the lesion is treated with drug-coated balloons.[25] A main message of this paper, however, is the great and unchanged percentage of secondary events in IFE patients with diabetes. The percentage of secondary peripheral interventions and three year mortality remained high and calls for improved treatment strategies.

Strengths and limitations

This study has some limitations. First, as guidelines changed over the last decade, it is likely that different patients have been selected for surgical revascularization. Moreover, this is expected to have affected the number of re-interventions during follow-up. The current study, unfortunately, could not provide information on conservative therapy applied in our cohorts. Secondly, due to the relatively small number of patients with diabetes we are less powered to find statistical significance for all time-dependent plaque changes. Third we did not have the possibility to investigate disease specific markers such as HbA1c. It would be interesting to look at severity of diabetes in relation to plaque morphology. Lastly, the relative small sample size of diabetic patients in this population should be taken into consideration when interpreting these results. A major strength of the current study is the unique opportunity to study both the effect of diabetes and time-dependency on plaque characteristics and follow-up data. The fifteen yearlong collections of plaques and clinical data have provided essential insights into changes of the pathophysiological substrate of cardiovascular disease.

In conclusion, in patients with diabetes undergoing IFE we found a time-dependent stabilization of atherosclerotic plaque features including plaque hemorrhage in line with previous observations in patients with severe atherosclerosis. The presence of severely calcified lesions remained high and unchanged. Secondary event rate decreased over time in patients without diabetes, however, in patients with diabetes remained high. These findings stress the need for improvement of care in IFE patients with diabetes.

Take home messages:

- Over time iliofemoral plaques obtained from patients with diabetes showed signs of stabilization and a decrease in the percentage of plaque hemorrhages.
- Secondary cardiovascular outcomes did not improve over time in patients with diabetes in contrast to the non-diabetic iliofemoral population that showed a decrease in the amount of secondary peripheral interventions.
- Plaque calcification is the feature strongest associated to diabetes and plaques from patients with diabetes remained severely calcified over time.
- Changes in best medical treatment are likely to have contributed to these effects. Considering that in the past, patients with diabetes were already treated aggressively with regards to blood pressure and lipid control. In patients without diabetes past treatment strategies have been less aggressive.

Conflict of interest: All the authors have no conflict of interest.
Disclosures: none

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SUPPLEMENTAL MATERIAL

Supplemental Table 1. Baseline characteristics diabetic and non-diabetic patients

Supplemental Table 2. Male patients: 3-year cohort clinical outcomes diabetic and non-diabetic IFE patients during 3-year follow-up

Supplemental Table 3. Female patients: 3-year cohort clinical outcomes diabetic and non-diabetic IFE patients during 3-year follow-up

Supplemental Table 4. Plaque confounder correlation analysis of diabetic IFE-patients.

Supplemental Table 5. Secondary outcome confounder analysis.

Supplemental material is omitted because of space limitations.

10

Serial carotid endarterectomy: Do plaques change over time?

In preparation

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ABSTRACT

Background

Atherosclerotic disease is a systemic condition and affects multiple vascular beds in patients at risk. Considering the systemic nature of atherosclerosis, a similar characterization of lesions might be expected within the same patient. It is currently unknown if multiple plaques obtained from the same patient show comparable histological features.

Methods

The Athero-Express study, which has been initiated in 2002, is an ongoing longitudinal biobank which collects atherosclerotic plaques from patients undergoing carotid endarterectomy (CEA). The Athero-Express was screened for patients who underwent CEA of both carotid arteries at two separate moments in time allowing for the investigation of bilateral plaques from the same patient.

Results

In total, 56 patients were operated both on the left and right carotid artery. Indication for surgery was largely comparable for the subsequent surgical procedures. Timing between event and surgery did not differ (65.1 ± 79.9 vs. 77.6 ± 89.8 days (p=0.60)). The first removed plaque when compared to the second removed plaque showed a higher percentage of vulnerable plaque features such as intraplaque hemorrhage ((69.6% vs 48.2%) p=0.021) and moderate/heavy macrophage content (57.1% vs. 38.2%) p=0.043). The percentage of fibrous lesions was highest in the second removed plaque (21.4% vs. 39.3%) however, not reaching statistical significance (p=0.089). Plaque features such as calcifications, collagen content, and smooth muscle cells did not change over time. Over time duration of statin treatment increased and was highest at the moment of second endarterectomy (p=0.025). Features associated with a relative worsening of plaque characteristics towards a more vulnerable phenotype are diabetes and male sex.

Conclusion

In patients who underwent two subsequent carotid endarterectomies on two separate moments in time, the second dissected plaque revealed a lower incidence of features associated with plaque vulnerability. Statin treatment duration increased over time and might have contributed to this relative stabilization of atherosclerotic plaques.

INTRODUCTION

Cardiovascular disease (CVD) and in particular atherosclerosis is a systemic condition often affecting patients at multiple moments in time and in different vascular beds. Risk factors involved are manifold and comprise of genetic predisposition, aging and lifestyle choices. Since these risk factors are predominantly patient-specific and often hard to reverse, we hypothesized that in individual patients with multiple atherosclerotic lesions a large similarity in plaque characteristics would be present.

Studies on repeated histological assessments of atherosclerotic plaques within the same patients are scarce if present. Most studies on lesion characteristics within the same patient are based on intravascular imaging and compare symptomatic with asymptomatic lesions. By performing two carotid endarterectomies of advanced lesions within the same patient our study will provide two unique ``vascular biopsies'' permitting us to assess the temporal consistency of lesion characteristics that could be driven by systemic effects that may differ among individuals. Additionally, in some patients, cardiovascular risk management such as initiation of statin therapy occurs at the moment of the first endarterectomy. This enables us to study effects of medical therapy and possibly identify potential drivers of changes in plaque characteristics.

For this study, we used the Athero-Express biobank Study (AE).[1] The AE study was initiated in 2002 and has continuously collected over 2400 plaques obtained by carotid endarterectomy (CEA), and over time patients could have had multiple entries in the biobank. This longevity of the biobank offers the opportunity to investigate time-dependent trends in changes of plaque features within the same patient. For this study, all patients were included who underwent CEA and at a later time point also underwent CEA of the contralateral carotid artery. Patients appeared to match on symptomatic status which reduces the risk of confounding by indication since plaques from asymptomatic patients have been shown to be more stable.[2] We report that symptomatic plaques appeared to have fewer features that are considered to be destabilizing when operated for the second time. The increase in prevalence and duration of statin treatment and also changes in lifestyle management could explain these observed differences in plaque characteristics.

METHODS

Population

The Athero-Express Study is an ongoing, longitudinal plaque biobank, collecting plaques from patients operated to treat symptomatic and asymptomatic carotid artery stenosis.[1] Patient characteristics, preoperative blood samples, and three-year follow-up are collected for all patients included in the study. Patients are operated at the University Medical Center Utrecht (UMCU) or the St. Antonius Hospital Nieuwegein, both located in the Netherlands. The Athero-Express study started in March 2002 and has collected over 2400 plaque samples since. Medical ethics committees of both participating hospitals gave approval

for the study and all patients provided written informed consent. Indication for surgery is based on international guidelines for the treatment of asymptomatic and symptomatic carotid artery stenosis.[3–5] The Athero-Express Study was checked for patients who are operated on both left and right carotid artery at two different moments in time. Plaque characteristics from the first removed atherosclerotic plaque were compared with the contralateral (second) removed atherosclerotic plaque.

