Atherosclerotic plaque composition and clinical outcome

A translational study in patients undergoing carotid endarterectomy

Willem E. Hellings

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A translational study in patients undergoing carotid endarterectomy
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A translational study in patients undergoing carotid endarterectomy

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(Met een samenvatting in het Nederlands)

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Voor mijn ouders,
mijn oma, en
Audrey

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PART 1

INTRODUCTION AND STUDY DESIGN

CHAPTER 1

INTRODUCTION

The composition of the pathologic substrate of atherosclerosis, the atherosclerotic plaque, is not taken into account in daily clinical decision making. From cross-sectional pathology studies, we have learned that atherosclerotic plaques differ greatly in their composition, and that certain plaques may be more likely to cause clinical adverse events, such as a myocardial infarction or stroke. However, so far plaque composition does not influence the decision if invasive treatment is required in patients with arterial obstructive disease, and does not influence the choice of treatment modality. Furthermore, the prognostic significance of plaque composition is unknown, because no longitudinal studies have been conducted. This lack of prognostic research led us to conduct the studies presented in this thesis, which relate the composition of the atherosclerotic plaque to clinical outcome. In the introduction, we elaborate on the backgrounds, describe the study design and provide a short overview of the thesis.

Backgrounds

The classical concept that clinical manifestations of atherosclerosis are caused by gradual narrowing of the vessel lumen as a result of fat accumulation in the vascular wall has been abandoned for some time. It has become clear that atherosclerosis is a chronic systemic inflammatory disease.[1] Smoking and hypercholesterolemia give rise to focal activation of the endothelium. This facilitates influx and retention of low density lipoprotein (LDL), which elicits an inflammatory response in the vascular wall. The activated endothelium expresses several leukocyte adhesion molecules which allow attachment and infiltration of macrophages and T-cells. With their scavenger receptors and Toll-like receptors, macrophages recognize different ligands such as oxidized LDL. The internalization of lipids causes them to become macrophage foam cells, the typical cells of the atherosclerotic lesion. In response to ligand binding, macrophages produce several cytokines and proteases which further fuel the inflammatory process. This process promotes binding of LDL to the endothelium, giving rise to a vicious circle of lipid driven inflammation.[2] These events occur already at young age, but at that time it does not yet give rise to clinically symptomatic disease.

It is assumed that the clinical manifestations of advanced atherosclerotic disease are caused by destabilization of atherosclerotic lesions.[3-7] Pathology studies of people who suffered sudden coronary death revealed that myocardial infarction was caused by a thrombus which was superimposed on a disrupted atherosclerotic plaque. Importantly, in the majority of cases the thrombosed plaque did not cause a significant (≥50%) reduction of the vessel lumen before thrombus-induced occlusion occurred. The thrombosis was associated with disturbed integrity of the plaque surface which was in most cases caused by rupture of the fibrous cap which separates the lipid contents of the plaque from the circulating blood. Alternatively, the thrombus was superimposed on plaque erosion without clear signs of plaque rupture. Interestingly, all of these thrombosed plaques showed signs of inflammation.[8] Findings that systemic inflammation, measured as circulating levels of C-reactive protein, is (moderately) predictive of the occurrence of future cardiovascular events further supported the role of inflammation.[9,10] Considering this circumstantial evidence, it was postulated that plaques with marked inflammation, as well as plaques with a large lipid core covered with a thin fibrous cap, would be prone to future plaque disruption and cardiovascular events. Plaques showing these characteristics are therefore called "vulnerable plaques".[11]

Despite this knowledge, clinical application of plaque composition is lacking at present. While it has become apparent that the composition of atherosclerotic lesions may be very important, the only plaque characteristic of interest to the clinician is the percentage luminal narrowing it causes. When we compare this to another life threatening disease, the field of oncology, a huge difference becomes apparent.

Only considering percentage stenosis could at best be compared by only considering the size of a tumor, because we should be aware that arteries show adaptive expansive remodeling when part of the lumen is obstructed by a plaque, so that the size of the lumen does not necessarily correlate with the plaque burden.[12] Whereas the difference in clinical management between benign and malignant tumors is evident, clinicians in the cardiovascular field do not consider the composition of atherosclerotic plaques when taking treatment decisions. The various ways that the composition of tumors influences patient management points out that there may be a big role for atherosclerotic plaque composition in diagnosis, treatment allocation and estimation of prognosis. Therefore, there is a great need for a catch-up in the field of cardiovascular disease.

What could be the reason that plaque composition is not yet part of decision making in treating patients with atherosclerosis? There are several areas where knowledge is lacking **(Table I)**. For treatment allocation, it is important to investigate what influence the composition of the atherosclerotic plaque has on the efficiency of invasive treatment. In patients with symptomatic carotid artery disease, the number needed to treat to prevent one stroke is 6.[13,14] In asymptomatic stenosis, this number goes up to 19.[15,16] If we were able to select only the one patient who will suffer a stroke in the future, this would portray an enormous benefit in overall outcome since all complications induced by the 18 other operations could be avoided. Furthermore, this would greatly reduce health care costs. In current practice, there are a few traditional risk factors which are taken into account to decide whether a patient with an asymptomatic ≥70% carotid stenosis should be operated on, namely age and gender. Men and elderly patients benefit more from carotid endarterectomy (CEA) than women and younger patients.[17] Probably this is due to differences in carotid plaque composition in these subgroups. The first part of this thesis contains cross-sectional studies which investigate these differences.

Prognostic research in the field of atherosclerosis has hardly been conducted. All histology studies performed so far have been small and were largely performed on post-mortem material. No longitudinal studies have associated plaque composition with clinical outcome. In patients undergoing vascular surgery, vascular

Table I Missing knowledge

Missing knowledge in the clinical application of plaque composition
Natural history of atherosclerotic plaque progression
Influence of plaque composition on outcome of invasive treatment
Guidance of invasive treatment modality by plaque composition
Prognostic value of plaque composition for clinical outcome (histology)
Prognostic markers (e.g. protein, receptors) in atherosclerotic plaques

specimens excised during surgery are available for these purposes. Therefore, we have chosen to link the composition of excised plaques to outcome after vascular surgery. Although skeptics may argue that it is not logical that an excised plaque contains prognostic information, since it is not in the body of the patient anymore, we hypothesize that excised plaques may also contain prognostic information for the patient, since atherosclerosis is a systemic disease,[1,2] and autopsy studies have shown that plaque composition correlates between different arterial segments within one patient.[18-20]

Study design

The main focus of this thesis is the association between the composition of the carotid atherosclerotic plaque and clinical outcome after carotid endarterectomy. For this purpose, we have constructed a large longitudinal biobank called "Athero-Express" (Differential ATHEROsclerotic plaque EXPRESSion of mRNA and protein in relation to cardiovascular events and patient characteristics). The study design is depicted in **Figure I**. The biobank contains samples from different types of vascular surgery: carotid endarterectomy, femoral endarterectomy, and aneurysm surgery. This thesis will focus on the plaques obtained during carotid endarterectomy. During surgery, the plaque is transferred to the laboratory directly after excision. In the lab, the plaque is processed in a standardized method. The plaque is cut in segments of 5mm thickness along the longitudinal axis of the vessel. The segment with the largest plaque burden (called the culprit segment) is used for histology. The methods and validation of the histological examination will be addressed further in **Chapter 3**. The other segments are snap frozen in liquid nitrogen and stored at -80°C. The segments directly adjacent to the culprit segment are used for protein, RNA and DNA isolation, and the remaining segments are kept for future research.
Along with the collection of plaque material, the study collects blood samples and a clinical data set from the patients. Before the operation, patients are asked to participate in the study. Patients with a terminal malignancy and patients who are referred abroad immediately following surgery were excluded. The participants of the study provide a written informed consent and fill out an extensive cardiovascular questionnaire. Further patient data, such as presenting symptoms and degree of luminal narrowing of the carotid arteries are collected from the patient charts.
There are two features which distinguish this study from previous research. First, the study includes a large amount of patients. With an annual volume of approximately 200 carotid endarterectomy procedures per year in the two participating hospitals (UMC Utrecht and St. Antonius Hospital Nieuwegein, The Netherlands), the study can provide sufficient power to link plaque composition to clinical parameters. Second, and most important, the Athero-Express study is the first biobank

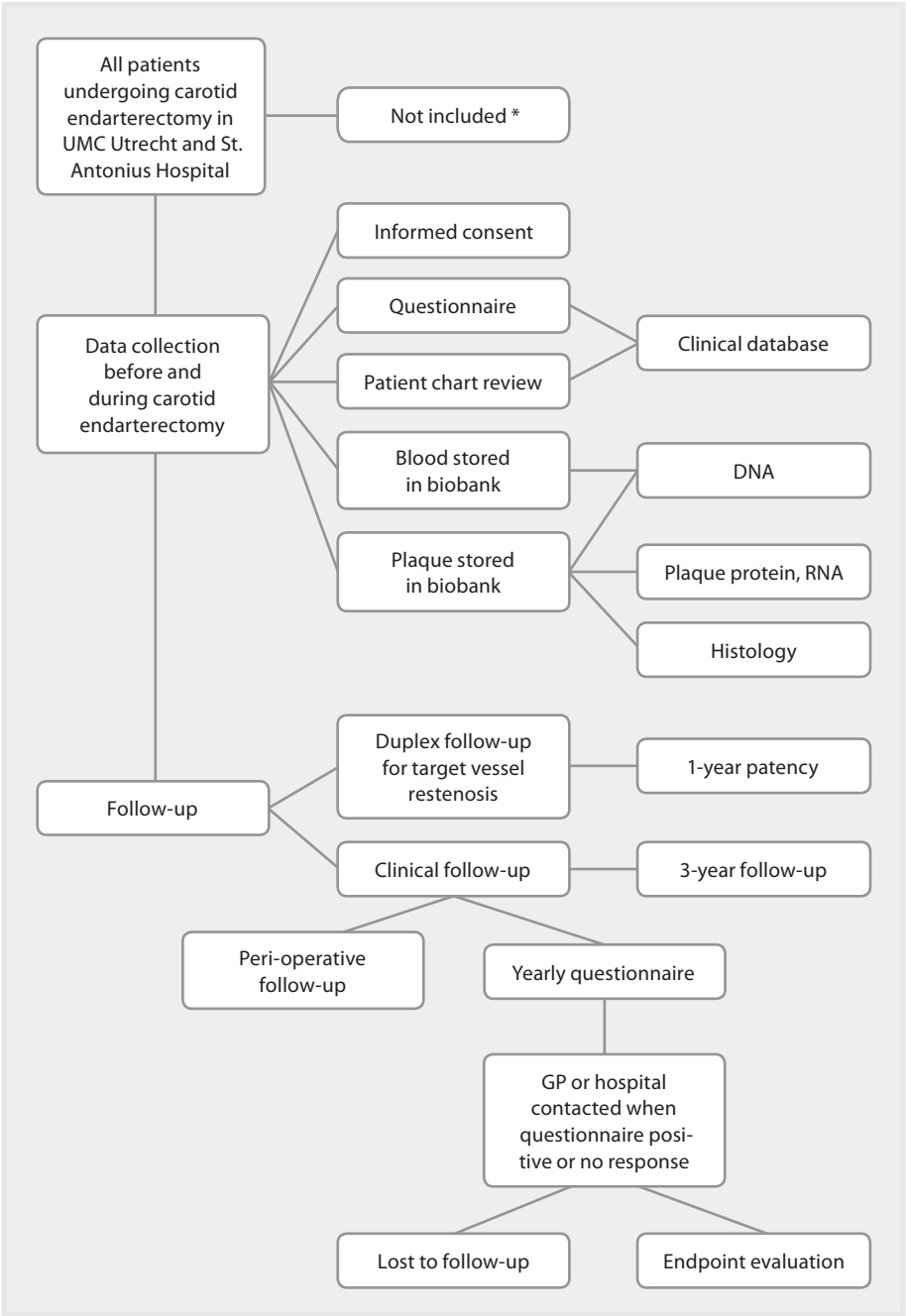

FIGURE I Study design of the Athero-Express study

* Exclusion criteria: terminal malignancy and referral abroad immediately following surgery

which is coupled to clinical and duplex follow-up. Every post-operative year, the patients are contacted with a standardized questionnaire to inform whether they have suffered any new complaints or have been hospitalized in the past year. If any of the questions is answered positively, the researchers initiate a validation process in order to assess reliably whether the patient has reached an endpoint. The primary endpoint of the Athero-Express is defined as the composite of any vascular event (vascular death, non-fatal myocardial infarction, non-fatal stroke) and any vascular intervention.

Overview of the thesis

The content of this thesis is outlined below. First, in **Chapter 2**, a review of the related literature is given and the rationale of the study is further elaborated upon. In **Chapter 3**, a systematic validation of the methods for histological examination of carotid endarterectomy specimens is presented, which forms the backbone of this thesis. The next part contains studies which relate the composition of the carotid plaque to the clinical presentation. The main focus of these studies is to investigate whether clinically important differences, such as symptomatic presentation **(Chapter 4)**, the gender difference **(Chapter 5)**, and the time interval between cerebrovascular symptoms and operation **(Chapter 6)**, are associated with plaque composition at the carotid bifurcation.

In part 3, the relation between plaque composition and restenosis after carotid endarterectomy is investigated. Restenosis is an important problem challenging the results of carotid endarterectomy, but the relation between the composition of the carotid atherosclerotic plaque and the occurrence of restenosis is unknown. Therefore, we prospectively investigated if the atherosclerotic plaque composition at baseline was predictive of the occurrence of restenosis during one year follow-up after the procedure **(Chapter 7)**. In **Chapter 8**, the histological appearance of the recurrent plaques after previous CEA is described and related to clinical presentation of restenosis.

In part 4, "The carotid atherosclerotic plaque and clinical outcome" the most important subject of this thesis is addressed, namely the relation between plaque composition at the time of carotid endarterectomy and the occurrence of adverse cardiovascular events during follow-up. First, in **Chapter 9** we present a summary of the literature about what is known about the relation between plaque composition and short term peri-interventional follow-up. This study focuses on carotid stenting but also addresses carotid endarterectomy. In **Chapter 10**, the predictive value of carotid plaque histology for the occurrence of cardiovascular events during follow-up is presented.

In part 5, we investigate the value of biomarkers in the carotid plaque protein for the prediction of clinical outcome. In **Chapter 11**, the expression of NGAL in carotid plaques and its secretion into the systemic circulation is studied. **Chapter 12**, dealing with Caveolin-1, is our first prospective plaque biomarker study. In this study we attempted to translate mechanistic knowledge obtained from experiments in Caveolin-1 knockout mouse and in vitro experiments into a clinically applicable plaque biomarker.
In **Chapter 13**, a systemic approach to identify predictive biomarkers in plaque protein is presented. Following a proteomics approach, 90 proteins were identified with differential expression between the plaques of patients who suffered an event during follow-up after carotid endarterectomy versus gender and age-matched controls. One of these proteins, Osteopontin, was then validated internally in the whole carotid patient group and externally in plaques obtained from patients undergoing femoral endarterectomy.
Part 6 **(Chapter 14-16)**, provides a general discussion and summary of the thesis, and additional information including a list of abbreviations.

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CHAPTER 2

Willem E. Hellings
Wouter Peeters
Frans L. Moll
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FROM VULNERABLE PLAQUE TO VULNERABLE PATIENT

THE SEARCH FOR BIOMARKERS OF PLAQUE DESTABILIZATION

There is a strong need for biomarkers to identify patients at risk for future cardiovascular events related with progressive atherosclerotic disease. Ideally, increasing knowledge in the mechanisms of atherosclerotic plaque destabilization should be translated in clinical practice. Currently, the following commonly followed strategies can be identified with the objective to detect either the local vulnerable plaque that is prone to rupture and gives rise to a thrombotic occlusion, or the systemic vulnerable patient, who has a high probability to suffer from an adverse clinical event. On the one hand, studies are ongoing to determine local atherosclerotic plaque characteristics to predict future local plaque rupture and subsequent vascular thrombosis. New imaging modalities are being developed and validated to detect these plaques in vivo. On the other hand, systemic approaches are pursued to discover serum biomarkers that are applicable to define patients at risk for future cardiovascular events. We propose a third original approach that is optional but yet unexplored, that is, to use local plaque characteristics as a biomarker not just for local plaque destabilization but for future cardiovascular events due to plaque progression in any vascular system. This review aims to provide an overview of the current standings of the identification of the vulnerable plaque and the vulnerable patient.

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Introduction

Atherosclerosis is the leading cause of mortality in the Western world. Despite advances in risk factor management, each year, 12 million patients die worldwide because of a cardiovascular event such as a myocardial infarction or a cerebrovascular accident.[1] To stratify patient groups who are eligible for treatment to prevent clinical manifestations of atherosclerosis, it is crucial to define risk factors and biomarkers with strong prognostic and diagnostic value. Traditional risk factors, combined with risk scores such as the Framingham score, can predict outcome for groups of patients but lack discriminative power to identify individual subjects who are at risk to experience a cardiovascular event in the near future.[2]

The knowledge on the pathophysiologic mechanisms of atherosclerotic plaque formation and destabilization is growing. However, the natural history of atherosclerotic plaque progression and complication is still unclear owing to the inherent drawback of the descriptive nature of pathology-based research and the lack of large animal models with spontaneous atherosclerotic disease that mimic advanced atherosclerosis in humans. In addition, atherosclerotic disease progresses over decades, which makes in vivo follow-up studies impractical. Still, the pressure is growing to identify plaques at risk for disruption and patients at risk to suffer from a cardiovascular event, such as a myocardial infarction or a cerebral vascular accident. The development and validation of plaque-stabilizing therapies is one of the major challenges of the pharmaceutical industry, but it is hampered by the lack of surrogate end points to prove efficacy of treatment. It is of importance to identify markers of progression of atherosclerotic disease that will facilitate drug development and drug efficacy in humans.