Atherosclerotic plaque assessment

Atherosclerotic plaques are immediately processed after removal by the vascular surgery team. Protocols have been reported previously.[1,6] In short, the atherosclerotic plaque is divided into segments of 5mm thickness along the longitudinal axis. The segment with the largest plaque burden is chosen as the culprit lesion and processed for immunohistochemical staining. Plaques were stained with Hematoxylin-Eosin (HE) for general overview including calcifications, Verhoeff-Van Gieson for staining of vascular elastic fibers (EVG), CD68 for macrophages, alpha-actin for smooth-muscle cells (SMC), pico-Sirius Red (PSR) for collagen, glycophorin (GLYCC) for plaque hemorrhage and CD34 for microvessels.[7] Plaques with a lipid core covering >40% of the plaque surface were considered atheromatous, plaques with a lipid core covering <40% but over >10% were classified fibroatheromatous and plaques with less than 10% fat were considered fibrous plaques. Intraobserver and interobserver variability have been previously determined and showed good reproducibility (κ 0.6-0.9).[6]

Statistical analyses

Multiple plaques were obtained from the same patients and therefore a paired sample analysis was performed. For continuous variables showing a parametric distribution a paired sample T-test and for nonparametric variables the Wilcoxon signed rank sum test were used. For categorical variables, the McNemar`s test was used to test differences between baseline characteristics and plaque features at two moments in time. Baseline characteristics of the remainder cohort will be given for a general impression of the biobank but will not be used in analyses. In a second analysis all patients who were treated of the contralateral artery for asymptomatic carotid stenosis to correct for possible confounding by indication. Sub-Analyses will be performed to ascertain which patient features associated with stabilization or destabilization of atherosclerotic plaques with regard to plaque hemorrhage and plaque fat content. In these sub-analyses, baseline characteristics will be compared using student`s T-test and Mann-Whitney U-test for parametric and nonparametric continuous variables when appropriate. Pearson Chi-square test was used to test dichotomous variables between groups. SPSS version 21.0 (SPSS Inc, Chicago, IL) was used for all statistical analyses.

RESULTS

Patient characteristics

In total 1,888 carotid artery plaques were screened for the current study, 112 plaques were identified from 56 patients operated on both the left and right carotid artery. Baseline characteristics of the cohort stratified by first and second carotid endarterectomy are reported in Table 1. Reported p-values are provided for differences in baseline characteristics between the moment of first and second contralateral plaque removal. Allremainingcases (1776) were not included for analyses but are reported in Table 1 for a general overview of the current study group in relation to the total AE cohort. Meantime between first and second carotid endarterectomy was 1.5 years and more patients were treated for asymptomatic carotid stenosis at second plaque removal when compared with the indication of surgery at first plaque removal (p=0.04). Timing between the event and surgical procedure was similar between both groups. Other baseline characteristics did not change over time; however, an overall trend of higher prescriptions of medication at second plaque removal is visible. Total cholesterol and blood pressure showed a slight improvement over time, however, none reaching statistical significance. Table 2 depicts a more detailed presentation on how indication for surgery shifted in patients between first and contralateral carotid endarterectomy. Striking is that almost half of all patients (25/51) had similar symptomatology as the indication for first and contralateral carotid endarterectomy.

Plaque composition

The overall plaque phenotype showed stabilization over time with only 12 (21.4%) fibrous plaques at first carotid endarterectomy compared to 22 (39.3%) fibrous plaques at contralateral carotid endarterectomy ((p=0.089) Table 3). Plaque hemorrhage was more often observed at first removed plaque (69.6%) when compared to second plaque removal (48.2%(p=0.021)). In Figure 1 changes in plaque hemorrhage are presented, illustrating that in 32% of all patients the second plaque no longer showed signs of plaque hemorrhage while the first plaque did. Moreover, only 10% of patients without plaque hemorrhage at first endarterectomy did show plaque hemorrhage at the second removed plaque. A similar figure was constructed for relative changes in plaque fat content and has been added to the supplemental (Supplemental Figure 1). In Figure 2 histological plaque staining of one patient operated on both carotids is shown. This figure shows that the first plaque, removed from the left carotid artery, resembles a thin-capped fibro-atheroma. The second plaque, removed from the right carotid artery, shows a relative stable plaque less prone to rupture. For continuous measures of plaque characteristics, the total percentage of macrophage staining was highest in the first plaque removal group (0.47% IQR: 0.12-1.06) when compared with second plaque removal group (0.22% IQR: 0.05-0.59) however, likely due to small sample size not reaching statistical significance (p=0.096). Other continuous plaque characteristics such as microvessels and percentage of smooth muscle cell staining did not differ between first and second plaque removal. Since plaques in asymptomatic patients show more stable features and more asymptomatic patients are present in the second plaque removal group we performed sub-analyses on all symptomatic patients only

Table 1. Patient characteristics stratified by first and contralateral plaque removal.

Patient characteristics	Athero-Express	First plaque removal	Contralateral plaque removal	P-value[a]
	n=1776	*n*=56	*n*=56	
Male sex, n(%)	1196(67.3)	47(83.9)	47(83.9)	-
Age in years, mean ± SD	69.0±9.3	67.7±9.2	69.1±9.6	2.04E-07
BMI, mean ± SD	26.3±4.0	26.9±3.2	27.0±3.3	0.76
Left carotid artery, yes n(%)	946(53.3)	26(46.4)	30(53.6)	-
Right carotid artery, yes n(%)	830(46.7)	30(53.6)	26(46.4)	-
Smoker current, yes n(%) [†]	607(34.9)	16(29.6)	15 (27.8)	0.50
Diabetes, yes n(%)	401(22.6)	18(32.1)	18 (32.1)	1.00
Hypertension, yes n(%) [†]	1261(73.2)	43(78.2)	45(80.4)	1.00
Hypercholesterolaemia, yes n(%) [†]	1080(66.4)	42 (80.8)	45 (84.9)	0.50
History of CAD, yes n(%)	553(31.2)	17(30.4)	21(37.5)	0.13
History of PAD, yes n(%)	368(20.8)	17(30.4)	18(32.1)	1.00
Symptom categories n(%)				0.04
Ø Asymptomatic	242 (13.7)	5 (8.9)	16(28.6)	
Ø TIA	770(43.6)	23(41.1)	17(30.4)	
Ø Stroke	488(27.6)	15(26.8)	9(16.1)	
Ø Ocular	268(15.2)	13(23.2)	14(25.0)	
Systolic blood pressure in mm/Hg mean ± SD	154±26	160±27	158±31	0.33
Diastolic blood pressure in mm/Hg mean ± SD	82±14	86±11	84±15	0.19
Time event to operation, mean ± SD	59.3±71.4	65.1±79.9	77.6±89.8	0.60
eGFR in mL/min/1.73 m2, mean ± SD	72.0±20.7	72.1±18.0	71.8±19.4	0.33
Triglycerides in mmol/L, mean ± SD	1.7±1.0	1.7±1.1	1.8±1.2	0.63
Total cholesterol in mmol/L, mean ± SD	4.5±1.2	4.6±1.3	4.5±1.1	0.46
HDL in mmol/L, mean ± SD	1.1±0.4	1.1±0.4	1.1±0.3	0.07
LDL in mmol/L, mean ± SD	2.6±1.0	2.5±0.7	2.5±0.8	0.74
Statin use, yes n(%)	1343(75.7)	47 (83.9)	47(83.9)	1.00
Antiplatelet use, yes n(%)	1574(88.9)	47(83.9)	50 (89.3)	0.45
Anti-coagulant use, yes n(%)	212(12.0)	8(14.3)	8(14.3)	1.00
Beta-blocker use, yes n(%)	776(43.8)	27(48.2)	31(55.4)	0.45

[a] P-value was calculated for differences in baseline characteristics at first and contralateral endarterectomy. BMI: Body mass index. CAD: coronary artery disease. PAD: peripheral arterial disease. TIA: Transient ischemic attack. eGFR: estimated glomular filtration rate. [†]Some data were missing for these variables.

to demonstrate that symptomatic status is not confounding the demonstrated time-dependent differences (Table 4). As illustrated by these stratified analyses the changes between first and contralateral plaque are similar to those seen in the total cohort.

Potential drivers of plaque destabilization

The cohort was then stratified on basis of plaque changes to assess which patient characteristics associated with destabilization or stabilization of histological plaque features.

Table 2. Operation indication changes between first and contralateral carotid endarterectomy.

Indication first CEA	n(%)	n(%)	Change	Indication contralateral CEA
Ø Asymptomatic	5 (8.9)	4(80%)	Ø Asymptomatic	16(28.6) Asymptomatic
		1(20%)	Ø TIA	17(30.4) TIA
		-	Ø Stroke	9(16.1) Ocular
		-	Ø Ocular	14(25.0) Stroke
Ø TIA	23(41.1)	5(22%)	Ø Asymptomatic	
		11(48%)	Ø TIA	
		3(13%)	Ø Stroke	
		4(17%)	Ø Ocular	
Ø Ocular	13(23.2)	3(23%)	Ø Asymptomatic	
		1(8%)	Ø TIA	
		-	Ø Stroke	
		9(69%)	Ø Ocular	
Ø Stroke	15(26.8)	4(27%)	Ø Asymptomatic	
		5(33%)	Ø TIA	
		5(33%)	Ø Stroke	
		1(7%)	Ø Ocular	

Table 3. Semi quantitative and continuous plaque characteristics stratified by first and contralateral plaque removal.