Different approaches are followed for this purpose. First, imaging modalities are being developed and validated to accurately characterize atherosclerotic plaques. This involves the noninvasive and invasive determination of atherosclerotic plaque characteristics that are considered to destabilize plaques and cause subsequent clinical events. Second, serologic biomarkers are being investigated as a measure of patient vulnerability, that is, to identify those who are more prone to suffer from a cardiovascular event.

This review will provide an overview of the current standing of the identification of the vulnerable plaque and patient, based on the current knowledge of the pathophysiology of atherosclerosis. In addition, novel strategies to identify biomarkers identifying patients at risk will be discussed.

The classically defined vulnerable plaque

It has been established that acute clinical manifestations of atherosclerotic disease, such as myocardial infarction or stroke, are not the result of slowly progressing luminal narrowing. Instead, these are a consequence of acute disruption (rupture or erosion) of the atherosclerotic plaque, leading to exposure of thrombogenic plaque components to the bloodstream, with superimposed thrombus formation.[3] The newly formed thrombus suddenly accelerates the degree of luminal stenosis or may totally occlude the lumen, giving rise to a myocardial infarction. On the other hand, superimposed thrombus can cause distal embolization, e.g. in symptomatic atherosclerotic carotid disease.[4]

Histologic examination of atherosclerotic plaques obtained postmortem or during endarterectomy has identified plaque characteristics associated with adverse clinical events. When these characteristics are found in asymptomatic plaques, they are thought to confer vulnerability to becoming symptomatic of the plaque. Accordingly, the vulnerable plaque, also referred to as unstable plaque or high-risk plaque, is defined as a plaque with a high risk to cause local thrombosis and thereby unstable clinical syndromes such as unstable angina, myocardial infarction, or cerebral vascular accidents.[5] There are basically three types of vulnerable plaques: First, the typical vulnerable plaque is described as the plaque prone to rupture. It has a large lipid core that is covered by a fragile fibrous cap. Rupture of this cap exposes the contents of the lipid pool to the blood, which can trigger local thrombosis. Second, the plaque with superficial erosion is considered vulnerable. The loss of endothelial coverage in eroded plaques results in direct contact of the blood with the underlying connective tissue, which can lead to thrombus formation. Third, the plaque with a calcified nodule protruding into the lumen is considered at high risk to induce thrombus. The classically defined vulnerable plaque, or rupture prone plaque, which is the most common vulnerable phenotype, will be discussed in more detail. It is described by a well-defined set of histopathologic features, such as a large lipid

Table I Histological characteristics of the vulnerable plaque vs. the stable plaque

Vulnerable plaque	Stable plaque
Large lipid core (>40% of the plaque)	Small or absent lipid core
Thin fibrous cap, depleted of smooth muscle cells	Thick fibrous cap or no necrotic core
Infiltration of inflammatory cells, particularly macrophages	No or minor infiltration of inflammatory cells
Neovascularization, intraplaque hemorrhage	No neovascularization, no intraplaque hemorrhage
Outward remodeling	No remodeling or constrictive remodeling

core, presence of inflammatory cells, and paucity of smooth muscle cells and fibrous tissue **(Table I)**.

First, the rupture prone vulnerable plaque has a large lipid core, which is composed of cholesterol and lipids remaining from death foam cells.[6] It is the most thrombogenic part of the atherosclerotic plaque that contains oxidized lipids, and it is loaded with tissue factor produced by macrophages.[7] The amount of fibrous tissue, such as collagen, which is also related to the cap thickness, is proportionally less. Autopsy series have shown that the size of the lipid core is bigger in ruptured plaques than in non-ruptured plaques.[8] A large lipid core is disadvantageous from a biomechanical point of view, because the soft core is unable to carry the mechanical forces on the plaque, which then are concentrated in the fibrous cap overlying the atheroma.[9]

Second, thinning of the fibrous cap increases vulnerability to plaque rupture. Strongly decreased cap thickness is observed near the site of rupture.[10] The cap consists of extracellular matrix components such as collagen, proteoglycans and elastin, and harbors smooth muscle cells and inflammatory cells. The thickness of the cap is the net effect of the balance between matrix synthesis by smooth muscle cells and matrix degradation by protease activity and local inflammation. Different factors contribute towards thinning of the cap in advanced atherosclerotic plaques: first, matrix synthesis is diminished because of decreased smooth muscle cell content, possibly caused by inflammation-induced apoptosis.[11] Second, matrix breakdown is increased as a result of overproduction of proteases by inflammatory cells.

Third, infiltration with inflammatory cells is another hallmark of the classically defined vulnerable plaque, especially infiltration of macrophages which vastly outnumber other inflammatory cells like mast-cells, T-cells and neutrophils. Macrophage infiltration of the fibrous cap is associated with rupture.[12] They weaken the fibrous cap by secretion and activation of matrix-degrading proteases, leading to disruption of the balance between these proteases and their natural inhibitors such as tissue inhibitors of metalloproteinases (TIMPs) and cystatin. Proteases, such as matrix metalloproteinase (MMP) -1,-3,-8 and -9, are highly overexpressed in vulnerable and ruptured plaques compared to the stable plaques, and they are particularly active in the vulnerable regions of the plaque, such as the cap.[13]

Fourth, vulnerable plaques are characterized by intimal neovascularization. Intraplaque angiogenesis contributes to plaque formation, plaque progression and intraplaque hemorrhages, which ensue as a consequence of rupture of neovessels, in some cases followed by plaque rupture.[14] Neovascularization also plays a crucial role in the recruitment of inflammatory cells into the plaque.[15] In addition, intraplaque bleeding serves as a mechanism for build-up of the lipid core, because the erythrocyte membranes contain large amounts of cholesterol.[16]

Table II Imaging modalities for detection of plaque composition

Precision of detection	Duplex			CT			MRI			IVUS			OCT		
	se(%)	sp(%)	ref	se(%)	sp(%)	ref	se(%)	sp(%)	ref	se(%)	sp(%)	ref	se(%)	sp(%)	ref
Lipid core	+		Gronholdt 1998 ECPSG [A] 1995	73		Becker 2003	67 92	78 95	Clarke 2006 Puppini 2006	59	97	Kume 2006	90 85	90 94	Yabushita 2002 Kume 2006
Fibrous components	56	88	Damme 1993	70 [B] 86 [C]		Becker 2003 Becker 2003	83	81	Clarke 2006	88	86	Kume 2006	71 79	98 99	Yabushita 2002 Kume 2006
Calcification	+		ECPSG [A] 1995	100 79	95	Becker 2003 Lau 2005	86 80	99 93	Clarke 2006 Puppini 2006	98	96	Kume 2006	95 96	97 88	Yabushita 2002 Kume 2006
Hemorrhage	75	87	Damme 1993	n.r.			92	100	Puppini 2006	n.r.			n.r.		
Fibrous cap	n.r.			n.r.			+		Hatsukami 2000 Puppini 2006	+		Jensen 2006	+		Kume 2006
Spatial resolution	0.5-1mm [D]			0.4 mm [E]			<0.1 mm [F]			0.1 mm [G]			0.01mm [H]		

Abbreviations: se, sensitivity; sp, specificity; CT, Computed Tomography; MRI, Magnetic Resonance Imaging; OCT, Optical Coherence Tomography; IVUS, Intravascular Ultrasound; n.r., not reported. When an imaging technique was able to detect a certain plaque characteristic, but this was not expressed in terms of sensitivity and specificity, this is indicated by a "+". [A] European Carotid Plaque Study Group; [B] "fibrous-atheroma"; [C] "fibrous-calcified"; [D] Vascular Diagnosis with Ultrasound, M Hennerici and D. Neuerburg-Heusler, 1998; [E] Computed body Tomography J.K.T. Lee and S.S. Sagel; [F] Clinical Magnetic Resonance Imaging 1990, Edelman and Hesselink; [G] Y. Kawase, ultrasound in med & boil 2006; [H] Bouma BE, Heart, 2003.

Finally, outward remodeling is considered as an additional characteristic of the vessel hiding the vulnerable plaque. Remodeling is an adaptive process that preserves the patency of the lumen when a plaque is formed. Although this seems beneficial, the presence of outward remodeling is associated with unstable phenomena such as inflammation and large necrotic core size and leads to greater biomechanical stress on the fibrous cap.[17, 18]

Imaging tools to detect the vulnerable plaque *in vivo*

Imaging techniques that enable visualization of structural and morphologic characteristics of the atherosclerotic plaque may be helpful to identify the characteristics of the vulnerable plaque in vivo and subsequently predict clinical outcome. These techniques either assess morphological characteristics of plaques or functional properties such as strain on the fibrotic cap (elastography) or macrophage infiltration (molecular imaging). We will shortly quote the most described techniques to characterize atherosclerotic plaques **(Table II)**.

Duplex ultrasound is a generally available imaging modality that is able to provide insights in lesion size, degree of obstruction at the site of the lesion, and intima-media thickness. Moreover, this technique is able to detect necrotic core size on the basis of plaque echogenicity: hyperechoic homogeneous plaques are more fibrous, whereas hypoechoic plaques are associated with a large lipid core.[19-21]

Computed tomography (CT) is another noninvasive imaging that has multiple applications: besides noninvasive detection of arterial stenosis (noninvasive CT angiography), it is well capable of detecting calcifications. Coronary CT enables computation of the coronary calcification score, which can be used as a measure of progression of atherosclerotic disease.[22] Despite the excellent detection of calcifications that correlates well with histopathologic findings (sensitivity of 79%–100% and specificity of 95% in coronary arteries),[23, 24] it has limited ability to identify other morphologic plaque characteristics such as size of the lipid core and thickness of the fibrous cap.[24]

Magnetic resonance imaging (MRI) is an emerging technique that can identify a range of important aspects of the atherosclerotic lesion, such as plaque size, lipid core size, calcifications, fibrous tissue, and thickness of the fibrous cap.[25] Puppini et al. have identified the lipid core with a sensitivity and specificity of 91.6% and 95%, respectively.[26] This modality could also identify calcifications with a sensitivity of 80% and a specificity of 94%. Furthermore, fibrous components can be identified with a sensitivity of 83% and a specificity of 81% by this modality.[27]

Intravascular ultrasound (IVUS) is a catheter-based imaging technique that provides high-resolution ultrasound (30 MHz) images of both the lumen and the wall of an artery. It is the only imaging modality that provides images in which varia-

tions in arterial geometry and atherosclerotic plaque along the artery can be observed simultaneously in vivo.[28] Besides revealing information of the lumen area, plaque area, and vessel area, this imaging modality may identify morphologic plaque components (lipid core, calcifications, and fibrosis) through differences in echogenicity.[29]

Optical coherence tomography (OCT) is a promising new technique capable of accurate detection of plaque composition (sensitivity and specificity for necrotic core size >90%).[30] Fibrous tissue can be detected with a sensitivity of 79% and specificity of 99%.[31] Because of its high resolution, it is also capable of identifying macrophage infiltration, showing excellent correlations with macrophage infiltration determined by histology.[32] However, penetration depth is limited.

Intravascular ultrasound–based elastography has been introduced to asses the local mechanical (elastic) properties of the arterial wall. The rationale of this technique is that tissue elements differing in hardness will be compressed differently by mechanical pressures. Hard tissues, containing collagen or calcifications, are compressed less compared with soft lipid–rich tissue. Schaar et al. have demonstrated in a postmortem study that intravascular elastography can accurately diagnose vulnerable plaques (large lipid core, thin cap, and heavy macrophage infiltration), with positive predictive value of 88% and negative predictive value of 89%.[33]

Molecular Imaging

The conventional imaging techniques are particularly based on anatomic and physiologic heterogeneity to provide image differences. Molecular imaging will help to identify specific molecular targets, pathways, and molecular processes by using radioactive-labeled molecules, which are specifically associated with the destabilization of the atherosclerotic plaque.[34] The identification of specific molecular atherosclerotic radiolabeled markers with different imaging techniques (MRI, positron emission tomography, or single photon emission CT) enables detection of the disease in an early stage and discrimination between active and inactive elements and stages of the disease. Examples of targets for molecular imaging are macrophages and markers for apoptosis, both of which are thought to be important in plaque destabilization. Animal studies have demonstrated that contrast-enhanced MRI with iron oxide particles shows characteristic signal intensity changes that correlate with iron accumulation within intraplaque macrophages. In a prospective study, Trivedi et al. have identified intraplaque macrophages by contrast-enhanced MRI in symptomatic patients.[35] Imaging of apoptosis in carotid atherosclerotic plaques has been successfully performed by Kietselaer et al. who performed single photon emission CT–based plaque imaging using technetium-99m–labeled annexin V as a visualization agent to detect apoptosis in atherosclerotic plaques in vivo.[36] Patients with recently symptomatic plaques (<1 week before imaging) showed contrast en-

hancement by technetium-labeled annexin V, whereas plaques of patients who had not recently been symptomatic showed no contrast enhancement.

The classically defined vulnerable plaque: the gold standard to identify patients at risk for cardiovascular events?

Theoretically, plaque characteristics assessed by local plaque imaging may identify lesions that are considered highly vulnerable, regardless of whether the plaque causes hemodynamically significant stenosis. However, it is unknown if the previously mentioned vulnerable plaque characteristics hide positive predictive value to identify plaques that are prone to rupture. The fact that strong associations between certain "vulnerable" plaque characteristics and plaque rupture are observed in cross-sectional studies, does suggest, but not prove, that plaques hiding these features actually have a higher risk to rupture. Prospective evidence regarding the predictive value of vulnerable plaque characteristics for future events is currently lacking. In addition, evidence from observational studies suggests that the positive predictive value of the earlier defined plaque characteristics to define vulnerable plaques at risk to cause future events may be low, because 1) lipid rich, inflammatory plaques are also frequently observed in asymptomatic patients, 2) plaques lacking typical vulnerable histopathologic characteristics are able to cause clinical events, and 3) plaque rupture itself is often asymptomatic. In the next paragraphs, we will discuss these arguments shortly.

Autopsy series have revealed that lipid-rich inflammatory plaques are not only found in patients with clinical manifestations, but also in asymptomatic patients. In an autopsy series of 124 coronary arteries from elderly patients with non-cardiovascular cause of death, 41% of cross sections showed cap infiltration with macrophages in non-ruptured coronary arteries. In 71% of the coronary plaques, one or more cross-sections showed cap infiltration with macrophages.[37] Another post-mortem study has demonstrated that a large lipid pool and a combination of a large lipid pool and macrophage infiltration are also frequently observed in asymptomatic plaques: 25% and 20%, respectively.[38] Apparently, inflammation and a large lipid core, the main hallmarks of the vulnerable plaque, are not very specific phenomena. These data suggest that the positive predictive value of these classically defined vulnerable plaque characteristics for plaque rupture and subsequent thrombotic events is likely to be low.

Another finding is that the classically defined rupture prone vulnerable plaques are not mandatory for formation of a luminal thrombus and subsequent events. In a subset of patients, especially among the young and women, clinically manifest events caused by luminal thrombosis are not triggered by plaque rupture. Instead, formation of the thrombus is initiated by a focal plaque erosion or endothelial de-

nudation. These eroded plaques are not as heavily inflamed as the ruptured plaques and posses a more fibrous phenotype.[39] Eroded plaques can also be considered vulnerable, but they lack specific features (e.g. large lipid core) that would facilitate non-invasive discrimination from non-eroded plaques.

Another limitation of the use of the traditional pathologic definitions of the vulnerable plaque for patients at risk is that plaque rupture can be asymptomatic. Asymptomatic plaque ruptures in the coronary arteries have been observed in 9% of healthy patients and 22% of patients with diabetes and hypertension.[40] Healed ruptures distant from the culprit lesion are a common finding in patients suffering acute coronary death,[41] and almost 20% of (asymptomatic) carotid arteries show signs of plaque rupture at post-mortem examination.[42]

The natural history of plaque rupture and the pathological definition of the vulnerable plaque

Although imaging techniques may be perfectly capable of revealing certain plaque characteristics; these will be of little value unless this specific imaged characteristic is prospectively shown to indicate a risk for future plaque disruption with subsequent clinical events. At present, the predictive value of the traditionally defined vulnerable plaque characteristics (large lipid core, thin fibrous cap and macrophage infiltration) for the occurrence of adverse events is actually unknown. Studies investigating the predictive value of imaged plaque parameters have been conducted, for example, Integrated Biomarker and Imaging Study I. This study investigated IVUS, IVUS-based elastography and angiography at baseline and after 6 months of follow-up in patients referred for percutaneous coronary intervention for stable or unstable angina.[43] The main finding was a decrease in the number of high-strain spots by elastography after follow-up, but the other imaging assessments showed no changes. The number of patients (90) was too small to be able to link imaging characteristics to adverse outcomes. The PROSPECT trial is a larger trial that investigates the predictive value of IVUS, angiography and virtual histology of the complete coronary tree for the occurrence of future cardiovascular events, with a follow up of 2.5 years. The initial enrollment of 700 patients has been completed in 2006, and follow-up is still ongoing. Prospective plaque imaging studies will help to understand the natural history of atherosclerotic disease and may enable us to define plaque characteristics which predict that a plaque will become symptomatic in the future. However the use of measures like large lipid core and the presence of inflammatory cells as surrogate markers for plaque vulnerability merits careful consideration, considering the high prevalence of vulnerable plaque characteristics in non-event related coronary arteries.