Semi-quantitative plaque characteristics	Athero-Express	First plaque removal	Contralateral plaque removal	P-value[a]
	n=1776	*n*=56	*n*=56	
Ø Fibrous plaque, n(%)	529(29.8)	12(21.4)	22(39.3)	0.089
Ø Fibroatheromatous plaque, n(%)	795(44.8)	31(55.4)	22(39.3)	
Ø Atheromatous, n(%)	450(25.4)	13(23.2)	12(21.4)	0.763
Moderate/heavy calcifications, n(%)	850(48.0	26(47.3)	29(51.8)	0.678
Moderate/heavy collagen, n(%)	1417(80.1)	46(82.1)	50(89.3)	0.424
Moderate/heavy macrophages, % (n)	933(52.8)	32(57.1)	21(38.2)	**0.043**
Presence of intraplaque hemorrhage, n(%)	1045(59.0)	39(69.6)	27(48.2)	**0.023**
Continuous quantified plaque characteristics				
% of positive macrophage staining per plaque, (median IQR)	0.32 (0.07-1.02)	0.47 (0.12-1.06)	0.22 (0.05-0.59)	0.096
Mean number of microvessels per hotspot mean, SD	8.23 (6.43)	9.38 (7.44)	8.45 (5.87)	0.328
% of positive SMC staining per plaque, (median IQR)	2.14 (0.46-2.78)	1.73 (0.41-2.13)	2.40 (0.39-2.32)	0.428

[a] P-value was calculated for differences between first and contralateral plaque. IQR; interquartile range, SMC; Smooth Muscle Cell. Bold values were considered statistically significant with a P<0.05.

Figure 1. Changes in plaque hemorrhage between first and contralateral endarterectomy.

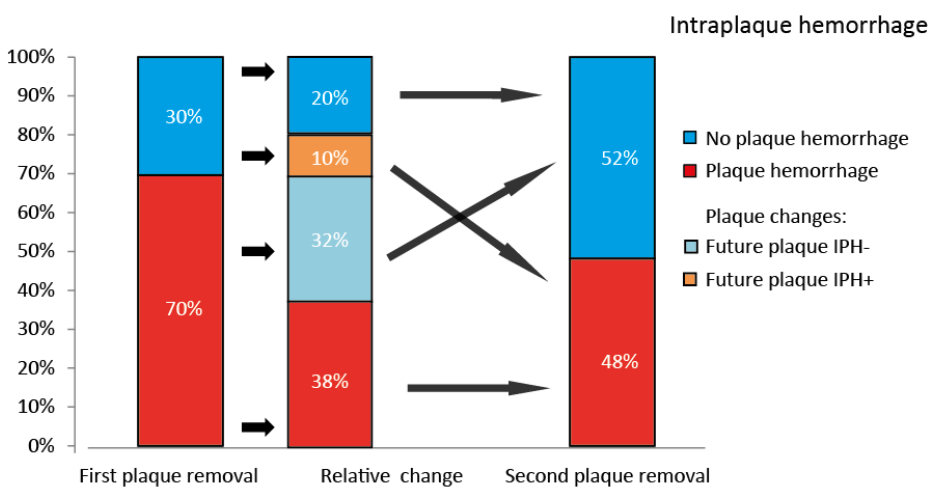
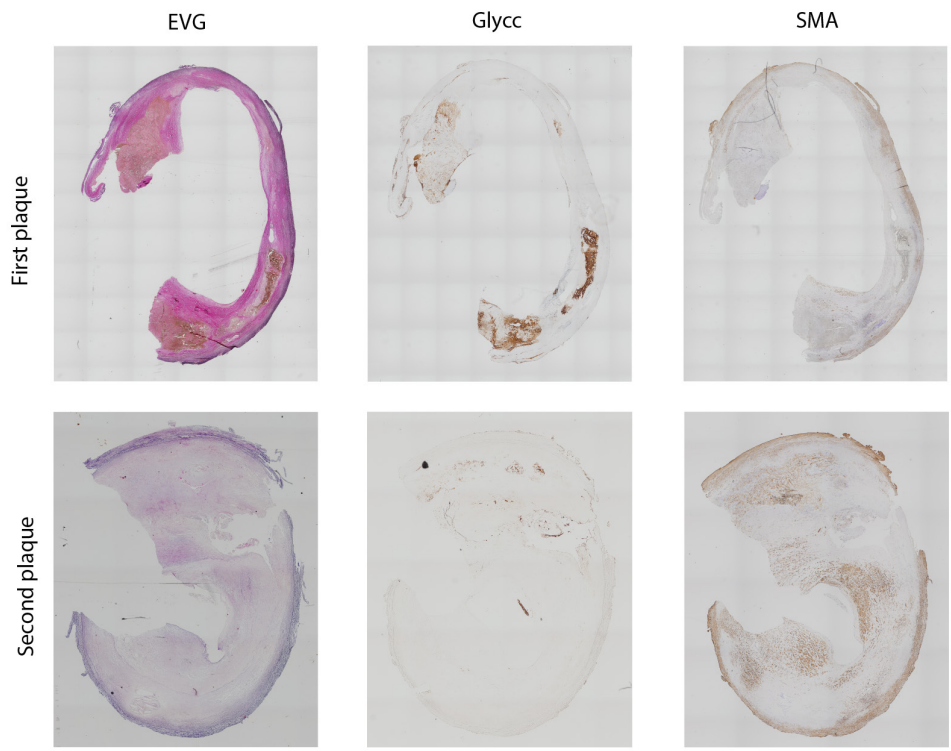


Table 4. Semiquantitative and continuous plaque characteristics stratified by first and contralateral plaque removal. (paired analyses excluding asymptomatic patients)

Semi-quantitative plaque characteristics	**Athero-Express**	**First plaque removal**	**Contralateral plaque removal**	**P-value** [a]
	n=1776	*n*=40	*n*=40	
Ø Fibrous plaque, n(%)	529(29.8)	8(20.0)	16(40.0)	0.096
Ø Fibroatheromatous plaque, n(%)	795 (44.8)	22(55.0)	15(37.5)	
Ø Atheromatous, n(%)	450(25.4)	10(25.0)	9(22.5)	1.000
Moderate/heavy calcifications, n(%)	850 (48.0	21(53.8)	21(52.5)	1.000
Moderate/heavy collagen, n(%)	1417(80.1)	33(82.5)	36(90.0)	0.508
Moderate/heavy macrophages % (n)	933(52.8)	22(55.0)	15(38.5)	0.118
Presence of intraplaque hemorrhage, n(%)	1045(59.0)	31(77.5)	21(52.5)	**0.021**
Continuous quantified plaque characteristics				
% of positive macrophage staining per plaque, (median IQR)	0.32 (0.07-1.02)	0.39 (0.10-0.78)	0.21 (0.039-0.43)	0.357
Mean number of microvessels per hotspot mean, SD	8.23 (6.43)	10.13 (7.66)	7.42 (5.13)	0.055
% of positive SMC staining per plaque, (median IQR)	2.14 (0.46-2.78)	1.04 (0.41-2.13)	1.20 (0.24-2.17)	0.541

[a] P-value was calculated for difference between first and contralateral plaque. IQR; interquartile range, SMC; Smooth Muscle Cell. Bold values were considered statistically significant with a P<0.05.

Figure 2. Examples of plaque histology at first and second endarterectomy.



Example of histological plaque staining of two atherosclerotic plaques obtained from one patient. First plaque shows example of thin-capped fibro-atheroma. Second plaque shows a relative stable plaque without a large necrotic core. EVG; Elastin von Giesen, GLYCC; Glycophorine, SMA; smooth muscle actin.

This stratification was carried out for changes in plaque fat content (Supplemental Table 1) and changes in plaque hemorrhage (Supplemental Table 2). Patients that showed plaque fat decrease were often older and female as opposed to patients that showed plaque fat increase. Medical treatment was highest in patients with plaque destabilization possibly reflecting their severity of atherosclerotic disease.

Duration of statin treatment was highest at second endarterectomy as shown in Table 5. Patients with initiation of statin treatment at least one year before endarterectomy was highest at the contralateral carotid endarterectomy (71.4%) when compared to the first carotid endarterectomy ((45.8%) p=0.025). Contralateral plaques that showed an increase in the prevalence of plaque hemorrhage compared to the first plaque were often obtained from older male patients treated for diabetes. Overall medical therapy was high in all groups and showed no absolute differences. These sub-analyses on potential drivers of plaque stabilization should be interpreted with caution since some strata only contain few patients. A schematic representation of changes in plaque fat content and plaque hemorrhage are proved in Figure 1 and Supplemental Figure 2.

Table 5. Duration of statin treatment stratified by fist and contralateral carotid endarterectomy.

Duration of statin treatment	First carotid endarterectomy N=48	Contralateral carotid endarterectomy N=49	P-value
Ø Statin treatment initiated at least 1 year before carotid endarterectomy, n(%)	22(45.8)	35(71.4)	**0.025**
Ø Statin treatment initiated in year of endarterectomy, n(%)	17(35.4)	7(14.3)	
Ø Not on statin treatment, n(%)	9(18.8)	7(14.3)	

Bold values were considered statistically significant with a P<0.05.

DISCUSSION

To our best knowledge, this is the first study reporting on repeated measures of advanced plaque characteristics in patients undergoing endarterectomy of both carotid arteries. The study shows that the first removed plaque is characterized by a higher incidence of vulnerable plaque features such as intraplaque hemorrhage, macrophages, and lipid content. Atherosclerotic plaques removed at a later moment in time, despite similar timing and comparable neurologic symptomatology, showed a lower incidence of vulnerable plaque characteristics. These histological differences between first and contralateral plaque removal suggest a systemic stabilization of atherosclerotic lesions in patients undergoing endarterectomy of both carotid arteries.

Our observations are in agreement with earlier reports that revealed time-dependent shifts in plaque characteristics that have occurred over the last decade in Western society. The incidence of manifestations of cardiovascular disease is changing, with a decline in stroke and ST-segment elevated myocardial infarctions (STEMI) and a rise in non-ST-segment elevation myocardial infarctions (NSTEMI).[8–10] The explanation for this change is considered to be caused by differences in the pathophysiological mechanisms leading up to manifestations of cardiovascular disease.[11] Symptomatic and asymptomatic atherosclerotic plaques have shifted from plaques characterized by a lipid-rich necrotic core, intraplaque hemorrhage, abundant inflammation and a thin fibrous cap prone to rupture, to a fibrous plaque with low-fat content, low inflammation and which is less prone to rupture.[12] Since atherosclerosis is a systemic condition this local occurring plaque stabilization is also believed to reflect systemic stabilization of the vasculature.[13] Stabilization of atherosclerotic plaques and subsequently changes in manifestations of CVD are believed to largely result from improved risk prevention starting in the 1980s. Effective pharmacological agents such as aggressive lipid-lowering drugs and antihypertensive agents have successfully contributed to a 50% decrease in age-adjusted CVD-mortality.[14]

Earlier work in our biobank has revealed a time-dependent decline in vulnerable atherosclerotic plaque characteristics over the last decade.[12] However, we could not show significant differences over time in the prescription of medication-use at the moment of carotid endarterectomy. A high percentage of medication-use was found in patients operated on both carotid arteries; this is best exemplified when compared with the lower percentage of drug-use in the remainder cohort. However, to establish if the duration of therapy could have influenced our findings of plaque stabilization, we checked the start dates of all prescribed statins in patients in our cohort. We were able to acquire the medication start date in 87 percent of all patients and listed these findings in Table 5. Duration of statin treatment >1 year was most frequently observed in patients undergoing their second endarterectomy (71.4%) when compared to patients at first endarterectomy ((45.8%) p=0.025). The longer duration of prescribed drug-therapy was part to be expected since the patients in the contralateral group were on average one and a half year older at the time of their second endarterectomyand statin-treatment initiated in a large part of patients at first endarterectomy. Animal studies have shown that statin-treatment can reduce lipid-size, decrease inflammation and supplement the overlying fibrous cap.[15] Moreover, in humans, imaging studies have shown that lipid-lowering therapy has decreased plaque lipid content and increased the amount of fibrous tissue making plaques less prone to rupture.[16,17] With the current study we are unable to prove causality, however, we do demonstrate that plaques showed features of stabilization over time concomitantly with a longer duration of statin treatment.

A systemic component of atherosclerotic lesion vulnerability has been suggested in a variety of previous publications.[18,19] Studies have shown strong associations between carotid plaque vulnerability and stability of lesions in the coronary circulation. The presence of unstable carotid plaques was 23.2% in patients with unstable angina, compared to 3.2% in patients with stable angina (p<0.001). Moreover circulating biomarkers of inflammation such as CRP were highest in patients with unstable plaques (7.55ml/L) when compared to patients with stable plaques (3.94mg/L (p<0.05)).[18] In patients after first myocardial infarction, a high risk for recurrent events exists, and studies have shown that ischemic injury accelerated atherosclerotic disease progression.[20] In Apoe–/– mice, atherosclerotic lesions increased in size, showed more advanced morphology and systemic components such as monocyte recruitment persisted over twelve weeks.

These studies all show that cardiovascular events and plaque vulnerability have a strong systemic component which is influenced by inflammation and can arguably catalyst inflammation. It is currently unknown if plaque vulnerability itself is a cause or solely a consequence of systemic inflammation. It is conceivable that the removal of a vulnerable inflammatory lesion could exert systemic effects such as the stabilization of other atherosclerotic lesions. Removal of the first vulnerable lesion in our patients might have contributed to a relative stabilization of lesions elsewhere in the vasculature.

Limitations and strengths

The presented results in this study are somewhat controversial since on the one hand, we show a stabilization of atherosclerotic plaques in patients treated at two different moments for carotid stenosis, while on the other hand these patients did develop bilateral carotid stenosis with subsequent symptoms requiring two endarterectomies. One could argue that it's hard to speak in terms of plaque stabilization in patients who develop new

symptoms of the contralateral artery. Another important limitation is the limited power due to the small sample size of this study. It is quite uncommon for patients to undergo carotid endarterectomy of both the carotid arteries and therefore assembly of a larger cohort was not possible at the current time. Despite the relative small number of patients we are able to show consistent changes in plaque traits over time. An important strength of this study is the unique opportunity to study atherosclerotic changes on a histological level within the same patient. Most evidence of plaque changes over time has come from imaging techniques which still enclose serious limitations. Moreover, histological and autopsy-studies only provide information of atherosclerotic disease severity at one moment in time and don`t allow for multiple ''vascular biopsies'' in time.

CONCLUSION

In patients who underwent bilateral carotid endarterectomy on two separate moments in time, the second removed plaque showed a lower incidence of features associated with plaque vulnerability. It appeared that over time and within the same patient, plaques requiring surgical endarterectomy were more stable. Statin treatment increased over time and might have contributed to this relative stabilization.

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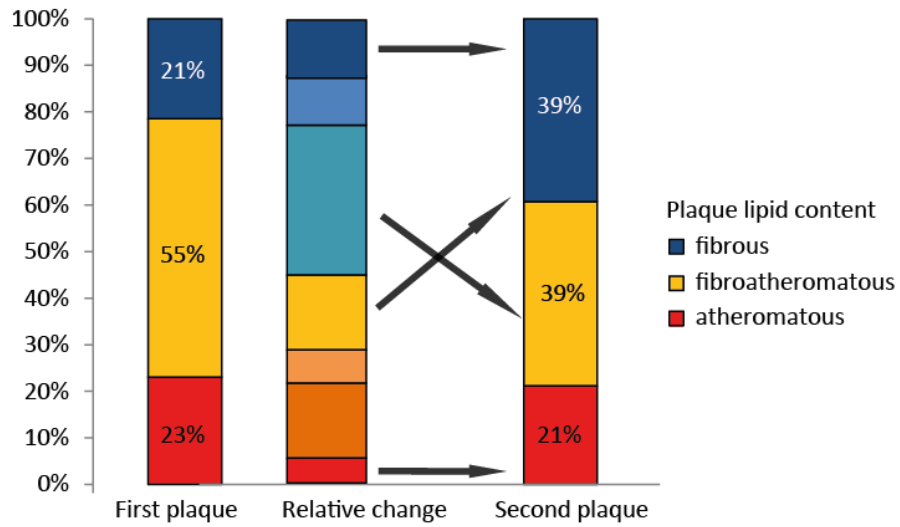
SUPPLEMENTAL

Supplemental Figure 1. Changes in plaque fat content between first and contralateral endarterectomy.