The vulnerable patient

A problem of many imaging modalities is the capability to visualize only a certain part of the vascular tree. Because plaques, especially vulnerable plaques, tend to be associated with compensatory remodeling, many potentially dangerous plaques will not be detected by standard imaging techniques such as angiography.[44] Therefore, with presently available imaging techniques, one can only speculate where to look for the classically defined vulnerable plaques. In addition, as mentioned before, it is unknown if the detection of a large lipid core with inflammatory cells with an imaging modality will hold a strong promise for a future in prognostic research. Therefore, the potential of this "local determinants to predict local outcome" approach **(Figure I)** may be limited.

To reliably predict if a patient will develop a cardiovascular event, we can consider many other factors besides local plaque characteristics. Systemic risk factors for atherosclerosis such as smoking, diabetes, hypercholesterolemia, sex, and age have been appreciated for a long time.

In their key article, Naghavi et al. introduce the term vulnerable patients: "patients in whom disruption of a vulnerable plaque is likely to result in a clinical event".[45] These vulnerable patients could be recognized by different factors, such as vulnerable plaques, thrombogenecity of the blood, and vulnerability of the myocardium.

Detection of the vulnerable patient should ideally be easy to perform, with high availability and by noninvasive means. In clinical practice, the search for the vulnerable patient should be performed by a cost-effective stepwise approach. The most logical way to start is careful examination of clinical characteristics such as traditional risk factors and cholesterol levels. In this aspect, a useful diagnostic test such as the ankle brachial index should not be ignored because it is a very easy test to perform and it possesses good prognostic value.[46] Patients at high risk could than be referred for screening by noninvasive imaging techniques. This is basically the strategy as proposed by the Screening for Heart Attack Prevention and Education task force report.[47] Essentially, the strategy as currently proposed builds upon traditional risk factors in combination with plaque measurements such as intima-media thickness and coronary calcium score.

At present, the "systemic determinants to predict systemic outcome" approach **(Figure I)** enables risk stratification but is not able to discriminate between individual patients who will or will not suffer a clinical event such as a myocardial infarction or a stroke. Although screening for atherosclerosis seems promising, it may be more effective when it does not only incorporate known risk factors, but it is also extended by new specific markers identifying patients at risk for cardiovascular events. Therefore, new developments are needed to find these markers. The following section addresses developments and strategies that are used in the search of new markers.

Systemic biomarkers to identify patients at risk for cardiovascular events

One of the easiest new approaches that could be incorporated in clinical practice is to test a peripheral blood sample for the presence of a specific atherosclerosis marker. Different study setups have been applied to investigate such markers, (1) comparing nonatherosclerotic with atherosclerotic patients, (2) comparing stable atherosclerosis vs. unstable atherosclerosis, and (3) relating baseline values of biomarkers to future clinical events. The latter is the most laborious study design, but also by far the most appropriate to test the value of a biomarker to identify vulnerable patients. Because atherosclerosis is a systemic inflammatory disease, known inflammatory proteins and acute phase proteins have been examined to identify atherosclerotic lesions. The most extensively studied serum biomarker in atherosclerotic disease is C-reactive protein (CRP), often referred to as high-sensitivity CRP. Presence of this acute-phase protein in the serum is greatly increased after infection or trauma, but in the absence of these events, it has a value as a surrogate biomarker for atherosclerosis. It is associated with traditional cardiovascular risk factors, such as smoking and obesity, but has an independent predictive value for the occurrence of cardiovascular events.[48] Treatment with statins, known to reduce plaque inflammation, is followed by a decrease in high-sensitivity CRP levels.[49] Nevertheless, predictive value of CRP as a surrogate marker for atherosclerosis is only moderate (less than that of the traditional risk factors), and CRP testing has not been widely accepted in the clinical setting.[50] In addition, many other inflammatory markers have been examined in relation to atherosclerotic disease, including interleukin 6 (IL-6), IL-18, monocyte chemoattractant protein-1, tumor necrosis factor, soluble CD40 ligand, and immunoglobulins.[51-55] However, none of these biomarkers have surpassed the predictive value of CRP, and routine clinical use cannot be recommended. A more extensive review of currently known biomarkers that are associated with atherosclerotic disease and adverse outcome can be found elsewhere.[56]

In the search for new biomarkers, one of the approaches is to develop a serum test for molecules that are known to be present in unstable plaques or that are involved in the mechanisms of plaque destabilization. High serum levels of plaque-derived markers could represent a high burden of unstable plaques and thereby help identify the vulnerable patient. It has been shown that proteins derived from unstable plaques can be secreted into the bloodstream, especially in the case of a disrupted plaque, and thus be retrieved in the circulating blood. It was shown that in patients undergoing percutaneous coronary interventions, blood samples distal from the coronary plaque contained increased amounts of IL-6, suggesting secretion of IL-6 from the plaques.[57] Interestingly, IL-6 has been identified as a possible biomarker,

and IL-6 release from plaques is able to induce CRP in the liver. The family of MMPs, of which especially MMP-9 plays an important role in plaque destabilization, are an interesting target to identify the vulnerable patient. In a follow-up study, blood MMP-9 levels at baseline were found to be associated with future cardiovascular events. However, the predictive value was moderate (comparable or less than CRP) and correcting for known risk factors, and CRP disturbed the association between MMP-9 and clinical outcome.[58] Another example is pregnancy-associated plasma protein A, which seems to be specifically expressed in ruptured and unstable plaques. Plasma levels of this protein strongly correlated with plaque levels, and a recent study documented an increased incidence of the combined end point of death or acute coronary syndrome in patients with chronic stable coronary artery disease.[59]

Besides being a surrogate marker for inflammation and plaque instability, biomarkers can also relate to the vulnerable blood, another ingredient of the vulnerable patient. The most widely investigated markers are D-dimer and fibrinogen.[60,61]

The currently known biomarkers potentially offer the possibility of risk stratification, but the "magic bullet" to identify the vulnerable patient that hides many vulnerable plaques has not been found. Therefore, the search for new biomarkers is ongoing. Because atherosclerosis is a multifactorial disease, it is probably simplistic to assume that a single systemic biomarker would suffice. Instead, several biomarkers could be combined in a multimarker test. With currently available biomarkers, even the use of multiple biomarkers only adds moderate predictive value to traditional cardiovascular risk factors. A cohort of 3209 patients derived from the Framingham Heart Study was tested for 10 biomarkers, but the relative hazard for cardiovascular events during 7.4 years of follow-up was no more than 1.84 for the patients with the highest quintile of multimarker scores compared with the lowest two quintiles.[62] This underlines the need for more specific prognostic biomarkers to identify the patients at high risk to suffer a cardiovascular event.

Local plaque characteristics in relation to systemic clinical outcome: from plaque to patient

As mentioned before, previously determined characteristics of the local atherosclerotic plaque could hold a certain predictive value for local plaque rupture; however, subjects who never suffered an event also hide plaques with inflammatory properties. Alternatively, instead of searching for local plaque characteristics predicting local rupture, or a systemic biomarker predicting systemic events, there is a third option: the search for local plaque characteristics predictive of systemic cardiovascular events ("local determinants for systemic outcome") **(Figure I)**. There is accumulating evidence that the processes causing plaque destabilization are not limited to a sin-

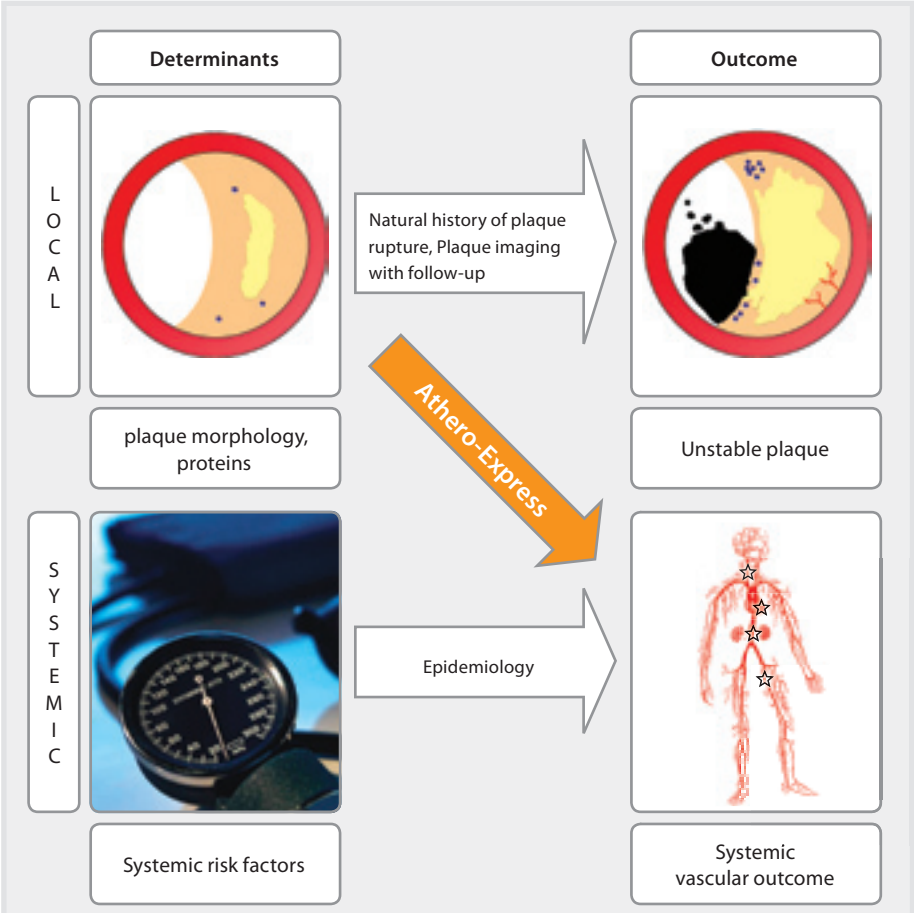

Figure I Overview of study designs to identify markers to predict future cardiovascular events

Traditionally, systemic risk factors are used to define the risk of cardiovascular events (systemic determinants to predict systemic outcome; epidemiology). An upcoming approach is the identification of vulnerable plaques with plaque imaging coupled to follow-up (local determinants for local outcome; natural history of plaque rupture). A novel approach will be identification of local plaque characteristics (morphology, protein expression, genes) that are predictive of systemic outcome (local determinants for systemic outcome; tissue epidemiology)

gle culprit plaque but are diffusely present throughout the vascular tree. Mauriello et al. showed that in 16 patients who died from myocardial infarction, inflammation was not only evident in the culprit lesion but throughout the entire coronary tree compared with patients dying from noncardiac causes (n=14).[63] Plaques throughout the coronary tree, either ruptured or vulnerable (defined as fibrous cap thinner than 65 µm) but also stable (cap >65 µm), showed a threefold increase in inflammatory cell density compared with (mostly stable) plaques in the coronary tree of patients with stable angina of asymptomatic patients. Other studies have shown that changes in the vascular wall are not confined to the coronary tree. When coronary disease presents with unstable clinical symptoms, carotid plaque morphology as measured by duplex ultrasound is also more unstable. In patients with unstable angina, 23.2% of patients demonstrated an unstable carotid plaque compared with 3.2 patients with stable angina.[64] Furthermore, a prospective study of 5393 carotid angiograms showed that patients with irregular carotid plaques had increased risk of nonstroke cardiovascular death during follow-up compared with patients with smooth plaques, which could not be explained by differences in traditional cardiovascular risk factors.[65] These studies all lend support to the idea that instability of the vascular wall is a systemic process rather than only local inflammation, and that the molecular structure of the atherosclerotic vascular wall at one site could hold information about the stability of the whole system. Thus, theoretically, inflammatory mediators or other markers of instability measured in one territory of the vascular tree could provide a fingerprint of the degree of stability of the whole atherosclerotic arterial system. Local atherosclerotic vascular tissue, obtained by endarterectomy or atherectomy, could thus help to find markers associated with generalized plaque vulnerability of the vascular tree. This novel approach is currently being investigated by the Athero-Express study, which already has included atherosclerotic specimens of more than 1000 patients undergoing carotid endarterectomy to test the predictive value of plaque characteristics of locally obtained plaque specimen for the occurrence of systemic adverse cardiovascular events.[66] All patients undergo a 3-year follow-up. Patients fill in questionnaires and donate blood at inclusion. The study was initiated in 2002 and does not suffer from a lack of power: approximately 20% of all patients reach a hard clinical end point within 3 years.

Technical development and novel approaches to discover new targets to identify the vulnerable patient

The use of biomarkers and new imaging tools to identify the vulnerable patient with prospectively new defined plaque characteristics actually depend on current knowledge of mechanisms of atherosclerosis. The search for biomarkers as per-

formed currently could be considered a "fishing expedition," in which candidate proteins are picked and tested for their ability to identify the vulnerable patient. This approach then brings target genes and proteins into atherosclerotic animal models and, one hopes, to clinical practice. This time-consuming approach is cumbersome and mostly involves the value of known candidate targets, and validation is hampered by the differences between atherosclerotic animal models and humans.

Advancing technology enables the study of larger numbers of genes and proteins simultaneously. The unraveling of the total human genome has stimulated the wide use of strategies that investigate cells and tissues for the total genome (genomics) or proteome (proteomics). Microarrays can be used to investigate expression of thousands of genes at once. Proteomics cover the entire protein spectrum found in humans.[67] Such an approach could very well be used to identify serum markers for atherosclerosis.[68] However, it is still a challenge to perform proteomics studies on sera because of the large amounts of albumin and other serum proteins in comparison with relatively low expressed biomarker targets. Therefore, research on proteomic techniques should be encouraged. When these techniques have advanced sufficiently to enable reliable proteomics research on blood samples, researchers possessing serum samples of longitudinal patient cohorts should be encouraged to perform proteomics on these samples to identify new prognostic serum markers. Alternatively, proteomic studies on plaque levels may provide new biomarker targets. In the Athero-Express study, protein expression in the atherosclerotic plaque from patients undergoing carotid endarterectomy is linked to clinical follow-up after the operation. Differentially expressed proteins between cases (who develop adverse cardiovascular events during follow-up after carotid endarterectomy) and controls will be identified by proteomics on protein isolated from the carotid plaques. Selection of cases and controls is done irrespective of plaque characteristics at baseline. This approach will help us to redefine or fine-tune the definition of the plaques that make the patient vulnerable. This research will yield new target proteins and may identify protein targets that have a predictive value as a serologic marker, but more importantly, the identified target proteins can also be used for molecular imaging.

Summary

The search for markers of the vulnerable plaque and vulnerable patient represents an exciting new research field. On the one hand, studies are ongoing to prospectively determine local atherosclerotic plaque characteristics to predict future plaque rupture and subsequent adverse events. Newly developed imaging modalities can be used to detect these plaques in vivo. On the other hand, systemic approaches are pursued to discover serum biomarkers for cardiovascular disease, which can be used to define patients at risk for future cardiovascular events. New approaches with genomics and proteomics studies provide opportunities to discover new targets to identify patients at high risk for cardiovascular events.

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CHAPTER 3

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INTRAOBSERVER AND INTEROBSERVER VARIABILITY AND SPATIAL DIFFERENCES IN HISTOLOGIC EXAMINATION OF CAROTID ENDARTERECTOMY SPECIMENS

Introduction Studies using histologic examination and protein analysis of atherosclerotic plaques are increasingly being performed, but reproducibility of plaque histology and variation of plaque composition among different parts of the plaque, which are key to reliability of these studies, are relatively unexplored. Therefore, this study investigated the intraobserver and interobserver variability of plaque histology and spatial variability in plaque composition.

Methods Atherosclerotic plaques (n = 100) obtained during carotid endarterectomy were divided into 0.5-cm segments. Paraffin sections were stained and semiquantitatively analyzed (four categories: no, minor, moderate, and heavy) for fat, macrophages, smooth muscle cells, collagen, calcification, thrombus and overall phenotype. First, to determine the intraobserver and interobserver reproducibility, two independent observers independently analyzed the plaques. Second, to investigate spatial variability in plaque composition, histologic appearances of the culprit lesions (0-segment) were compared with the histologic appearances of adjacent (+5 mm) and more distant (+10 mm) plaque segments of 30 specimens.

Results The κ values for intraobserver variability of fat, macrophages, smooth muscle cells, collagen, calcifications, thrombus, and overall phenotype were 0.83, 0.85, 0.71, 0.63, 0.81, 0.80, and 0.86, respectively, and κ values for interobserver variability were 0.68, 0.74, 0.54, 0.59, 0.82, 0.75, and 0.71, respectively. Comparison of the histologic scorings of adjacent segments revealed a mean κ of 0.40 (range, 0.33 to 0.60). When the culprit segment was compared with the more distant segment, the mean κ was 0.24; however, in 91% of cases, the difference between the culprit segment and the distal segment was one category or less.

Conclusion Semiquantitative analysis of carotid atherosclerotic plaque histology was well reproducible, both intraobserver and interobserver. Although variation between different plaque segments in histologic appearance was observed, differences were small in almost all cases. Variability in histologic examination needs to be taken into account in studies comparing plaque imaging with histopathology and plaque research studies.

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Introduction

The percentage carotid artery stenosis has traditionally been considered the main determinant of stroke risk related to carotid artery disease. It has recently become evident that composition of the atherosclerotic lesion is also an important determinant of clinical presentation. Three large studies have convincingly shown that symptomatic presentation of carotid artery disease is associated with specific histologic features of the carotid plaque such as a large lipid core, rupture of the fibrous cap, and increased inflammatory activity.[1-3] This is comparable with earlier findings in the coronary tree, where the same characteristics have been linked with unstable clinical presentation such as myocardial infarction and sudden cardiac death.[4-8]

The increasing knowledge on plaque pathophysiology has the potential to be clinically applied; for example, noninvasive imaging of these plaque characteristics could, in the future, aid in selection of the most appropriate treatment. Furthermore, it is currently being investigated if the plaque histology itself holds prognostic information.[9] When histologic characteristics of endarterectomy specimens could be identified that are related to a highly increased risk of restenosis, patients whose plaque histology showed these characteristics could undergo a more aggressive duplex surveillance scheme. For all of these applications, it is important to investigate the reliability of the histologic examination.