Fibrous plaques; plaque with lipid core <10%, fibroatheromatous plaques; plaques with lipid core <40% >10, atheromatous plaques; plaques with lipid core >40%.

Supplemental Table 1. Plaque changes with regard of relative change in plaque fat content.

Patient characteristics	Second plaque lower fat-content than first plaque	Plaque fat-content unchanged	Second plaque higher fat- content than first plaque	p-value
	n=24	*n*=19	*n*=13	
Male sex, n(%)	17 (70.8)	18(94.7)	12(92.3)	0.068
Age in years, mean ± SD	69.0±9.8	68.0±8.1	65.0±10.1	0.729
BMI, mean ± SD	26.2±3.5	25.8±2.4	27.5±3.6	0.174
Smoker current, yes n(%) †	5(21.7)	8(42.1)	3 (25.0)	0.328
Diabetes, yes n(%)	8(33.3)	6(31.6)	4(30.8)	0.985
Hypertension, yes n(%) †	19(82.6)	16(84.2)	8 (61.5)	0.249
Hypercholesterolaemia, yes n(%) †	19(86.4)	14(73.7)	9(81.8)	0.587
History of CAD, yes n(%)	8(33.3)	7(36.8)	2(15.4)	0.395
History of PAD, yes n(%)	5(20.8)	8(42.1)	4(30.8)	0.321
Symptom categories n(%)				0.335
Ø Asymptomatic	3(12.5)	1(5.3)	1(7.7)	
Ø TIA	9(37.5)	11(57.9)	5(38.5)	
Ø Stroke	7(29.2)	4(21.1)	3(23.1)	
Ø Ocular	5(20.8)	3(15.8)	4(30.8)	
eGFR in mL/min/1.73 m2, mean ± SD	77.03±16.97	73.28±20.08	73.85±16.01	0.252
Triglycerides in mmol/L, mean ± SD	1.67±1.41	1.80±0.77	1.85±0.86	0.893
Total cholesterol in mmol/L, mean ± SD	4.70±1.64	4.76±0.83	4.39±0.90	0.738
HDL in mmol/L, mean ± SD	1.19±0.45	1.10±0.28	1.08±0.29	0.650
LDL in mmol/L, mean ± SD	2.44±0.68	2.75±0.79	2.42±0.69	0.457
Statin use, yes n(%)	19(79.2)	15(78.9)	13(100.0)	0.198
Antiplatelet use, yes n(%)	12(92.3)	15(78.9)	23(95.8)	0.190
Anti-coagulant use, yes n(%)	2(8.3)	4(21.4)	2(15.4)	0.492
Beta-blocker use, yes n(%)	12(50.2)	12(63.2)	7(53.8)	0.684

Baseline characteristics of CEA patients stratified by plaque change. BMI: Body mass index. CAD: coronary artery disease. PAD: peripheral arterial disease. eGFR: estimated glomular filtration rate.

Supplemental Table 2. Plaque changes with regard of relative change in plaque hemorrhage.

Patient characteristics	**Second plaque no more plaque hemorrhage**	**Plaque unchanged**	**Second plaque new onset of plaque hemorrhage**	**p-value**
	n=18	*n*=32	*n*=6	
Male sex, n(%)	16(88.9)	25(78.1)	6(100.0)	0.320
Age in years, mean ± SD	67.0±9.6	67.2±9.2	70.0±9.5	0.786
BMI, mean (SD)	26.9±3.3	26.7±3.5	27.7±2.9	0.821
Smoker current, yes n(%) †	2(11.8)	11(35.5)	3(50.0)	0.116
Diabetes, yes n(%)	6(33.6)	7(21.9)	5(83.5)	**0.012**
Hypertension, yes n(%) †	17(94.4)	23(74.2)	3(50.0)	0.053
Hypercholesterolaemia, yes n(%) †	16(94.1)	12(76.7)	3(60.0)	0.160
History of CAD, yes n(%)	3(16.7)	13(40.6)	1(16.7)	0.155
History of PAD, yes n(%)	5(27.8)	9(28.1)	3(50.0)	0.541
Symptom categories n(%)				0.916
Ø Asymptomatic	1(5.6)	3(9.4)	1(16.7)	
Ø TIA	13(72.2)	12(37.5)	-	
Ø Stroke	2(11.1)	10(31.3)	2 (33.3)	
Ø Ocular	2(11.1)	7(21.9)	3(50.0)	
Glucose (mmol/L) mean ± SD	6.82±2.38	6.14±1.74	9.20±4.78	0.068
eGFR in mL/min/1.73 m2, mean ± SD	74.5±16.4	73.0±17.0	56.6±23.5	0.117
Triglycerides in mmol/L, mean ± SD	1.5±0.6	1.9±1.3	1.8±0.8	0.448
Total cholesterol in mmol/L, mean ± SD	4.3±1.1	4.9±1.4	4.1±0.7	0.148
HDL in mmol/L, mean ± SD	1.19±0.39	1.11±0.38	0.98±0.20	0.528
LDL in mmol/L, mean ± SD	2.21±0.62	2.72±0.70	2.25±0.83	0.053
Statin use, yes n(%)	15(83.3)	27(84.4)	5(83.5)	0.994
Antiplatelet use, yes n(%)	16(88.9)	29(90.6)	5(83.5)	0.867
Anti-coagulant use, yes n(%)	1(5.6)	5(15.6)	2(33.3)	0.229
Beta-blocker use, yes n(%)	10(55.6)	17(53.1)	4(66.7)	0.829

Baseline characteristics of CEA patients stratified by plaque change. BMI: Body mass index. CAD: coronary artery disease. PAD: peripheral arterial disease. eGFR: estimated glomular filtration rate.

PART

five

SUMMARY AND DISCUSSION

11

Summary, General discussion and
Future perspectives

Pursuing a moving target in an ever-changing landscape

Over the last decade, the major underlying culprit of CVD; the atherosclerotic plaque, has shown a strong stabilization over time.[1,2] Vulnerable plaque features such as large lipid core, intraplaque hemorrhage, and high macrophage content strongly decreased over time. This changing feature of atherosclerotic disease occurred in both the total population as in the individual patient.[3] Together with drops in inflammatory biomarkers it appears that two main drivers of atherosclerotic disease, lipids and inflammation, are now somewhat silenced. In first-world countries the current decline in major manifestation of CVD such as stroke and myocardial infarction is expected to be attributable to this stabilization.[4] Rigorous lipid and blood pressure control, government legislation such as a public smoking ban and dietary reductions of salt and trans fats are likely causing these effects. Apart from disease traits changing over time, twenty-first century treatment options have also arrived. Technological advancements have brought us to the endovascular era in which fully personalized fenestrated and branched arterial stents are becoming routine practice. Together with state of the art pharmaceutical agents such as antibodies targeting PCSK9 or interleukin-1β the cardiovascular field remains dynamic as ever.[5,6]

However, considering all these changes it is essential to keep re-evaluating treatment strategies. In order to keep our eyes on the ball we should ask ourselves; are results obtained in passed clinical trials still translatable to the patients we treat today? Outcomes from trials comparing best medical treatment with invasive procedures performed decades ago, likely not represent current clinical practice. A considerable strong point of this thesis and of ongoing biobanks in general is that we can study these time-dependent effects over decades of research investments which is discussed in **chapter 2**.[7] The continuous collection of biological specimens enabled us to understand the disease process affecting patients we treat today instead of decades ago. In **chapter 3** we describe a large follow-up study, we show that despite the fact that plaques stabilized over time and cardiovascular risk management greatly improved, secondary cardiovascular event-rate did not change.[8] Secondary event rate remained high at approximately twenty-five percent during three-year follow-up and warrants our full efforts for future reduction. A possible explanation for this lack of decline could be that the patients that do develop symptoms today, despite optimal preventive care, are those that are least responsive to current preventive treatment. This evidence could therefore point to a poorly understood patient group for whom new treatment strategies may have to be designed. Another explanation could be that in these patients atherosclerotic disease burden is already too advanced and therefore less amenable for treatment. In order to tackle this problem, it could be that our definition of good health is no longer up-to-date. Perhaps the situation in which disease manifestations are absent no longer suffices as a surrogate for good health. Early detection and more important prevention of preclinical conditions will therefore become increasingly more important in the near future.