Present data on reproducibility of atherosclerotic plaque histology are scarce. To our knowledge, one study has been published that systematically investigated the re-

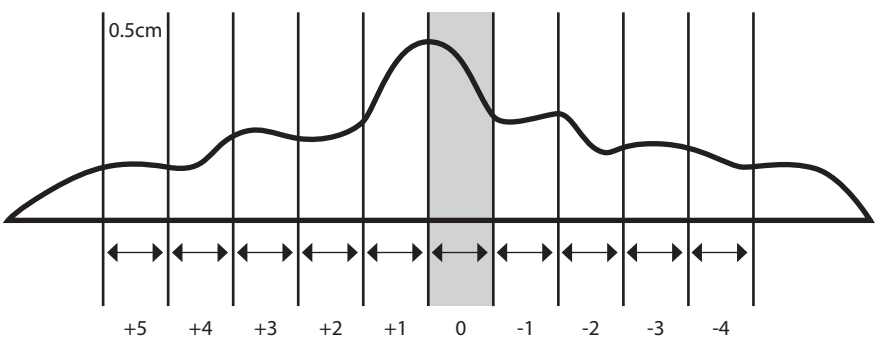

Figure I Schematic representation of plaque segmentation

Each transverse segment measures approximately 0.5 cm. The zero-segment, the reference segment which is routinely used for assessment of plaque histology in the Athero-Express study, is defined as the segment with greatest plaque burden. The adjacent segments at both sides are called +1, +2 etc., at one side and -1, -2, etc., at the other side.

Table I Semi-quantitative assessment of plaque histology

Histological characteristic	Staining	Categories			
Fat (lipid core)	HE, Picro-Sirius	<10% fat	10% - 40%	> 40% fat	
Macrophage infiltration	CD68	No	Minor	Moderate	heavy
SMC infiltration	Alpha Actin	No	Minor	Moderate	heavy
Collagen	Picro-Sirius	No	Minor	Moderate	heavy
Calcifications	HE, Hematoxilin[A]	No	Minor	Moderate	heavy
Thrombus	HE, EvG	No	Minor	Moderate	heavy
Overall phenotype	HE, Picro-Sirius	Fibrous	F-ath	Atheromatous	

Abbreviations: EvG, Elastin von Gieson; HE, Hematoxylin and eosin; SMC, Smooth muscle cell.
[A]Hematoxylin was used as counterstaining in CD68 and Alpha-Actin stainings.

producibility of carotid plaque histology.[10] This study showed good reproducibility of plaque histology between observers, and the authors concluded that examination of a single culprit segment of the plaque was reasonably representative for the plaque as a whole.[3] Hematoxylin and eosin was the only histologic staining used, however, and the reproducibility of scoring of immunohistochemical stainings, for example to assess macrophage infiltration, was not investigated.
The current study therefore investigated the reproducibility of the plaque histology. For this purpose, two substudies were performed. First, sections were repeatedly examined to determine intraobserver and interobserver variability, and semiquantitative ratings based on visual estimation were compared with computer-aided analyses. Second, the variability of scorings along different segments of the plaque was examined.

Methods

Patients

A random subset of 100 carotid endarterectomy specimens from the Athero-Express study was included.[9] The study has been approved by the Institutional Review Boards of the two participating hospitals, and written informed consent was obtained from all patients. The patients had significant carotid artery stenosis as assessed by preoperative duplex examination: 3% had 50% to 69% stenosis and 97% had 70% to 99% stenosis. Most patients (85%) were symptomatic and had symptoms of amaurosis fugax, transient ischemic attack (TIA), or stroke.

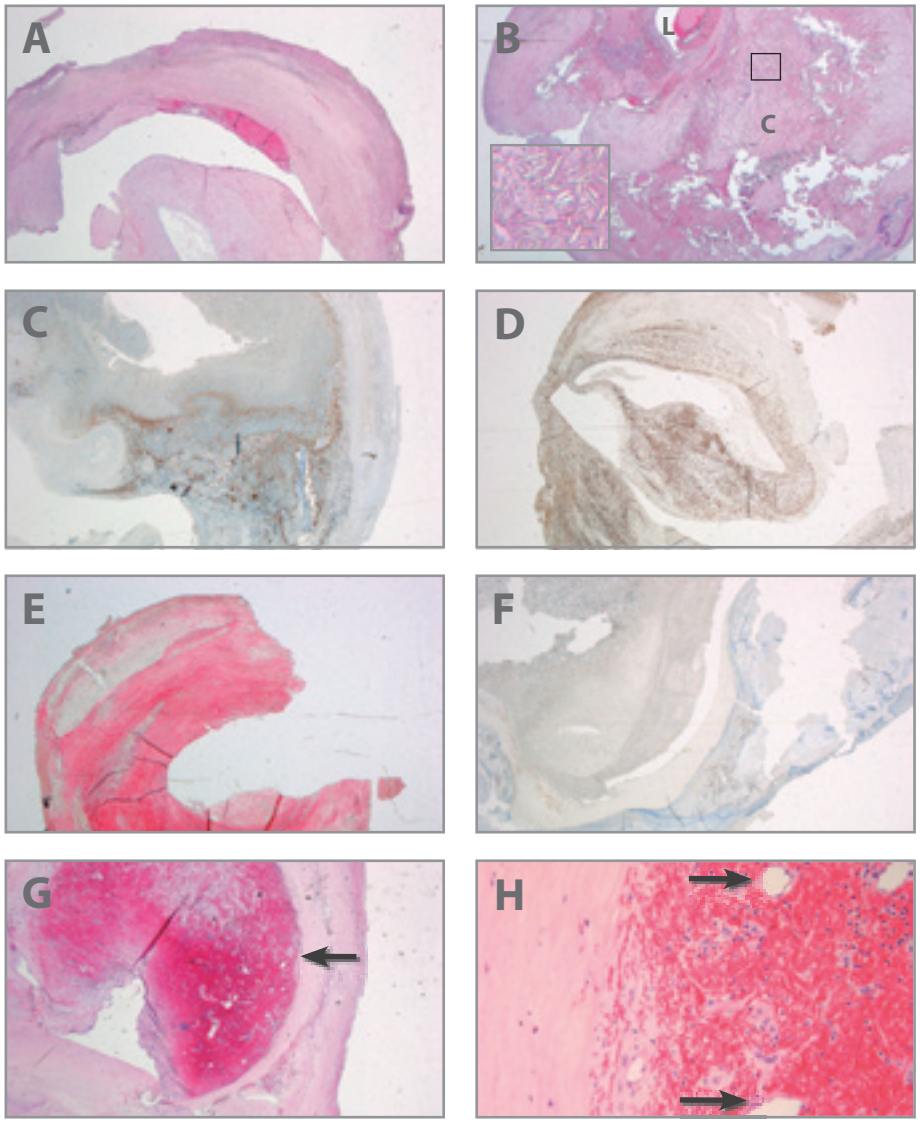

Figure II Representative carotid plaque histology

Images A-G were photographed at 20x magnification. **A:** Hematoxilin and Eosin (HE) staining showing a plaque with no lipid core. **B:** HE staining, large lipid core; L = lumen; C = core, inset shows cholesterol crystals which were clearly visible at higher magnification. **C:** CD68: heavy macrophage infiltration. **D:** Alpha-Actin: heavy smooth muscle cell infiltration **E:** Picro-Sirius Red: heavy collagen **F:** Hematoxilin: heavy calcifications (blue) **G:** H&E: thrombus (arrows) **H:** HE (200x magnification) At higher magnification signs of organization of the thrombus can be appreciated: angiomatosis (arrows) and influx of inflammatory cells

Plaque processing

All carotid endarterectomies were performed by an experienced vascular surgeon or by a trainee under specialist supervision. Before the carotid artery was cross-clamped, 5000 U of heparin was administered intravenously. The plaques were carefully dissected in toto and directly transferred to the laboratory after excision. The plaques were dissected by a dedicated technician into 5-mm-thick cross-sectional segments along the longitudinal axis of the vessel. The plaque segment showing the largest plaque burden as determined by visual assessment of plaque macroscopy was called the zero segment **(Figure I)**. The rationale is that the segment of a plaque with largest plaque burden is generally the plaque segment where most vulnerable characteristics, such as large lipid core and inflammation, are present.[11] This was not necessarily the middle segment of the endarterectomy specimen.
Adjacent segments (5-mm thickness) were numbered sequentially: +1, +2, etc, at one side and –1, –2, etc, at the other side. Segments used for the analysis were fixed in formalin, quickly decalcified in ethylenediaminetetraacetic acid, and embedded in paraffin. Cross-sections of 5-µm thickness were cut on a microtome and used for staining with hematoxylin and eosin (H&E), elastin von Gieson (EvG), picrosirius red, and immmunohistochemical stainings for α-actin and CD68 **(Figure II)**.

Semiquantitative assessment

Table I gives an overview of all semiquantitative assessments and the stainings used for each item. The definitions of each staining category have been published previously.[9] Briefly, all scorings are based on visual estimates and are rated on ordinal scales. Overall phenotype is established by overall appearance: the typical atheromatous plaque has a large lipid core (high fat content, defined as >40% of plaque area) and high macrophage infiltration with low smooth muscle cell and collagen content, whereas the typical fibrous plaque has a small (<10% of plaque area) or absent lipid core, low macrophage infiltration, and high smooth muscle cell and collagen content. The fibrous-atheromatous phenotype is an intermediate between the two other phenotypes.

Quantitative assessment

The sections were visualized under a microscope at 40x magnification and digitally captured on a computer workstation with a 3-megapixel camera. Three representative fields were identified in each section. These fields contained only intima; lumen, and fragments of media were avoided. The percentage of plaque area occupied by the specific staining (CD68 for macrophages and α-actin for smooth muscle cells) was measured automatically in each of these fields, based on color thresholds (AnalySIS 3.2, Soft Imaging Systems GmbH, Münster, Germany). The mean of the

Table II Intra- and inter-observer variability of semi-quantitative plaque examination

Histological characteristic	Categories[A]	Intraobserver variability		Agreement	Interobserver variability		Agreement
		κ (95% CI) quadratic weighted	κ (95% CI) linear weighted		κ (95% CI) quadratic weighted	κ (95% CI) linear weighted	
Fat	3	0.83 (0.69 - 0.96)	0.77 (0.65 - 0.89)	83%	0.68 (0.49 - 0.87)	0.60 (0.48 - 0.73)	71%
MO infiltration	4	0.85 (0.59 - 1.00)	0.78 (0.67 - 0.89)	83%	0.74 (0.46 - 1.00)	0.63 (0.50 - 0.75)	73%
SMC infiltration	4	0.71 (0.24 - 1.00)	0.63 (0.48 - 0.79)	78%	0.54 (0.12 - 0.95)	0.42 (0.25 - 0.57)	66%
Collagen	4	0.63 (0.19 - 1.00)	0.54 (0.40 - 0.68)	68%	0.59 (0.33 - 0.85)	0.51 (0.38 - 0.67)	69%
Calcification	4	0.81 (0.65 - 0.98)	0.75 (0.64 - 0.85)	76%	0.82 (0.74 - 0.90)	0.71 (0.61 - 0.81)	70%
Thrombus	4	0.80 (0.64 - 0.95)	0.68 (0.55 - 0.80)	71%	0.75 (0.52 - 0.98)	0.65 (0.53 - 0.76)	71%
Overall phenotype	3	0.86 (0.78 - 0.94)	0.79 (0.68 - 0.89)	82%	0.71 (0.55 - 0.87)	0.62 (0.49 - 0.74)	71%

Abbreviations: MO, macrophage; SMC, smooth muscle cell.

[A]Categories are specified in Table I.

three fields was calculated and used as the representation of percentage of positive staining in the section.

Reproducibility

To determine interobserver reproducibility, two independent observers assessed the zero segments of the plaques. The observers did not have access to scorings of the other observer. All cross-sections were blinded so that patient characteristics were not available to the observers. To determine intraobserver reproducibility, the second observer reassessed the sections in a random order about 2 months after the first assessment. The initial ratings of the second observer were compared with the repeated assessment of the same observer. The observer was blinded for earlier scorings of the plaques. Macrophage and smooth muscle cell infiltration were also measured by computer-aided analysis in the sections from the zero segment and compared with the semiquantitative ratings.

Inter-segment variation

In a subset of 30 plaques, the zero segment, the directly adjacent segment, and the next segment underwent histologic examination by the second observer. Per

plaque, either segments 0, +1, +2, or segments 0, –1, –2 were examined. The examination of these segments was blinded and was performed in a random order. Comparison was made between adjacent segments (0 vs. +1 and +1 vs. +2) and between nonadjacent segments (0 vs. +2).

Data analysis

Weighted kappa (κ) statistics were used as the measure of agreement between different observations. Two different weightings were employed: First, linear weights are presented, in which the weights are calculated as [1 – abs(i – j)/(m – 1)] and second, quadratic weights are presented, in which the weights are calculated as $1 - abs(i - j)^2/(m - 1)^2]$, with abs(i – j), the number of categories difference between observers; m, the number of categories, and m – 1, maximum difference between two observations.[12] When four semiquantitative categories are used, linear weightings for disagreement (no difference between observers), and difference of 0, 1, 2, or 3 categories between observers are 0, 1/3, 2/3, and 1, respectively. With quadratic weighting, these numbers are 0, 1/9, 4/9, and 1, respectively. This clarifies that small disagreements affect the quadratic-weighted κ less than linear-weighted κ.

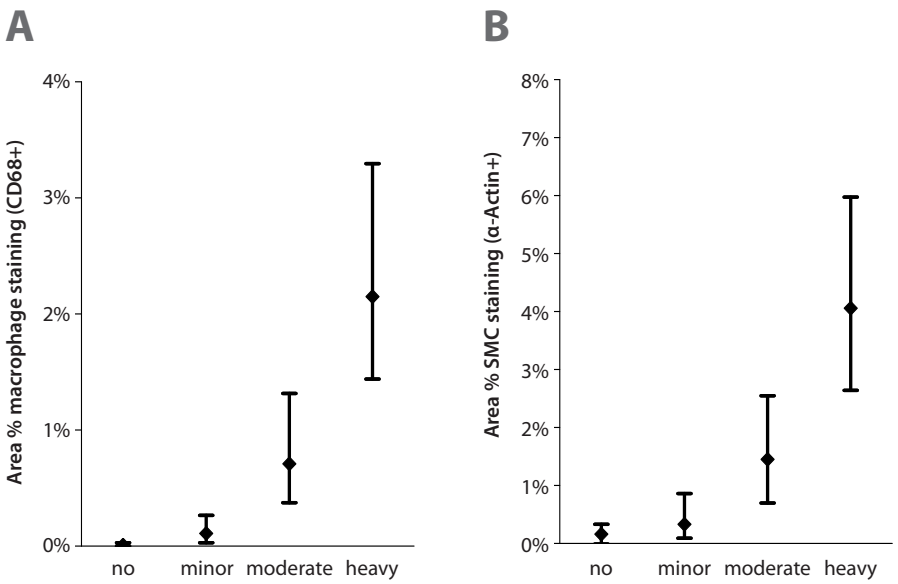

Figure III Semiquantitative vs. computer-aided measurements

Comparison of semi-quantitative assessments, expressed as "no" – "minor" – "moderate" – "heavy" (x-axis) and the computer-aided measurements, expressed as the percentage of plaque area occupied by the specific immunohistochemical staining in three representative fields (y-axis). **A:** Macrophage infiltration (CD68+) **B:** Smooth Muscle Cell Infiltration (Alpha-Actin+)

Given κ values are quadratic-weighted unless otherwise specified. In addition, the percentages of sections in which both observations were exactly equal are given (percentage agreement).

The Spearman correlation was used to test the relation between semiquantitative and quantitative measurements. For comparison of different plaque segments, quadratic-weighted κ were used. The κ values for comparison of different segments were also expressed with correction for the intraobserver κ, because perfect alikeness of two adjacent segments could theoretically not result in a κ value higher than the intraobserver κ. Applying generally accepted definitions, κ values ≤0 indicate no agreement; 0 to 0.2, slight agreement; 0.2 to 0.4, fair agreement; 0.4 to 0.6, moderate agreement; 0.6 to 0.8, good agreement; and 0.8 to 1, excellent agreement.[13]

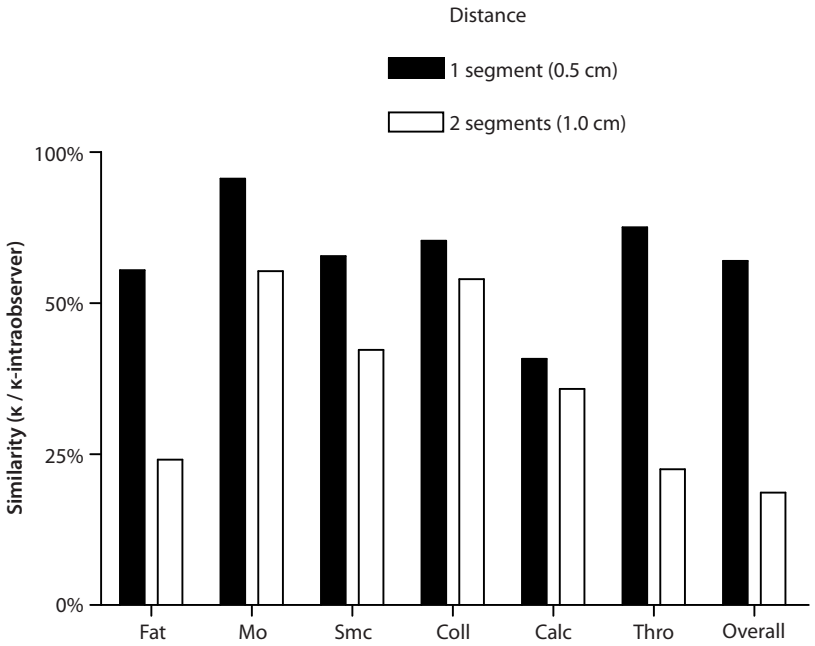

Figure IV Inter-segment variability of histological examination of the plaque

Abbreviations: MO, macrophage infiltration; SMC, smooth muscle cell infiltration; coll, collagen; calc, calcifications; thro, thrombus; overall, overall phenotype.