Preventive versus reactionary medicine

The greater part of this thesis has been carried out by studies performed with data from the Athero-Express Study.[9] The average age of patients in the study is sixty-nine years old and eighty-six percent of all patients are symptomatic, meaning that they have recently developed some form of neurological failure (transient ischemic attack, amaurosis fugax or stroke). In these patients the goal of carotid endarterectomy is to prevent new neurological symptoms from occurring. Therefore, we could state that carotid endarterectomy classifies as a preventive measure. But is this really the type of preventive medicine we should be aiming for? Is it not our primary goal to prevent neurological events from occurring in the first place? Especially considering that the retirement age in the Netherlands is sixty-seven years old a very large part of our study population was denied from these much deserved carefree. Moreover, since atherosclerosis is a systemic condition other vessels such as the coronary or the femoral arteries are often affected as well. In this stage, after decades of plaque development it is unlikely that complex and calcified lesions will show any medication induced regression. It is therefore of paramount importance to detect patients at risk for cardiovascular disease earlier in life.

Fortunately, the arsenal of biomarkers that can be used to gather insights into cardiovascular function and prognosis is rapidly increasing. Moreover, biomarkers that reflect function of organs that strongly interact with the vasculature are readily available.

In addition there are great efforts made into understanding cardiovascular genetic risk by studying the human genome. An increasing number of cardiovascular risk loci have been identified with the help of large genome-wide-association studies. Even though the average effect-size of cardiovascular risk-loci is relatively small, polygenic risk scores can be used to obtain a much enhanced impression of genetic predisposition.

When traditional risk factors and imaging techniques are combined with novel biomarkers and in-depth genetic information, true patient-tailored risk assessment tools can be constructed. These state-of-the-art applications will revolutionize personalized medicine and help identify patients in need for pre-emptive measures but as important help discard unnecessary checks and medication. In patients at risk, small lifestyle changes will be initiated early in life and show the strongest long-term effects.

Treatment disparities

Although the beneficial effect of carotid endarterectomy for symptomatic patients is well-established, these favorable effects are less clear for patients with carotid stenosis that have not (yet) developed neurological symptoms (asymptomatic patients). It is unclear what part of this population will eventually develop neurological symptoms when improved best medical therapy is applied. Moreover, CEA itself is not without risk and procedural strokes are seen in around 1-3% of all procedures.[10] It is therefore a difficult matter what to decide for and worldwide large disparities exist on how to treat these patients. In this thesis we highlight this disparity in **chapter 4** by comparing Dutch with North American carotid endarterectomy patients.[11] In the Dutch cohort only thirteen percent of all patients were asymptomatic, while in the North American cohort this percentage was fifty-seven.

Upon review of the different guidelines we found that while both European and American guidelines support surgical treatment of asymptomatic carotid stenosis, in the European guidelines at least one risk factor associated with long-term stroke is required.[10,12] Moreover, the European guidelines discourage CEA for patients > 75 years, especially in asymptomatic women in which CEA-benefit is less clear. This indicates that the benefit to risk ratio in asymptomatic patients without additional risk factors is insufficient.
Interestingly the US guidelines leave more room for interpretation and likely resulting in a large part of the CEAs performed on asymptomatic patients. Moreover, it appears that different incentives such as financial compensation and provider-induced are at play here. In a recent study into treatment systems (comparing fee-for-service physicians in the private sector with salary based military physicians), patients in the private sector were two times more likely to undergo carotid revascularization instead of receiving best medical treatment alone.[13] These motivations in which patients' medical interest is not at heart are a danger to modern healthcare systems. Patients encountered with medial problems often find themselves in a situation of dependence and this trust should be guaranteed in order to keep patients from turning to charlatans. Fortunately, two large randomized trials comparing different treatment options (carotid endarterectomy, carotid stenting and best medical treatment alone) in asymptomatic patients are currently carried out.[14,15] Hopefully, the results from these trials will give a more definite answer to this dilemma.

Circulating biomarkers in cardiovascular disease

In this thesis the relation of atherosclerotic plaque composition with circulating biomarkers and clinical outcomes was studied, thereby increasing our understanding of atherosclerosis. In part three of this thesis we studied several different biomarkers and their link with plaque composition and outcomes after carotid endarterectomy.[16] In **chapter 6** we show that decreased kidney function is a strong prognostic factor for the occurrence of secondary cardiovascular events and which specific plaque features associate with decreased kidney function in CEA patients. Interestingly we could not correlate decreased kidney function with abundant inflammation in the plaque, while inflammation is the proposed mediator for kidney-vasculature interaction. However, we did find a correlation with intraplaque hemorrhage and coagulation pathways indicating that perhaps coagulation is involved in plaque vulnerability and poor outcome in patients with decreased kidney function.
In **chapter 8** we focused on an often reported hematological parameter, the red cell distribution width (RDW), and its prognostic role in patients undergoing major cardiovascular surgery.[17] There we show that this marker of anisocytosis is a strong predictor for inflammatory events following cardiovascular surgery. Moreover, we provide insight in hematopoietic tissue activity and their association with the RDW. We show a strong correlation of RDW with organ activity of the spleen and bone marrow and thereby likely reflecting a state of low grade inflammation.
In **chapter 7** we studied the role of two major sex hormones, testosterone and estradiol, and their role in male patients undergoing carotid endarterectomy.[18] We show that although the individual levels have limited prognostic value the testosterone to estradiol ratio (T/E2) is more promising. Patients with low T/E2 had a higher risk of major cardiovascular events after CEA and showed increased inflammation in both plaque and blood. From a treatment-perspective normalization of sex-hormones could serve as a practical tool. First, since elevated estradiol levels are likely the result of increased aromatase activity in white fat tissue, weight loss should strongly be considered. Efforts such as supervised exercise programs and help from dieticians should be made to reduce excess abdominal fat. Second, restoring testosterone levels can be considered in patients on the lower end of the testosterone curve, hormone replacement therapy however, should be done with great caution since too high levels will bring about a whole range of new problems.

Exponential data growth and pushing the boundaries of science

Unfortunately, adding information alone does not directly translate into better healthcare, prevention of disease and improvements in health-related quality of life. The annual growth of scientific output is close to 8-9 percent and thereby doubles every nine years.[19] This increase in scientific publications illustrates current modern society and embodies our pursuit of knowledge and understanding. All these achievements have brought about great accomplishments such as modern medicine and goes hand-in-hand with exponential data production. The amount of newly generated information can come across as bewildering. The size and complexity of these data-collections are outgrowing the cognitive capabilities of the human brain. Navigating through this constantly densifying forest of ''Big Data'' with the help of a basic map simply does not suffice any longer. More and more we are in need of ''smart'' systems that do not only display the thousand results that match your search term but rather help us understand, analyze and guides us through this dense forest of information. Data-infrastructures comparable to a Google Map that commences with an overview of the body and allows for zooming in to the anatomical level, thereby stepwise increasing the number of details displayed, could serve as a tool in this.

Future perspectives

One of the biggest challenges for modern medicine is how to deal with quickly rising healthcare expenditures. Costs of healthcare systems have quickly grown due to ageing of the population and an increase in chronical illnesses. Fortunately, this is not the only feature that changed over time. The internet has radically transformed the way we communicate and brought information to the masses. With the press of a button on a device carried around most hours of the day we are now able to access information on almost everything and everyone at any time. Modern patients are more informed than ever which helps in coming to an agreement for the individual treatment plan. Current easy access to communication and information tools is solely used as an addition to the traditional ways we see and treat patients. It is likely that in the near future a large part of the work done in the outpatient clinic can be replaced by automated and supervised algorithms. Algorithms using Big Data and Machine Learning perform increasingly better and in some areas already outperform doctors.[20] The implementation of these techniques will certainly not replace doctors but they can be used to reduce a large part of routine patient-doctor-

visits. Automated algorithms will help decide whether medication should be altered or when a visit to the nearest hospital is necessary. This ambitious goal can be achieved by help of other future devices. The widespread introduction and adaption of wearable biosensors for instance will accommodate in this need. The continuous collection of vital parameters combined with a ''live'' biomarker feed can help further improve decision algorithms and decide when hospital visits is unnecessary but more important, calls for an intervention when health risks arise. This will help restrict expenses by reducing non-essential hospital visits and help tackle medical problems at the earliest stage necessary.