The similarity between different segments expressed as percentage of the kappa value of the intraobserver repeatability, which is the theoretical maximum when two adjacent segments are completely equal: 100% indicates perfect similarity, 0% indicates no similarity, negative values (not observed) indicate inverse associations. The similarity is compared between adjacent segments (inter-segment distance "1") and between the reference segment and a non-adjacent segment (inter-segment distance "2").

Results

The semiquantitative analysis of plaque histology was well reproducible, with a mean κ of 0.78 **(Table II)**. Intraobserver reproducibility showed highest κ values for macrophage infiltration (0.85) and overall phenotype (0.86). Percentage exact agreement was 68% to 83%. Reproducibility between observers was slightly lower than intraobserver reproducibility: on average, κ was 0.69. The mean percentage interobserver agreement was 70% compared with 77% intraobserver agreement. Most of the disagreements between the observers were minor (1 category): in the intraobserver assessments, disagreements of two categories were observed in 0.8% of cases and disagreements of 3 categories in 0.1% of cases. This observation was comparable in interobserver analysis: difference of two categories was observed in 0.9% of cases and disagreements of three categories in 0.2% of cases. Moreover, ratings for macrophages and smooth muscle cells did not show any differences greater than one category in either intraobserver or interobserver analyses.

The reliability of the scorings of macrophage and smooth muscle cell presence was confirmed by comparison with quantitative analyses. For macrophages, semiquantitative and quantitative measurements were very well correlated ($R = 0.77$; $P < .0005$), and there was no overlap between the 25th and 75th percentiles of subsequent scoring categories **(Figure III-A)**. The cutoff values between semiquantitative scoring categories were approximately 0.03% (no–minor), 0.3% (minor–moderate), and 1.4% (moderate–heavy) of plaque area. With increasing semiquantitative ratings, the quantitative measurements increased more than linear. For smooth muscle cells, the same relations were found. Although the quantitative measurements of subsequent semiquantitative rating categories overlapped a little more, the semiquantitative ratings compared very well with the quantitative ratings overall ($R = 0.67$; $P < .0005$; **Figure III-B**). In general, the area occupied by smooth muscle cells amounted to more than twofold the area occupied by macrophages (mean, 2.3% vs 0.89%). The cutoff points between semiquantitative scoring categories for smooth muscle cells were therefore higher: 0.2% (no–minor), 0.7% (minor–moderate), and 2.5% (moderate–heavy) of plaque area.

The variation in plaque histology between different plaque segments was investigated. Average κ between adjacent segments was 0.40, and the percentage of exact agreement between adjacent segments was 60% compared with 77% for intraobserver analysis of the same segment. Similarity between the adjacent segments was good for macrophage infiltration (0.60), moderate for fat (0.46), smooth muscle cell infiltration (0.41), collagen (0.46), and overall phenotype (0.50), and fair for thrombus (0.33) and calcifications (0.38). Because assessment of the similarity of segments is hampered by intraobserver variation, the similarity of segments was corrected for the intraobserver variability (**Figure IV**).

In the distant segments (two-segment distance from the reference segment), similarity was generally less compared with the reference segment, but most items still showed fair to moderate agreement (**Figure IV**; average κ, 0.24). For all histologic assessments, similarity decreased with increasing distance. Still, in the distal segments, most differences with the reference segment were minor (one category), and 91% showed exact agreement or at maximum one category difference.

Discussion

The present study shows that histologic examination of carotid plaque histology is well reproducible. The current study did not achieve 100% reproducibility. However, even clinical standards such as angiography sometimes show considerable variation, but this does not mean that the diagnostic tool can not be used reliably.[14] Moreover, comparison of the semiquantitative analysis with quantitative measurements of smooth muscle cell and macrophage infiltration showed that each semiquantitative scoring category corresponded with a distinct range of quantitative measurements. We further found that differences in plaque characteristics between adjacent segments are relatively small. When the distance was bigger, however, the similarity consistently decreased.

The results of the current study are comparable with the previously published results from Lovett et al.[10] The current data have added value because macrophage infiltration, smooth muscle cell infiltration, and collagen are also included in the analysis and because our results are backed up by quantitative computer-based measurements. The previous study found good intraobserver and interobserver reproducibility for repeated assessment in the same sections of lipid core size, calcifications, and thrombus, which is comparable with our results. Findings on intersegment difference were also comparable. The current study adds the intersegment difference in macrophages, smooth muscle cells, and collagen. Compared with the other plaque characteristics, macrophages and smooth muscle cells showed low variability between segments.

Clinical implications

The interpretation of the extent of variability depends greatly on the underlying purpose. On the one hand, plaque imaging is an upcoming field, and validation of these imaging modalities requires comparison with the gold standard of plaque histology. On the other hand, histologic examination of atherosclerotic plaques is used by studies linking the plaque with clinical characteristics. For both purposes, it is very important to quantify the amount of variability in histologic assessment.

Implications for validation of plaque imaging studies

Preoperative noninvasive imaging of the plaque has the potential to help guide the choice of treatment modality. Supporting this is that the composition of symptomatic plaques is different from asymptomatic plaques. Symptomatic presentation is related to a large lipid core, infiltration of macrophages, plaque rupture, and thrombus.[1-3,15] Some studies have already shown that the size of the lipid core can be determined reliably with magnetic resonance imaging.[16-18] To be clinically applicable, the imaging technique needs to be validated against the gold standard of plaque histology.

The present study clarifies that the accuracy of plaque imaging may be underestimated because of variability in histologic assessment of plaques. The observed variability between imaging modalities and histology is the sum of (1) variability and imperfections of the imaging technique and (2) the variability in assessment of plaque histology. When the latter is not taken into account, the ability of imaging techniques to identify plaque characteristics noninvasively will be underestimated.

From our current results, we can make the following recommendations with regard to validation of plaque imaging against histology. First, sections along the whole endarterectomy specimen need to be taken, especially when thrombus or calcifications are studied, which show marked variablility within the plaque. Optimally, the site where histological sections are taken should be documented and matched to the images following a standardized protocol. Second, we recommend histological assessment of each section by two independent observers, in order to minimize variability. Studies investigating plaque imaging should report the variability in their histological assessments and consider this in their conclusions.

Implications for plaque research and biobanking

The study of endarterectomy specimens without involvement of imaging is of interest for two reasons. First, the study of endarterectomy specimens may reveal insights into pathophysiology of the disease and underlying mechanisms. Second, the endarterectomy specimen may hide prognostic information that could predict risk of restenosis and risk of adverse vascular events during follow-up. The latter is currently under investigation in the Athero-Express study.[9]

The interpretation of the current results may be different for these biobanking studies then for validation of plaque imaging. For plaque research studies, a perfect segment-to-segment match is not needed. When one wants to investigate the difference in plaque composition between patient groups, it is sufficient to study a large cohort with less extensive sampling per plaque. This will minimize the probability of chance findings and also provide power to perform multivariable statistical analysis to correct for confounders. It is unlikely that with the currently observed degree of variation, clear differences between patient groups would be missed in large series of endarterectomy specimens. This is underlined by the strong differ-

ences we observed between plaque histology of men compared with women in 450 patients.[19] Furthermore, for biobanking studies, we recommend that histologic assessment needs to be done by two independent observers, that the sampling of the plaques is performed by following a standardized protocol, and that variability in histologic examination is reported.

When biobanking would succeed in identifying predictive markers in the atherosclerotic plaque, such as for restenosis, the possibility of comparable assessment at different laboratories would be warranted. Semiquantitative histologic assessment is well reproducible within one laboratory but may be difficult to reproduce by others. The semiquantitative methods are well suited for research studies, but quantitative techniques such as computer-based analysis or measurement of protein markers by enzyme-linked immunosorbent assay (eg, macrophage markers) will be more suitable for extrapolation of results to other centers.

Another issue for biobanking studies is the comparison of plaque histology with protein expression within the plaques. In the Athero-Express study, the segments adjacent to the zero segment are regularly used for protein extraction.[9] The protein extracts can be used for determination of different proteins, such as inflammatory markers (interleukin-6 and -8 are routinely determined) and matrix metalloproteinases (MMP-2, MMP-8, MMP-9). The current study shows that intersegment differences in histology are relatively small, especially for the smooth muscle cells and macrophages, the main cell types responsible for production of several substances (eg, cytokines and proteases) in atherosclerotic plaques. Therefore, the use of the plaque segment adjacent to the reference segment for comparisons within plaques (histology in the reference segment vs protein in the adjacent segment) is a valid approach. This is underlined by the fact that well-known associations, for example between macrophage infiltration and production of MMP-9,[20] could be readily confirmed when the histology in the zero segment was compared with protein analysis from the adjacent segment.[21] In distant segments, however, the differences in histology compared with the reference segment were larger. This indicates that when histopathology is compared with protein analyses within a plaque, this is best done in adjacent segments.

Strengths and limitations

This study contained a random sample of plaques taken from an ongoing consecutive series of carotid endarterectomies. Macroscopy was not used to select specimens, and therefore, this study provides a real-world comparison. To assess variability of different observations, the weighted κ statistic was used. Weightings are arbitrary, however, and thus we attempted to give optimal insight into our data by providing two different weightings.

A potential limitation of the current study is that plaque rupture was not assessed. In our experience, the plaque morphology is disrupted by surgical trauma in many

cases, thus making assessment of cap integrity difficult. In line with these observations, plaque rupture had relatively low reproducibility in the study by Lovett et al.[10] We have not included cap rupture in our own plaque assessments for this reason. Assessments of plaque rupture are probably much more accurate in postmortem series where vessels can be examined with the plaque in situ.

Conclusion

Semiquantitative analysis of carotid atherosclerotic plaque histology is well reproducible. Although variation between different plaque segments in histologic appearance was observed, differences were small in almost all cases. Variability in histologic examination needs to be taken into account in studies that compare plaque imaging with histopathology and plaque research studies.

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PART 2

THE CAROTID ATHEROSCLEROTIC PLAQUE AND CLINICAL PRESENTATION

CHAPTER 4

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CAROTID ATHEROSCLEROTIC PLAQUES IN PATIENTS WITH TRANSIENT ISCHEMIC ATTACKS AND STROKE HAVE UNSTABLE CHARACTERISTICS COMPARED WITH PLAQUES IN ASYMPTOMATIC AND AMAUROSIS FUGAX PATIENTS

Introduction Atherosclerotic carotid artery disease is responsible for a variety of clinical presentations, ranging from asymptomatic to cerebral ischemic events. Considering the upcoming use of noninvasive imaging modalities, plaque characteristics could serve as a marker in the selection of patients eligible for carotid endarterectomy (CEA). This would be more likely if characteristics corresponded with clinical manifestations and were predictive of future events. In this study, we hypothesized that plaque characteristics correlate with the clinical presentation of carotid artery disease.

Methods We included 404 patients undergoing a carotid endarterectomy (CEA). Ipsilateral clinical symptoms and duplex measurements were recorded. Patients could be asymptomatic (23.5%) or symptomatic with stroke (26.5%), transient ischemic attack (TIA) (36.1%), or amaurosis fugax (AFX) (13.9%). Plaques were stained and semi-quantitatively analyzed for the presence of macrophages, smooth muscle cells, collagen, calcifications, and thrombus. Plaques were categorized in three phenotypes by their overall presentation and the amount of fat. In addition, plaque matrix metalloproteinase (MMP) activity and cytokines expressions were measured.

Results Fibrous, fibro-atheromatous, and atheromatous plaques were observed in 30.2%, 35.6%, and 34.2%, respectively. Atheromatous plaques were more prevalent in patients with stroke and TIA compared with asymptomatic patients or patients with AFX ($P = .001$). Collagen staining was less evident in patients with TIA and stroke compared with asymptomatic patients or patients with AFX ($P < .001$). Plaques of patients with TIA and stroke showed significantly higher activity levels of MMP-8 and MMP-9 and higher levels of interleukin-8 compared with asymptomatic and AFX patients.

Conclusion Plaque phenotype of patients with TIA is comparable to that of patients with stroke; whereas, the plaque phenotype of patients with AFX resembles the plaque phenotype of asymptomatic patients. Follow-up studies should be encouraged to determine whether plaque characteristics visualized by imaging techniques might help to identify patients most likely to benefit from CEA.

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Introduction

Carotid endarterectomy (CEA) is a widely accepted method of treating carotid stenosis.[1-3] Indications for CEA are based on the percentage of stenosis and clinical manifestation of carotid artery disease, both of which are determinants of the success rate of carotid endarterectomy.[3-5] Symptoms associated with carotid atherosclerotic disease are (minor) stroke, transient ischemic attack (TIA), and amaurosis fugax (AFX). Although AFX and TIA are both considered symptomatic, the prognosis is better for patients with AFX.[6] With the upcoming development of imaging techniques to visualize the atherosclerotic plaque, it would be of interest if plaque characteristics were related to future cerebral events. Selection of patients for CEA could be made by plaque phenotype determined, for instance, by magnetic resonance imaging.

From coronary artery disease, we already know that atheromatous inflammatory plaques (presence of inflammatory cells and high levels of cytokines and proteases) are associated with unstable coronary syndromes, whereas fibrous plaques are associated with stable syndromes.[7-10] An analogy with coronary artery disease suggests that carotid plaque characteristics correlate with the clinical manifestations. Indeed, carotid plaque characteristics have been analyzed previously in relation to symptomatic and asymptomatic carotid patients.[11-16] However, these studies have mainly focused on single plaque characteristics (ulceration, thrombus, or calcification) and pooled all symptomatic patients without discriminating between patients with AFX, TIA, or stroke. Other studies that related carotid plaque characteristics to clinical symptoms were based on plaque morphology visualized by imaging techniques in relatively small patient populations.[17]

In the present study, we hypothesized that plaque characteristics were associated with different clinical manifestations of carotid artery occlusive disease. For this purpose, we used the Athero-Express biobank, which includes patients undergoing CEA.[18] We report that plaques from symptomatic patients with TIA or stroke display more atheromatous characteristics and protease activity and less fibrous tissue compared with asymptomatic patients. Surprisingly, plaques from patients with AFX demonstrate strong similarities with plaques from asymptomatic patients.

Patient and Methods

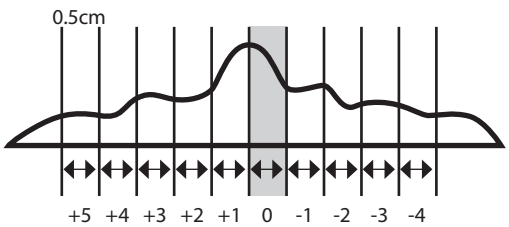
Baseline data

Baseline characteristics were retrieved from patient records and questionnaires. The questionnaire included a history of cardiovascular and peripheral artery disease, risk factors, physical activity, and family history of vascular disease. At baseline, a blood sample was withdrawn before operation. Laboratory studies routinely as-

sessed total cholesterol, triglycerides, high-density lipoprotein, and low-density lipoprotein. Before surgery, patients were examined by a neurologist. The clinical symptoms of carotid artery disease, date of onset, and latest symptoms were documented. These data were used to calculate the symptom-free interval (time passed since the latest presentation of symptoms and CEA). Patients were categorized into four groups by clinical presentation:
(1) ipsilateral asymptomatic (no symptoms occurred), (2) TIA, defined as an ipsilateral focal ischemic neurologic deficit of abrupt onset lasting at least 30 seconds and resolved completely within 24 hours,[2] (3) ipsilateral stroke (stokes were classified as disabling or nondisabling, based on the modified Rankin scale),[19] (4) AFX, or transient monocular blindness, which was defined as ipsilateral partial or complete visual-field loss in one eye which was of ischemic origin, lasted <24 hours, and was followed by complete recovery.[6]

Figure I Plaque processing

The plaque obtained during carotid endarterectomy is divided into 0.5-cm segments. The culprit lesion is designated 0 and is used for histopathologic staining. Other segments are used for protein and DNA/RNA isolation or kept at –80°C.



Carotid Endarterectomy

Patients were monitored during CEA using transcranial Doppler (TCD) scans (if a window was available) and online electroencephalogram (EEG) registration. A shunt was selectively used based on EEG and TCD scan criteria, as described in earlier reports.[20,21] Before cross-clamping, a bolus of heparin (5000 IU) was administered intravenously. All endarterectomies were performed by an open, noneversion technique, with careful dissection of the bifurcation into the internal and external carotid arteries. Atherosclerotic plaque was harvested and transported to the laboratory immediately after dissection.

Atherosclerotic plaque analysis

In the laboratory, the atherosclerotic segment was dissected into 0.5-cm segments. The culprit lesion, which was defined as the segment with the largest plaque mass, was numbered 0 and the adjacent segments –1 and +1 **(Figure I)**. The –1 and +1 segments, and all subsequently numbered segments (–2, +2, –3, etc) were immediately frozen in liquid nitrogen and stored in –80° in numbered metal cups. Segment 0 was fixed in 4% formaldehyde and paraffin embedded. From each paraffin-embed-

ded segment, 15 sections (5-µm thickness) were cut on a microtome for histologic immunostaining.

Plaque phenotyping

All stained sections were examined microscopically and digitally stored on CD-ROM at 20x and 40x magnification. Two observers independently scored all stainings semi-quantitatively, as described previously.[22] Plaques were categorized as no/minor staining or moderate/heavy staining for the stains listed below:

- Picro-Sirius Red (collagen using polarized light microscopy)
- CD68-positive cells (reflection of inflammatory cells)
- α-Actin-positive cells (reflection of the presence of smooth muscle cells)
- Hematoxylin (thrombus and calcifications)

For thrombus the following criteria were used: (1) no signs of earlier intraplaque thrombus formation, (2) signs of earlier thrombus formation (fibrin deposition). The percentage of atheroma of the total area of the plaque was visually estimated using the Picro-sirius red with polarized light and hematoxylin stains. Three groups were considered based on the percentage of atheroma in the plaque being present: fibrous plaques containing <10% fat; fibro-atheromatous, 10% to 40%; or atheromatous, >40% fat.

RNA and protein were isolated from all +1 or –1 segments. For this purpose, frozen arterial segments were crushed in liquid nitrogen. Protein was isolated using two techniques. One part of segment one was treated with 1 mL Tripure Isolation Reagent (Boehringer, Mannheim, Germany) according to the manufacturer's protocol. The other part was dissolved in TrisHCl (pH, 7.5 at 4°C).

For 133 patients, matrix metalloproteinase (MMP)-8 and MMP-9 activities were measured in isolated protein using the Biotrak activity assays RPN 2635, and RPN 2634, respectively (Amersham Biosciences, Buckinghamshire, United Kingdom).[23,24] Interleukin (IL)-6 and -8 was measured in 293 protein isolates from segment one. All measurements were done with a multiplex suspension array system according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, Calif).

Data analysis

Symptom categories were associated with plaque characteristics using Chi Square and the Fisher's exact test. All continuous variables were distributed nonparametrically. Testing was performed by the Mann-Whitney test and the test. A model was created in which plaques were given points based upon the vulnerable plaque characteristics as defined in the literature.[25,26] Single points were given for plaque characteristics that are supposed to be involved in plaque instability: moderate or heavy presence of macrophages; no (or minor) collagen staining, no (or minor)

presence of smooth muscle cells, and >10% fat. A value of $P < 0.05$ was considered statistically significant.

Results

The baseline characteristics are presented in **Table I**. Patients were divided into aforementioned groups by their clinical symptoms: asymptomatic (n = 95), AFX (n = 56), TIA (n = 146), and (minor) stroke (n = 107). Except for the history of prior carotid intervention, which was more prevalent in the asymptomatic patients, the patient groups did not differ. To exclude bias, additional analyses were performed with omission of patients with restenotic lesions. Excluding these patients did not

Table I Baseline characteristics

	Asymptomatic		AFX		TIA		Stroke		P
Number of patients	95		56		146		107		
Age (sd)	66.8	(8.6)	66.2	(9.4)	67.5	(9.3)	67.7	(8.3)	0.7
Female	32	34%	16	27%	38	31%	33	31%	0.7
Hypertension	57	71%	24	62%	71	63%	57	69%	0.6
Diabetes	18	22%	8	19%	22	19%	13	16%	0.8
PTCA / CABG	27	31%	6	13%	24	19%	19	22%	0.07
Peripheral vascular operation	12	14%	1	2%	11	9%	9	11%	0.17
Prior ipsilateral carotid intervention	10	11%	0	0%	4	3%	2	2%	0.002
Current smoker	22	28%	13	30%	32	27%	21	27%	1.0
Smoking in the past	49	61%	29	64%	72	62%	55	72%	0.6
Never smoked	11	14%	3	7%	14	12%	7	9%	0.6
Hypercholesterolemia	48	70%	28	62%	69	59%	48	61%	0.5
Cholesterol mmol/l (sd)	5.2	(1.2)	5.0	(1.2)	5.1	(1.2)	4.9	(1.1)	0.5
HDL mmol/l (sd)	1.2	(0.4)	1.2	(0.3)	1.2	(0.4)	1.1	(0.3)	0.6
LDL mmol/l (sd)	3.1	(1.0)	2.9	(1.1)	3.1	(1.0)	2.9	(1.0)	0.6
Triglycerides mmol/l (sd)	2.2	(1.3)	1.9	(0.8)	2.1	(1.1)	2.1	(1.0)	0.8
Statin use	52	59%	37	70%	90	65%	65	68%	0.5
Duplex stenosis									
50 - 64%	1	1%	1	2%	1	1%	5	6%	0.22
65 - 89%	25	30%	15	34%	46	40%	32	38%	
90 - 100%	56	68%	28	64%	69	59%	47	56%	

Abbreviations: AFX, Amaurosis fugax; TIA, transient ischemic attack; PTCA, percutaneous transluminal coronary angioplasty; CABG, coronary artery bypass graft; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
All values are mean+/- SD or absolute number of patients in each group with calculated percentage.

Table II Plaque characteristics compared to symptoms

		Asym	AFX	TIA	Stroke	P-value
MMP [A]	MMP-8	4.6 (1.7-8.0)	6.0 (2.0-8.8)	7.0 (3.4-10.5)	9.6 (3.7-16)	0.02
	MMP-9	1.3 (0.4-3.2)	1.9 (0.9-5.7)	2.7 (1.1-6.8)	2.5 (0.9-5.2)	0.03
IL[A]	Il-6	6.4 (2.1-12.9)	6.1 (1.1-17.1)	13.0 (4.8-30.4)	11.8 (3.7-30.4)	0.12
	Il-8	19.0 (0-53.6)	18.1 (0-118)	72.9 (27-171)	43.5 (2-182)	<0.001
Collagen	Minor	15.2%	16.1%	30.5%	22.4%	
	Moderate	50%	48.2%	54.9%	60.7%	<0.001
	Heavy	34.8%	35.7%	14.6%	16.8%	
Lipid core	Fibrous	41.1%	46.4%	22.6%	22.4%	
	F-ath	28.4%	35.7%	36.3%	41.1%	0.001
	Atheromatous	30.5%	17.9%	41.1%	36.4%	

[A]MMP and IL data are displayed as median and interquartile range (pg/ml).

influence the results (data not shown). The grades of duplex lumenal stenosis did not differ among the categories of clinical symptoms (see **Table I**). There was no difference in symptom-free intervals between the various categories of symptoms.

Figure II shows the relations between plaque characteristics and clinical symptoms. Surprisingly, patients with AFX and asymptomatic patients revealed similar plaque characteristics. In asymptomatic patients and patients with AFX, fibrous plaques were observed more frequently compared with patients with TIA and stroke (41.1% and 46.4% vs 22.6% and 22.4%; P = .001) **(Figure II and Table II)**. Accordingly, strong collagen staining was observed more frequently in asymptomatic patients and patients with AFX compared with TIA and stroke patients (34.8% and 35.7% vs 14.6% and 16.8%; P < .001) **(Figure II)**. In addition, high levels of MMP-8, MMP-9, and IL-8 correlated significantly with patients with TIA and stroke compared with those with AFX and asymptomatic patients **(Table II)**. No statistically significant differences were observed between the clinical symptoms and stainings for macrophages, smooth muscle cells, calcification, and the presence of thrombus formation.

No relation was observed between the symptom free-interval and plaque characteristics **(Figure III)**. However, a slight difference was noted in plaque characteristics in 11 patients who underwent immediate CEA compared with patients operated on at regularly scheduled points in time. The indications for these immediate procedures included repetitive TIA (n = 5), recent stroke and TIA (n = 5), or repetitive AFX (n = 1). Ninety-one percent (10/11) of these patients had a fibro-atheromatous or atheromatous plaque phenotype in contrast with 69% (272/393) of the regular operated patients (P = .12). The patient with repetitive AFX had a fibrous plaque. Eighty-two percent (9/11) of these patients had moderate or heavy macrophage-rich areas in their plaques compared with 53% (182/388) for all the patients operated on regu-

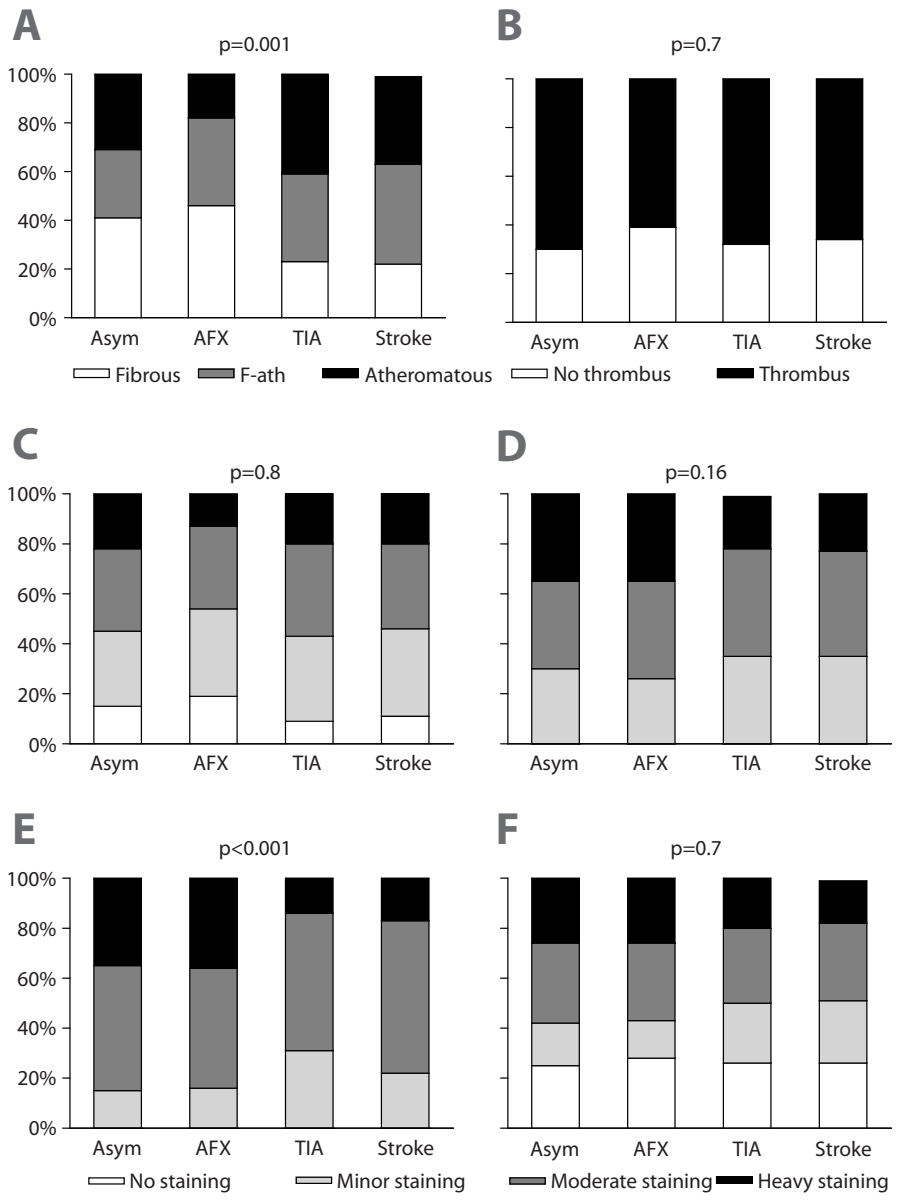


Figure II Clinical symptoms and plaque characteristics

This figure shows different plaque characteristics present at the culprit lesion, for different symptomatic categories. All data are displayed in percentages. AFX, Amaurosis fugax; TIA, transient ischemic attack. **A:** plaque overall phenotype; **B:** luminal thrombus; **C:** macrophages; **D:** smooth muscle cells; **E:** collagen; **F:** calcification.

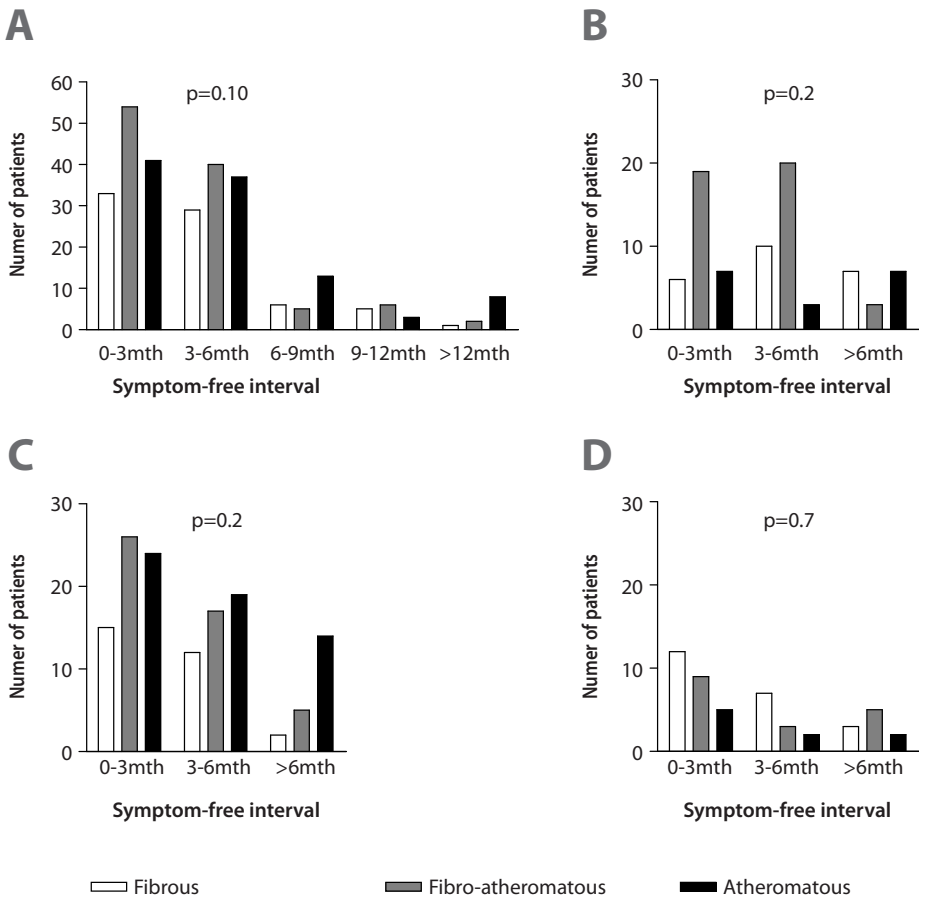


Figure III Symptom-free interval and plaque phenotype

Number of patients with specific plaque phenotype and symptom free interval. P-values indicate that no differences are observed between distributions of plaque phenotype and increasing symptom-free interval for **A:** all patients, **B:** stroke, **C:** transient ischemic attack (TIA), **D:** and amaurosis fugax (AFX), respectively.

larly (P = .05). **Figure IV** demonstrates the association between symptoms and the cumulative score for plaque vulnerability. This graph illustrates a consistent relation between histologic markers for plaque vulnerability and the prevalence of TIA and stroke (P < .001).

Discussion

Endarterectomy has been described as a safe procedure to reduce the risk of stroke in symptomatic and asymptomatic individuals who have carotid atherosclerosis. However, most asymptomatic patients will remain free of future cerebral ischemic events even if they are not operated on. Hence, most asymptomatic patients may unnecessarily become exposed to operation-related risks of CEA.[2]

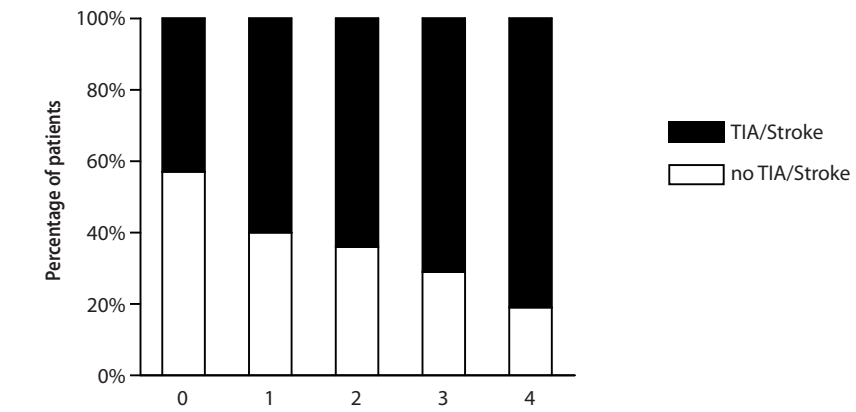
Plaque characteristics could be a useful marker in the selection of patients eligible for CEA—if plaque characteristics are correlated with clinical symptoms and future events. With the upcoming development of imaging techniques to visualize the atherosclerotic plaque, it may well become possible to characterize plaques non-invasively.

The primary findings of our study are: (1) Patients with AFX have plaque characteristics that are comparable with asymptomatic patients. (2) Asymptomatic patients and patients with AFX have a decreased prevalence of atheromatous plaques and an increased prevalence of collagen-rich plaques compared with patients who have TIA or stroke. (3) Plaques from patients with TIA or strokes have higher plaque levels of MMP-8, MMP-9, and IL-8. (4) The overall score for plaque characteristics of the vulnerable plaque increased for patients with TIA or stroke.