In addition tech-companies are now working on the so called 'digital twin'' which can be used for real-life simulations. By combining vast amounts of biomedical information with detailed individual patient data a simulation will be made that can make a perfect prediction of the near future. For patients with carotid artery disease, the EU-consortium TAXINOMISIS has been assembled with this precise goal. By integrating clinical data, circulating biomarkers, plaque and cerebral imaging data with computational modelling, a multiscale risk stratification model will be developed. Risk prediction will become a dynamic process in which live data input will show the effects of initiated medical treatment or lifestyle choices made. And perhaps, after all these endeavors, eyes will finally be opened.

Ian van Koeverden, August 2018

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12

Samenvatting

Slagaderverkalking

Hart- en vaatziekten (HVZ) is een verzamelnaam die gebruikt wordt voor verschillende aandoeningen van het hart of het vaatstelsel. Een van de grootste onderliggende ziekteprocessen van problemen voorkomend in het vaatstelsel is slagaderverkalking. Slagaderverkalking, ook wel atherosclerose genoemd, is een door vet en ontsteking veroorzaakte ziekte van de vaatwand. Atherosclerose wordt beïnvloed door een breed scala aan factoren. Enkele belangrijke risicofactoren zijn: hoge bloeddruk, hoog cholesterol, roken, suikerziekte, overgewicht, beperkte lichaamsbeweging en genetische aanleg. Tijdens dit ziekteproces slibt een slagader langzaam dicht door de opbouw van plaque in de slagaderwand. Hierdoor kunnen klachten optreden in het orgaan die door dat bloedvat van zuurstofrijk bloed moet worden voorzien. Deze klachten kunnen acuut zijn, zoals in het geval van het hartinfarct, waarbij er door een stolsel op de plaque of door een scheur in de plaque een bloedvat lokaal afsluit waardoor een deel van het hart niet meer van bloed wordt voorzien. In het geval van beroerte lijkt het vaker te gebeuren dat een stolsel los schiet van een plaque in de halsslagader en deze verderop in het brein voor problemen zorgt. Daarnaast is het ook mogelijk dat de plaque zelf scheurt en losse fragmenten van de plaque verderop in het brein terechtkomen. Slagaderverkalking kan zich echter ook minder acuut presenteren zoals in het geval bij etalagebenen. Interessant genoeg is het ontwikkelen van slagaderverkalking niet specifiek voor de huidige westerse samenleving. In de overblijfselen van gemummificeerde Egyptenaren, die ruim drie duizend jaar geleden leefden, zijn namelijk al forse atherosclerotische afwijkingen gevonden. Of deze afwijkingen ook klachten gaven is onduidelijk.

Opvallend genoeg is recent in een nu levende inheemse Zuid-Amerikaanse bevolkingsgroep van jagers-verzamelaars de laagste hoeveelheid slagaderverkalking gevonden. Deze bijzondere inheemse bevolking met een levenswijze van jagen, verzamelen, vissen en landbouw lijkt grotendeels beschermd voor het ontwikkelen van slagaderverkalking. Factoren zoals veel lichaamsbeweging, een gevarieerd dieet met veel groente en fruit kwamen overeen met lage bloeddrukken, een laag cholesterol, lage bloedsuikerwaarden en zeer weinig slagaderverkalking.

Slagaderverkalking bestuderen

Er zijn vele manieren om atherosclerose te bestuderen en een belangrijke manier waarop ik dit gedaan heb is met behulp van weefsel verkregen tijdens vaatchirurgische ingrepen. Tijdens een chirurgische procedure, genaamd ‘endarteriëctomie’, wordt een slagader geopend en vrij gemaakt van de slagaderverkalking ter plaatse. De slagaderverkalking, ook wel atherosclerotische plaque genoemd, die hierbij vrij komt worden door onderzoekers gretig verzameld voor verdere bestudering. In dit proefschrift is in het bijzonder gekeken naar slagaderverkalking voorkomend in de halsslagader, ook wel de ‘arteria carotis’ genoemd welke verzameld is in de Athero-Express biobank studie. In **hoofdstuk 1** staat de inleiding en de opbouw van dit proefschrift beschreven.

In **hoofdstuk 2** bespreken wij de successen en valkuilen van vijftien jaar weefsel verzamelen in onze biobank. Patiënten die geopereerd worden aan de halsslagader hebben in ruim 85 procent van de gevallen kort hiervoor neurologische uitval gehad, veroorzaakt door een ernstige vorm van halsslagaderverkalking. Het type neurologische uitval kan voorbijgaand zijn in een proces genoemd T.I.A. (transient ischemic attack) of blijvend in het geval van een beroerte. Een groot voordeel van het bestuderen van plaques van patiënten is dat het weefsel dat je bestudeert ook daadwerkelijk symptomen heeft gegeven bij de patiënt. De chirurgische ingreep waarbij de halsslagader vrij gemaakt wordt van plaque heet carotis endarteriëctomie (CEA). De CEA wordt uitgevoerd om nieuwe neurologische uitval te voorkomen. Helaas zijn chirurgische ingrepen vrijwel nooit zonder risico en is dit voor de CEA niet anders. Het risico voor het ontwikkelen van een beroerte tijdens of kort na de ingreep is in Nederland zo’n 1-2%. Verder bestaan er wereldwijd grote verschillen in de behandeling van patiënten met een halsslagadervernauwing welke wij bestudeerd hebben in **hoofdstuk 4**. In dit hoofdstuk hebben wij Amerikaanse CEA patiënten met Nederlandse CEA patiënten vergeleken. Uit deze studie kwam naar voren dat Amerikaanse CEA patiënten die nog nooit neurologische klachten hebben gehad (asymptomatisch), het beter doen dan de Nederlandse asymptomatische patiënten. De uitkomsten van symptomatische patiënten zijn echter gelijk tussen beide landen. Een sterke selectie-bias lijkt hier plaats te hebben gevonden, aangezien er in Nederland veel terughoudender wordt omgegaan met het opereren van asymptomatische patiënten terwijl dit niet het geval lijkt te zijn in de VS. Hierdoor werden betrekkelijk gezondere asymptomatische patiënten in de VS wel geopereerd, terwijl die in Nederland geen operatie ondergaan.

Verder is het belangrijk te weten dat atherosclerose een systemische ziekte is. Dit wil zeggen dat andere slagaders ook vaak aangedaan zijn. Inzicht verkrijgen in hoe dit ziekteproces zich in het lichaam manifesteert is van groot belang voor de arts en patiënt. Zo krijgen zij samen inzicht in het risico voor de patiënt en bepalen ze in welke mate verschillende therapieën zoals bloeddruk en cholesterol medicatie ingezet moeten worden. Het inzichtelijk maken van deze cardiovasculaire risico`s is een hele uitdaging en een van de doelen van mijn studies.

Een veranderend ziekteproces

Interessant genoeg is het proces van slagaderverkalking sterk veranderd over de afgelopen decennia. Zo liet de plaque in het verleden vaak nog een ernstige mate van instabiliteit zien zoals; veel vet, veel ontsteking, veel kalk en de aanwezigheid van bloedingen in de plaque. Deze instabiele kenmerken zorgen ervoor dat een plaque eerder scheurt. Tegenwoordig zien we deze instabiele eigenschappen steeds minder vaak terug. De plaque lijkt dus tegenwoordig wat gestabiliseerd te zijn. Deze stabilisatie over de tijd hebben we terug gezien in onze eigen weefselbank de Athero-Express en lijkt sterk samen te hangen met verbeteringen in het cardiovasculair risicoprofiel. Zo wordt er in de meer recente jaren minder gerookt, meer medicatie gebruikt zoals statines (cholesterolverlagers) en bloeddrukmedicatie en worden er ook lagere bloeddrukken en een lager cholesterol gemeten. Daarnaast zijn er ook andere zaken aan te wijzen die voor een verbetering in het cardiovasculair risicoprofiel hebben gezorgd, zoals een rookverbod in openbare gelegenheden en zoutbeperkingen in het dieet.