Asymptomatic and AFX patients reveal comparable plaque characteristics and have a decreased percentage of atheromatous plaques compared with TIA and stroke. The observed relation (increased prevalence of atheromatous plaques in symptomatic patients) is analogous to postmortem observations in coronary plaques of patients who died from a sudden coronary death.[27]

First, we hypothesize that these carotid atheromatous lesions are unstable and subsequently lead to plaque rupture and thrombosis.[28] When a plaque ruptures, embolization may occur, resulting in occlusion of an artery, arteriole, or capillary, and TIA or stroke. This hypothesis is supported by the fact that microemboli are more frequently recorded by TCD scans in symptomatic patients (53.8%) compared with asymptomatic patients (28.6%) with carotid artery stenosis.[29,30] Also in line with this hypothesis is the finding that patients with TIA and stroke have increased protease activity and IL-8 levels in their plaques. Increased protease activity and cytokine levels have been found to be related to plaque rupture.[10,31,32]

In clinical practice, AFX is also regarded to be a symptomatic disease. However, plaque characteristics differ between patients with AFX or TIA and stroke. Pathogenetically, this observed difference for AFX could be explained in two different ways. First, in patients with AFX, emboli may originate from carotid atherosclerotic plaques and be trapped in the retinal artery. These emboli must have a limited size to enter the relatively small ophthalmic artery. Considering their limited size, cerebral ischemic events are less likely to be induced by these microemboli. The fact that the retina is more sensitive to microemboli than the brain should also be con-



Plaque score items	0 points	1 point
Macrophages	no / minor	moderate / heavy
Collagen	moderate / heavy	no / minor
Smooth Muscle cells	moderate / heavy	no / minor
Fat	<10%	≥ 10%

Figure IV Plaque score model

Specific plaque characteristics based on the definition of the vulnerable plaque were given points. The percentage and number of patients with transient ischemic attack (TIA) and stroke related to received points are displayed.

sidered. This implies that a small platelet-fibrin embolus will more readily become clinically manifest in the retina than it would in the brain.[6] As we stated before, carotid atheromatous lesions are unstable and subsequently lead to plaque rupture and thrombosis.[28] It is possible that atheromatous plaques are more thrombogenic and cause larger emboli than fibrous plaques if they rupture.

Second, previous reports showed that AFX might be initiated by vasospasm.[33-35] Although carotid artery disease was excluded as a cause of AFX in these reports, it does not exclude the possibility that vasospasm-induced AFX is associated with the presence of carotid atherosclerotic disease. Carotid artery stenosis could induce a lower flow state or blood pressure drop, which makes patients more sensitive to va-

sospastic episodes. In addition, atherosclerotic disease is associated with endothelial dysfunction and impaired nitric oxide-dependent vasodilatory responses.

The current study demonstrates that more fibrous plaques are obtained from asymptomatic patients and patients with AFX compared with TIA or stroke patients. This result suggests that AFX should be considered as a separate carotid disease entity and supports the observation that patients with AFX have a better cerebral prognosis compared with patients who have TIA and stroke.[6] Pooling AFX with TIA and stroke may dilute differences in outcome variables when comparisons with asymptomatic patients are made.

No direct relation was observed between the symptom-free interval and plaque characteristics. We expected that a short, symptom-free interval in particular would be associated with a more unstable plaque phenotype compared with patients with a long symptom-free interval.[7-9,36] Although the numbers were small, we could not demonstrate a difference in plaque phenotype in relation to the symptom-free interval. A bias in this finding is a relatively short waiting list for TIA and AFX patients compared with asymptomatic and stroke patients. However, 11 patients undergoing immediate carotid surgery demonstrated differences in plaque characteristics compared with regularly scheduled patients. In this small group of patients, inflammatory plaques were more prevalent. Only the patient who suffered from repetitive AFX had a fibrous plaque.

A summation of histologic markers for plaque vulnerability (e.g., presence of atheroma, lack of collagen and smooth muscle cells, and presence of CD68-positive cells) was associated with the prevalence of TIA and stroke **(Figure IV)**. The presence of one vulnerable plaque characteristic (e.g., atheroma size) was already associated with an increased prevalence of TIA and stroke patients. Summating additional characteristics for plaque vulnerability strengthened this association with the occurrence of TIA and stroke in our population. This finding implicates a possible clinical application that needs further investigation.

Asymptomatic patients newly referred to the outpatient clinic could be screened for vulnerable plaque characteristics and followed during treatment. New imaging techniques may become available that are capable of detecting plaque characteristics associated with plaque vulnerability. For an overview of advances in vascular imaging development, we would like to refer to previously published reviews.[37]

Limitations

Plaque stainings were performed only on the culprit lesion, not on the entire plaque. To verify whether the observed relation was also evident for the total atherosclerotic plaque, we also analyzed segment four **(Figure I)** of 94 patients. Patients with TIA or stroke still revealed a significantly increased prevalence of lipid in the culprit lesion or segment four (when the segment with the strongest lipid staining

was counted) (data not shown). In this study, thrombus is only classified as present or absent. Thrombus size might well be related to clinical presentation.

Conclusions

Carotid plaque characteristics are associated with clinical symptoms. AFX and asymptomatic patients have comparable plaque characteristics in contrast with TIA and stroke patients. Plaques obtained from patients with TIA or stroke show an increased prevalence of plaques that are atheromatous and rich in MMP-8, MMP-9, and IL-8 and have a decreased prevalence of strong collagen. The results of the present study suggest that AFX differs from an etiologic point of view from TIA and stroke and that pooling these disease entities as inclusion criteria or end-point in clinical studies may induce a regression to the mean. Determining plaque characteristics by imaging techniques might be useful in the future to select patients for CEA.

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CHAPTER 5

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GENDER-ASSOCIATED DIFFERENCES IN PLAQUE PHENOTYPE OF PATIENTS UNDERGOING CAROTID ENDARTERECTOMY

Introduction Carotid endarterectomy to prevent a stroke is less beneficial for women compared with men. This benefit is lower in asymptomatic women compared with asymptomatic men or symptomatic patients. A possible explanation for this gender-associated difference in outcome could be found in the atherosclerotic carotid plaque phenotype. We hypothesize that women, especially asymptomatic women, have more stable plaques than men, resulting in a decreased benefit of surgical plaque removal.

Methods Carotid endarterectomy specimens of 450 consecutive patients (135 women, 315 men) were studied. The culprit lesions were semi-quantitatively analyzed for the presence of macrophages, smooth muscle cells, collagen, calcifications, and luminal thrombus. Plaques were categorized in three phenotypes according to overall presentation and the amount of fat. Protein was isolated from the plaques for determination of interleukin-6 (IL-6) and IL-8 concentrations and matrix metalloproteinase-8 (MMP-8) and MMP-9 activities.

Results Atheromatous plaques (>40% fat) were less frequently observed in women than in men (22% vs 40%; $P < .001$). In addition, plaques obtained from women more frequently revealed low macrophage staining (11% vs 18%; $P = .05$) and strong smooth muscle cell staining (38% vs 24%; $P = .001$). Compared with men, women had a lower plaque concentration of IL-8 ($P = .001$) and lower MMP-8 activity ($P = .01$). The observed differences were most pronounced in asymptomatic women, who showed the most stable plaques, with an atheromatous plaque in only 9% of cases compared with 39% in asymptomatic men ($P = .02$). In addition, a large proportion of plaques obtained from asymptomatic women showed high smooth muscle cell content (53% vs 30%; $P = .03$) and high collagen content (55% vs 24%; $P = .003$). All relations between gender and plaque characteristics, except for MMP-8, remained intact in a multivariate analysis, including clinical presentation and other cardiovascular risk factors.

Conclusion Carotid artery plaques obtained from women have a more stable, less inflammatory phenotype compared with men, independent of clinical presentation and cardiovascular risk profile. Asymptomatic women demonstrate the highest prevalence of stable plaques. These findings could explain why women benefit less from carotid endarterectomy compared with men.

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Introduction

Carotid endarterectomy (CEA) reduces the risk of stroke in both symptomatic and asymptomatic patients with high-grade carotid artery stenosis. The benefit of the operation in terms of stroke reduction differs among patient subgroups. Gender is a major determinant of the long-term outcome after carotid surgery.

It has been well established that CEA is more beneficial for men than for women. Carotid plaque associated stroke risk in patients on best medical treatment is higher in men than in women. After carotid surgery, stroke risk is reduced to comparable levels for both sexes, resulting in a larger reduction of stroke risk in men[1-3] This gender difference in outcome after CEA is evident in symptomatic and asymptomatic patients. Randomized trials suggest that although asymptomatic women still benefit from CEA, their benefit is smaller compared with asymptomatic men or symptomatic patients.[1,2,4]

Our understanding of these gender differences is incomplete. Different hypotheses have been raised that might account for the observed differences in outcome after carotid endarterectomy between men and women. Duplex analyses of the carotid artery before surgery have demonstrated that plaque volume is larger in men than in women at a comparable stenosis grade and that the plaque size is a predictor of clinical outcome.[5] Effects of hormones on atherosclerosis are becoming better known with increasing research,[6] but no direct pathophysiologic link has been recognized between hormones and outcome of carotid surgery.

The observed clinical differences may also be a direct reflection of carotid plaque characteristics. If women have plaques that are less prone to cause a stroke owing to distal embolization, then removal of such a plaque would be less beneficial. In coronary circulation, certain plaque characteristics are strongly associated with unstable clinical presentation. The vulnerable plaque that gives rise to myocardial infarction or unstable angina is defined as a plaque with high fat content, low structural components (thin fibrous cap, low smooth muscle cell and collagen content), high inflammatory cell content, and increased protease activity.[7-9] Recent large studies of endarterectomy specimens have shown that the pathophysiology of carotid artery disease is very similar to coronary artery disease. The vulnerable plaque characteristics known from coronary circulation have been linked to symptomatic presentation of carotid artery disease.[10-12] In addition, the association between plaque destabilization and matrix metalloproteinase-8 (MMP-8) and MMP-9 activity in carotid artery plaques has been reported.[13,14]

In this study we hypothesized that women who have been diagnosed with hemodynamically significant atherosclerotic carotid artery disease have more stable carotid plaques than men and that this is especially evident in women who are asymptomatic. This could explain the observation that CEA is less beneficial in women.

Patients and Methods

Athero-Express biobank

The Athero-Express is an ongoing longitudinal biobank study with the objective of studying the relation between plaque characteristics and the occurrence of future cardiovascular events.[15] All patients undergoing CEA in two participating Dutch hospitals are asked to participate in the study, with an inclusion rate of 94.6%. At baseline, patients complete an extensive questionnaire and blood is drawn and stored at −80°C. During CEA, the plaque is transferred to the laboratory and processed and stored according to a standardized protocol. After surgery, patients undergo duplex and clinical follow-up. The Medical Ethical Committees of the participating hospitals have approved the study, and all patients provided written informed consent. For the purpose of the current research question, we studied all consecutive patients undergoing CEA who were included in the Athero-Express study between April 2002 and November 2005.

Patient inclusion and pre-operative workup

The indication for CEA was based on the recommendations published by the Asymptomatic Carotid Surgery Trial (ACST) for asymptomatic patients and European Carotid Surgery Trial (ECST)/North American Symptomatic Carotid Endarterectomy Trial for symptomatic patients (NASCET).[2,16-18] All patients were examined by a neurologist for assessment of their preoperative neurologic status. The percentage of stenosis was determined with duplex ultrasonography, using duplex criteria as described by Strandness et al.[19,20] If the duplex investigation was not conclusive, an additional imaging technique (magnetic resonance angiography, computed tomography angiography, conventional angiography) was used to determine the level of carotid stenosis. Excluded were patients with a terminal malignancy and those who were referred back to a hospital outside The Netherlands immediately after surgery.

Baseline characteristics

Baseline data were obtained by chart review and from extensive questionnaires completed by the participating patients that included questions on history of cardiovascular disease, cardiovascular risk factors (smoking, hypertension, diabetes), and use of medication. Presenting symptoms and duplex stenosis were retrieved from patient charts. Symptom categories were asymptomatic, defined as no carotid territory ischemic symptoms; amaurosis fugax, defined as ipsilateral mono-ocular blindness of acute onset lasting <24 hours; cerebral transient ischemic attack (TIA), defined as ipsilateral focal neurologic deficit of acute onset lasting <24 hours; and

ipsilateral stroke. Lipid spectra were determined in blood specimens drawn at baseline.

Carotid Endarterectomy

CEA was performed under general anesthesia. Patients received 5000 IU heparin intravenously before cross-clamping. All endarterectomies were performed by an open, noneversion technique, with careful dissection of the bifurcation into the internal and external carotid arteries. The atherosclerotic plaque was immediately transferred to the laboratory after removal.

Plaque processing

The atherosclerotic plaque was dissected into 5-mm segments by a dedicated technician. The segment having the greatest plaque area was defined as the culprit lesion. This segment was fixated in formaldehyde 4%, decalcified for 1 week in ethylenediaminetetraacetic acid, and embedded in paraffin. The other segments were snap frozen in liquid nitrogen and stored at –80°C. Sections of 5-µm thickness were cut on a microtome for immunohistochemical staining. Plaques were characterized for macrophage content (CD68 staining), smooth muscle cell content (α-Actin staining), collagen content (Picro-sirius red), and extent of calcification (hematoxylin and eosin [HE] staining) and were analyzed semiquantitatively and scored as no, minor, moderate, and heavy staining, as reported previously.[15] Briefly, no and minor represent absent or minimal staining with few clustered cells, whereas moderate and heavy represent larger areas of positive staining. Presence of luminal thrombus (HE and elastin van Gieson staining) was scored as absent or present. The percentage of atheroma of the total area of the plaque was visually estimated using the Picrosirius red and HE stains. Three overall phenotypes were considered according to overall presentation and visual estimation of the percentage of atheroma in the plaques: fibrous plaques containing <10% fat, fibro-atheromatous, 10% to 40%; or atheromatous, >40% fat.

The scorings were done by observers blinded for patient characteristics. In addition, quantitative measurements were performed for macrophage and smooth muscle cell staining. For this purpose, images of plaque cross-sections were recorded onto a computer workstation using a microscope equipped with a digital camera. The images were captured and analyzed with AnalySIS 3.2 software (Soft Imaging System GmbH, Münster, Germany). The quantification was done as follows: in each plaque, three representative areas were defined and selected in such a way that no media (which was present in the specimen, in most cases) was included. The positive staining in these areas was measured as a percentage of total plaque area using AnalySIS software. The mean of these three measurements was used for further analysis.

Table I Baseline characteristics of patients undergoing carotid endarterectomy

	Women	Men	P-value
Number of patients	135	315	
Age, y (sd)	66.2 (9.3)	67.7 (8.5)	0.09
Hypertension	100 (74%)	211 (67%)	0.15
Diabetes	30 (22%)	62 (20%)	0.53
Prior vascular intervention	41 (30%)	128 (41%)	0.04
Prior ipsilateral carotid intervention	9 (7%)	11 (4%)	0.14
Smoking	41 (31%)	76 (25%)	0.20
Hypercholesteroleamia	68 (62%)	156 (62%)	0.96
HRT	6 (7%)		
Statin use	79 (66%)	182 (63%)	0.58
Aspirin use	106 (85%)	249 (85%)	0.90
Oral anticoagulation use	17 (14%)	48 (16%)	0.46
NSAID use	8 (6%)	12 (4%)	0.32
Cholesterol, mmol/l (sd)	5.4 (1.1)	4.9 (1.2)	0.006 *
HDL, mmol/l (sd)	1.3 (0.36)	1.1 (0.35)	0.004 *
LDL, mmol/l (sd)	3.2 (1.0)	2.9 (1.0)	0.04 *
Triglycerides, mmol/l (sd)	2.1 (1.2)	2.1 (1.0)	0.96
Symptoms			
Asymptomatic	33 (25%)	69 (22%)	
Amaurosis fugax	18 (13%)	45 (14%)	0.91
TIA	47 (35%)	118 (38%)	
Stroke	37 (27%)	83 (26%)	
Duplex stenosis			
50 - 64%	3 (2%)	9 (3%)	
65 - 89%	46 (36%)	112 (37%)	0.84
90 - 100%	79 (62%)	181 (60%)	

Abbreviations: HRT, Hormone replacement therapy; NSAID, nonsteroidal anti-inflammatory drug; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TIA, transient ischemic attack.
Categoric variables are presented as n (%); continuous variables as mean ± standard deviation.
* Statistically significant (P < .05).

Interleukin and matrix metalloproteinase measurements

The segment adjacent to the culprit lesion was used for protein isolation. This frozen segment was mechanically crushed in liquid nitrogen with a pestle and mortar. The protein isolation was done in two ways: (1) by using Tripure reagent (Boehringer Mannheim, Germany), according to the manufacturer's protocol and (2) by

Table II Comparison of carotid plaque histology between men and women

		Women	Men	P-value univar	P-value multivar[A]
Overall phenotype	fibrous	40.7%	24.4%		
(Semi-quantitative)	fibro-atheromatous	37.8%	35.2%	<0.001 *	0.006 *
	atheromatous	21.5%	40.3%		
Luminal thrombus	no	77.0%	70.6%	0.26	0.45
	yes	23.0%	29.4%		
	no	18%	10.6%		
Macrophages	minor	31.6%	33.2%	0.05 *	0.20
(Semi-quantitative)	moderate	36.8%	35.2%		
	heavy	13.5%	21%		
Macrophages	median area %	0.26	0.39	0.12	0.36
(Quantitative)	IQR	0.08 - 0.95	0.07 - 1.30		
	no	0.8%	1.9%		
Smooth muscle cells	minor	20.3%	32.9%	0.001 *	0.01 *
(Semi-quantitative)	moderate	41.4%	41.9%		
	heavy	37.6%	24.2%		
Smooth muscle cells	median area %	2.27	1.62	0.03 *	0.03 *
(Quantitative)	IQR	0.86 - 4.35	0.47 - 3.58		
	no	0.0%	0.3%		
Collagen	minor	19.4%	22.6%	0.08	0.39
(Semi-quantitative)	moderate	53.0%	57.6%		
	heavy	27.6%	19.4%		
Calcifications	no	29.6%	25.7%		
(Semi-quantitative)	minor	15.6%	24.8%	0.41	0.88
	moderate	28.9%	31.1%		
	heavy	25.9%	18.4%		

Abbreviation: IQR, interquartile range. Data presented as percentage or median (IQR), as noted.