In **hoofdstuk 3** is er gekeken of dit uiteindelijk ook een vermindering gaf in het aantal cardiovasculaire gebeurtenissen (hersen- of hartinfarcten) na een chirurgische ingreep, ook wel 'cardiovasculaire events' genoemd. Dit was inderdaad het geval, maar dan wel alleen voor patiënten geopereerd aan de liesslagader. Patiënten die een CEA ondergingen, maakten even vaak als in het verleden een cardiovasculair event door ondanks al deze verbeteringen. Een mogelijke verklaring hiervoor is dat ondanks alle preventieve therapieën er een selectie heeft opgetreden van patiënten die nu geopereerd worden. Patiënten die ondanks al deze verbeteringen in het risicomanagement nog steeds ziek worden, lijken mogelijk minder goed op huidige therapieën te reageren. Deze tijdsafhankelijke veranderingen in het atherosclerotische ziekteproces hebben wij verder bestudeerd in **hoofdstukken 9** en **10**.
In **hoofdstuk 9** hebben we in het bijzonder gekeken naar patiënten met diabetes (suikerziekte) die geopereerd werden aan de liesslagader. Aangezien diabetes een belangrijke risicofactor is voor hart- en vaatziekten, is het belangrijk te begrijpen hoe deze groep zich verhoudt tot de rest van de populatie. Wat wij in deze studie vonden, is dat juist in deze ''zieke'' populatie er veel minder plaque stabilisatie heeft opgetreden. Bovendien was er geen afname in cardiovasculaire events na de chirurgische ingreep.
In **hoofdstuk 10** is er gekeken naar patiënten die geopereerd werden aan zowel de linker als de rechter halsslagader om te zien of deze stabilisatie over de tijd ook heeft plaatsgevonden binnen dezelfde patiënt. Interessant genoeg was dit ook het geval en liet de eerst verwijderde plaque meer instabiele eigenschappen zien dan de plaque uit de halsslagader die vaak jaren later verwijderd werd. Een zekere vorm van plaque stabilisatie had dus ook in de individuele patiënt plaatsgevonden en een mogelijke reden hiervoor zou het gebruik van statines kunnen zijn dat duidelijk een verbetering liet zien.

Het begrijpen van cardiovasculair risico
Eerder werd al gezegd dat veel factoren hart- en vaatziekten kunnen beïnvloeden. Het is namelijk zo dat hart- en vaatziekten een multifactorieel ziektebeeld is. Wat wil zeggen dat er niet slechts één oorzaak is aan te wijzen voor het ziekteproces, zoals bijvoorbeeld wel het geval is bij bepaalde vormen van borstkanker veroorzaakt door een enkele genetische mutatie. Deze verscheidende risicofactoren zijn op diverse manieren te bestuderen en in dit proefschrift is dat gedaan middels biomarkers. Biomarkers, ook wel signaalstoffen genoemd, zijn factoren die je kunt meten in of aan je patiënt die ons vertellen wat er in het lichaam gaande is. Een voorbeeld van een vaak gemeten biomarker is de lichaamstemperatuur. Met deze simpele meting kunnen we bepalen of een patiënt koorts heeft en deze simpele test levert dus belangrijke informatie op over de gezondheidstoestand van een patiënt. De biomarkers die we in dit proefschrift bestudeerd hebben, zijn stoffen of cellen gemeten in het bloed, maar het achterliggende idee is hetzelfde.
In **hoofdstuk 6** hebben we bij 1796 patiënten het creatinine gemeten in het bloed. Dit eiwit kan gebruikt worden om de nierfunctie te bepalen en op deze manier konden we de relatie van nierfunctie met het vaatstelsel bestuderen. Uit deze studie komt naar voren dat patiënten met een verminderde nierfunctie vaker een bloeding in de plaque hebben, wat

een prognostisch slecht teken is. Deze patiënten zijn ook drie jaar gevolgd na hun chirurgische ingreep en ook hier kwam naar voren dat patiënten met een verminderde nierfunctie een sterk verhoogde kans op cardiovasculaire events hebben na hun ingreep en ook vaker komen te overlijden.
In **hoofdstuk 7** hebben wij gekeken naar de rol van de twee belangrijkste geslachtshormonen (testosteron en oestrogeen) in mannen en hun relatie met het vaatstelsel. In deze studie laten we zien dat bij mannen met een relatief laag testosteron maar hoog oestrogeen, de kans voor het optreden van nieuwe cardiovasculaire events tot twee keer verhoogd is. Daarnaast werd bij deze mannen meer ontsteking in zowel de plaque als in het bloed gevonden. Een belangrijke factor die deze relatie beïnvloedt is de lichaamsbouw van patiënten. Deze gevonden relaties zijn namelijk het sterkst in mannen met overgewicht.
In **hoofdstuk 8** hebben we vervolgens de voorspellende waarde van de biomarker rode bloedceldistributiebreedte (RDW) bestudeerd in patiënten die een grote (vaat)chirurgische ingreep ondergaan. De RDW zegt namelijk iets over de homogeniteit van de grootte van de rode bloedcellen. Bij een grote variatie hebben patiënten vaak veel kleine, maar ook veel grote rode bloedcellen en dit is in de literatuur geassocieerd met slechte uitkomsten. In deze studie hebben wij bekeken of een afwijkend RDW, gemeten nog voor de chirurgische ingreep, geassocieerd was met meer complicaties na de ingreep. Dit bleek inderdaad het geval te zijn en patiënten met een hoger preoperatief RDW ontwikkelden postoperatief vaker een pneumonie, nierfalen en sepsis. Ook kwamen zij vaker te overlijden dan patiënten met een normaal RDW. Om de RDW beter te begrijpen hebben we een cohort patiënten samengesteld die zowel een FDG PET-scan hebben gehad als de RDW-meting. Met een FDG PET-scan wordt weefselactiviteit middels een scan zichtbaar gemaakt en weefsels tonen een hoger signaal wanneer er een hoge weefselactiviteit plaatsvindt. Op deze manier hebben wij kunnen laten zien dat een verhoogd RDW samenhangt met een verhoogde activiteit van de milt en het beenmerg. Deze verhoogde activiteit wordt vaker gezien bij laaggradige ontstekingsziekten en daarom denken wij dan ook dat een verhoogd RDW deze laaggradige ontstekingsactiviteit weerspiegeld.
Deze studie laat dus zien dat we met behulp van een simpele biomarker inzichtelijk kunnen maken bij welke patiënten er verhoogde ziekteactiviteit aanwezig is. Deze biomarker helpt daarnaast met het identificeren van patiënten met een grote predispositie aanleg voor het ontwikkelen van complicaties.

CONCLUSIE

In de afgelopen drie jaar heb ik mij toegelegd op twee belangrijke onderwerpen in het cardiovasculair onderzoeksveld; tijdsafhankelijke veranderingen én het herkennen en begrijpen van cardiovasculair risico. Met dit proefschrift is een belangrijke bijdrage geleverd aan ons begrip over het atherosclerotisch ziektebeeld en de risico's voor de patiënt die wij vandaag behandelen in de kliniek. De praktijk is soms weerbarstig en de tijd zal ons leren of deze kennis zich vertaald in een overlevingsvoordeel voor de individuele patiënt. Hier zal ik mij in de toekomst voor blijven inzetten.

appendices

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CURRICULUM VITAE

Ian David van Koeverden was born on the 14th of August 1989 in Geldermalsen, the Netherlands, as the second oldest son of Jacques and Hennie. During childhood Ian grew up in a lively household together with his four brothers. At the age of 6 Ian started training different types of martial arts which resulted in a black belt in karate at the age of 18 and he has continued to practice contact sports ever since. Following six years of high school education at O.R.S. Lek en Linge he started his medical study at the Radboud University Nijmegen in September of 2008. During his medical study Ian moved to San Francisco to do a research scholarship at the University of California San Francisco under supervision of Professor Mehrdad Arjomandi. During this period he studied the effects of second hand smoke and air pollution on lung physiology. This internship resulted in the publication of three peer-reviewed articles and marks the starting point of his scientific career. When returned to the Netherlands Ian completed his medical training. After obtaining his master`s degree he started working as a surgical resident (not in training) at the St. Antonius Hospital in Nieuwegein under supervision of Dr. D. Boerma and Dr. P.M.N.Y.H. Go in 2015. After this residency Ian started as a PhD candidate at the Laboratory of Experimental Cardiology and the Vascular Surgery Department of the University Medical Center Utrecht. Under supervision of Professor Gerard Pasterkamp and Professor Gert Jan de Borst he studied the atherosclerotic plaque, circulating biomarkers and clinical outcomes in cardiovascular disease by use of the Athero-Express Study. The results of his research are presented in this thesis. In 2019 he will start with his training in General Surgery at the Jeroen Bosch Hospital in s`Hertogenbosch under supervision of Dr. K. Bosscha.

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