[A]Adjusted for symptom status, age, hypertension, diabetes, smoking, prior vascular intervention, prior ipsilateral carotid intervention, and cholesterol levels.

* Statistically significant (P < .05).

dissolving in 40 mM Tris-HCl (pH, 7.5) at 4°C. Protein from 301 plaques was available for analysis. Interleukin-6 (IL-6) and IL-8 concentrations were determined in all samples. The measurements were done on the Tris-isolated samples using a multiplex suspension array system according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, Calif). MMP-8 and MMP-9 activities were measured for a randomly selected group of 133 patients in Tripure isolated protein using Biotrak activity assays RPN 2635 and RPN 2634, respectively (Amersham Biosciences, Buckinghamshire, UK). MMP activities were expressed as an arbitrary unit. All measurements were done by investigators blinded for patient characteristics.

Data analysis

The statistical software SPSS 11.5 (SPSS Inc, Chicago, Ill) was used for data analysis. Continuous baseline variables are given as mean and standard deviation. All plaque measurements are expressed as median and interquartile range. Equal distribution of baseline variables was determined using the Chi Square test for discrete variables, the Student's t test for normally distributed continuous variables and the Mann-Whitney U test for nonnormally distributed continuous variables. The Mann-Whitney U test was used for comparison of plaque stainings, IL, and MMP levels between men and women and between combined groups according to gender and symptom status. The association between gender and plaque characteristics was adjusted for traditional cardiovascular risk factors, clinical presentation, and all baseline variables showing an association ($P < .20$) with gender, using multivariate logistic regression. For this purpose ordinal variables were dichotomized into two categories (no/minor staining vs moderate/heavy staining) and continuous variables were dichotomized at the median. Values of $P < .05$ were considered statistically significant.

Results

A total of 450 carotid plaques were obtained. The baseline characteristics are given in **Table I**. Clinical presentation was equal for men and women: 25% of women and 22% of men were asymptomatic. Women (n = 135) had higher total cholesterol, accompanied by a higher low-density-lipoprotein cholesterol as well as higher high-density-lipoprotein cholesterol. Use of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins), aspirin, and oral anticoagulants did not differ between men and women. The other baseline characteristics, including duplex stenosis, were also comparable.

The plaques obtained from women demonstrated a more fibrous phenotype compared with those obtained from men. Atheromatous plaques were present in 22% of women compared with 40% of men ($P < .001$; **Table II**). In 38% of women, a heavy

smooth muscle cell staining was present, compared with 24% in men (P = .001). Heavy macrophage staining was found in 14% of women compared with 21% in men (P = .05). Luminal thrombus, calcifications, and collagen staining did not differ consistently between men and women. Assessment of lipid spectra in relation to overall plaque phenotype did not reveal any associations (data not shown).

Plaque characteristics were compared between men and women within the symptom groups (asymptomatic vs TIA/stroke). This analysis shows that differences in plaque characteristics were comparable or even more evident in asymptomatic male and female patients **(Figure I)**. Plaque overall phenotype was more atheromatous in asymptomatic men than in asymptomatic women. Atheromatous plaques were found in 9% of asymptomatic women compared with 39% of asymptomatic men (P = .02) and 44% of symptomatic men (P < .001). This difference was also evident but less prominent when symptomatic women were compared with symptomatic men (27% vs 44%; P = .003). Smooth muscle cell staining also revealed strong differences within the asymptomatic group. High staining for smooth muscle cells was observed in 53% of asymptomatic women compared with 30% of asymptomatic men (P = .03) and 20% of symptomatic men (P < .001). High collagen staining was present in 53% of asymptomatic women compared with 22% of asymptomatic men (P = .003) and 15% of symptomatic men (P < .001). No gender-related difference in collagen staining was observed in the symptomatic patient group. Macrophage staining was not significantly different between men and women within the asymptomatic or symptomatic patient group.

The differences in plaque histology between men and women were paralleled by the inflammatory and protease activity in atherosclerotic plaques **(Table III)**. Women showed lower values of the proinflammatory cytokine IL-8 compared with men (25.9 vs 51.3 pg/mL; P = .001). Levels of proinflammatory cytokine IL-6 were not different between men and women. Protease activity was lower in women, with MMP-8 showing significantly lower values than in men (4.2 vs 7.1; P = .01), whereas MMP-9 activity was lower without reaching statistical significance (1.6 vs 2.6; P = .07). These differences were still present when men and women were subdivided into symptomatic and asymptomatic groups **(Table IV)**. Asymptomatic women showed lower levels of interleukins and MMPs compared with the other groups: IL-8 levels (14.1 vs 83.3 pg/mL; P < .001), MMP-8 activity (2.6 vs 9.2; P = .003), and MMP-9 activity (1.1 vs 2.9; P = .002) were significantly decreased compared with symptomatic men.

All associations between gender and plaque phenotype, except MMP-8, which were significant in univariate analysis, were also significant when adjusting for symptom status, age, hypertension, diabetes, smoking, prior vascular intervention, prior ipsilateral carotid intervention, and cholesterol levels. This suggests that the observed gender-associated differences in plaque characteristics are not caused by differences in cardiovascular risk factors or clinical presentation.

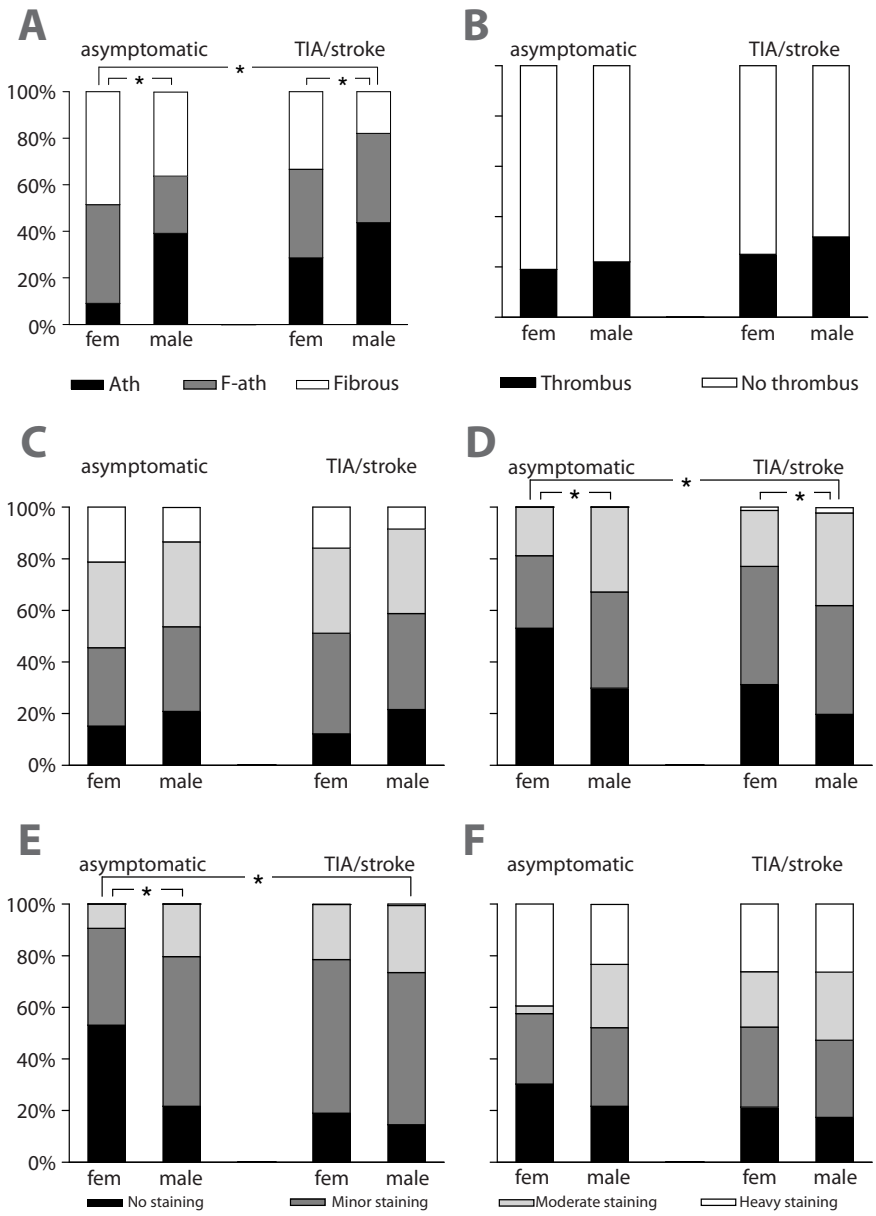

Figure I Comparison of carotid plaque histology between men and women, subdivided by symptom status: asymptomatic vs TIA/stroke

A: plaque overall phenotype; **B:** luminal thrombus; **C:** macrophages; **D:** smooth muscle cells; **E:** collagen; **F:** calcification. * $p<0.05$

Table III Interleukin and matrix metalloproteinase measurements in the plaque compared between men and women

		Women	Men	P-value univariate	P-value multivariate[A]
IL-6	median	6.7	8.8	0.2	0.89
	iqr	0.5 - 21.1	3.6 - 19.3		
IL-8	median	25.9	51.3	0.001*	0.01*
	iqr	0 - 59.3	8.8 - 147.4		
MMP-8	median	4.2	7.1	0.01*	0.34
	iqr	1.2 - 8.1	3.7 - 11.4		
MMP-9	median	1.6	2.6	0.07*	0.42
	iqr	0.9 - 3.1	0.8 - 6.4		

Abbreviations: IL, Interleukin; IQR, interquartile range; MMP, matrix metalloproteinase.
[A]adjusted for: symptom status, age, hypertension, diabetes, smoking, prior vascular intervention, prior ipsilateral carotid intervention, and cholesterol levels.
*p<0.05

Table IV Interleukin and matrix metalloproteinase measurements in the plaque compared between men and women, subdivided by symptom status

		Asymptomatic		Symptomatic		P-value	P-value	P-value
		Women	Men	Women	Men			
		1.	2.	3.	4.	1 vs. 2	3 vs. 4	1 vs. 4
IL-6	median	6.3	7.9	11.0	10.3	0.81	0.56	0.23
	iqr	1.6 - 23.4	3.6 - 12.0	1.0 - 23.8	3.9 - 23.8			
IL-8	median	14.1	17.2	41.9	83.3	0.18	0.01*	<0.001*
	iqr	0 - 31.6	0.5 - 53.6	7.5 - 90.8	25.6 - 178.7			
MMP-8	median	2.6	5.0	5.7	9.2	0.18	0.04*	0.003*
	iqr	0-6.6	2.5 - 8.5	2.1 - 8.8	4.5 - 12.7			
MMP-9	median	1.1	1.4	1.7	2.9	0.19	0.12	0.002*
	iqr	0.3 - 2.7	0.4 - 3.6	1.0 - 3.7	1.2 - 7.0			

Abbreviations: IL, Interleukin; IQR, interquartile range; MMP, matrix metalloproteinase.
* p<0.05

Discussion

The main finding of this study is that women undergoing CEA have more stable plaques compared with men. Plaques obtained from women contain less fat and macrophages and more smooth muscle cells. This is accompanied by lower IL-8 content and lower MMP-8 activity.

It has been recognized that histologic plaque characteristics are related to the clinical presentation of atherosclerotic coronary and carotid artery disease. To our knowledge, no study has reported on gender differences in carotid plaque phenotype, probably because of the number of patients required. In coronary artery disease, gender-associated differences in plaque morphology have been described that point to a higher prevalence of fresh thrombus and plaque rupture in men.[21,22] These studies did not, however, specifically address gender-related differences in plaque phenotype but focused on changes in plaque morphology in relation to clinical syndromes like acute myocardial infarction or coronary death.

Our present study indicates that women operated on for carotid artery disease show more stable atherosclerotic plaques than men. These results suggest that not just plaque volume but also plaque phenotype may be associated with adverse outcomes. Iemolo et al found that outward remodeling of the carotid artery was more evident in men than in women. [5] This outward remodeling was predictive of stroke and other cardiovascular events. Outward remodeling has previously been associated with unstable plaque characteristics, rendering the results from Iemolo et al very consistent with ours.[23]

IL-8 was significantly higher in men compared with women, but IL-6 showed no statistically significant difference. Most reduced levels of both cytokines were observed in the asymptomatic women. Both proinflammatory cytokines can be produced by a variety of cells within the atherosclerotic plaque and play an important role in atherosclerosis.[24] It remains to be elucidated whether a differential effect exists between IL-6 and IL-8, which could explain the fact that IL-8 has a stronger relation to gender and plaque instability than IL-6. In the current study, women showed significantly lower MMP-8 activity in their plaques than did men. MMP-9 was lower in asymptomatic women compared with symptomatic men. Experimental models and previous human endarterectomy series have shown that the presence of these MMPs contributes to plaque instability.[13,14] MMPs are important in cell migration, degradation of the fibrous cap, expansive remodeling, and intraplaque neovessel formation.[25] Their production can be directly inhibited by statins.[26] To our knowledge, no gender differences in MMP activity have been described in human atherosclerotic disease before. Two studies on rat aortas show higher MMP-9 production in male rat aortas compared with females, which could be partially reversed by estrogen treatment or transplantation of the artery into a female rat.[27,28] This suggests that there may be a direct effect of sex hormones

on MMP production contributing to attenuation of atherosclerotic disease in females. It is difficult to extrapolate these findings to our population because most of the women in our study cohort are postmenopausal. The observed differences in plaque phenotype could be due to estrogen exposure earlier in life resulting in a more stable plaque phenotype with inherent lower MMP-8 and MMP-9 activity compared with unstable plaques.

The gender-related differences we observed in plaque histology, inflammation, and protease activity were evident within all symptom categories. Therefore, the gender differences cannot merely be explained by different clinical presentation of the carotid artery disease; neither can they be attributed to differences in cardiovascular risk profiles, because all associations between gender and plaque characteristics, except MMP-8, remained intact when correcting for cardiovascular risk factors. The differences are most pronounced in the asymptomatic group: Among asymptomatic women, the prevalence of stable plaques is very high. Although speculative, one of the reasons for the large differences within the asymptomatic group compared with the symptomatic group is that when a plaque becomes symptomatic, some features have already changed, both in men and women. The asymptomatic group probably better represents the true gender-associated differences in plaque phenotype. Nevertheless, we also found gender-associated differences in the symptomatic patient group.

The more prevalent stable plaque phenotype found in women may explain why CEA is less effective in preventing a stroke in women. In all randomized, controlled trials of CEA, women have a lower baseline risk of stroke compared with men that is reduced to an equal level for both sexes after surgery. Thus, women benefit less from CEA then men.[1,2,16-18,29] Asymptomatic women benefit the least from CEA, as shown in the ACST and Asymptomatic Carotid Atherosclerosis Study trials.[1,2,4] Stable, fibrous plaques are less prone to cause ischemic events in the coronary and the carotid arteries.[7-12]

Our observations led us to hypothesize that removal of a stable plaque is less beneficial than removal of a vulnerable plaque, which is more frequently found in asymptomatic men and symptomatic patients. In addition, the aforementioned studies showed that women have a slightly higher perioperative risk than men, also contributing to gender differences in benefit of CEA. Interestingly, the presence of a fibrous plaque is associated with an increased amount of microembolization during CEA.[30]

Our results suggest that selecting patients for CEA on the basis of plaque characteristics may hold a promise for the future. This is especially true for patient groups with a small margin of benefit from the operation. We show that variations in plaque phenotype that exist within different patient groups are consistent with previously reported outcomes after CEA. Selection of asymptomatic women for CEA who have vulnerable, unstable plaques might improve the long-term outcome. High-risk

groups that benefit greatly from CEA, such as symptomatic men with high-grade stenosis, would probably benefit to a lesser extent from such a strategy. The new imaging techniques such as high-resolution magnetic resonance imaging, single photon emission computed tomography, and palpography may bring the potential of the observed differences in plaque level into clinical practice.[31-33]

Limitations

In the current study, we examined the segment with the largest plaque area and not the entire plaque. The rationale for this method is that the segment of the plaque with the largest plaque burden is the part with the most inflammation and the largest atheroma.[23] It has also been shown that assessment of the culprit segment is reasonably representative for the plaque as a whole.[34] In some cases, an important feature might be missed when only the culprit lesion is studied, potentially masking differences between groups. This drawback is overcome by the large number of patients in our study.

Conclusion

Women undergoing CEA have more stable carotid plaques than men, with lower fat, lower macrophage and higher smooth muscle cell content, and lower inflammatory and protease activity. This is not explained by clinical presentation and cardiovascular risk factors, suggesting an independent gender-related effect on carotid plaque phenotype of patients undergoing carotid endarterectomy. The gender-associated differences in plaque phenotype are most evident in asymptomatic women, which could explain why especially asymptomatic women have lower long-term stroke reduction after carotid endarterectomy.

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CHAPTER 6

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CAROTID ATHEROSCLEROTIC PLAQUES STABILIZE FOLLOWING STROKE

INSIGHTS INTO THE NATURAL PROCESS OF ATHEROSCLEROTIC PLAQUE STABILIZATION

Background Rupture of unstable atherosclerotic plaques is the pathological substrate for acute ischemic events. Cellular and molecular characteristics of the plaque that may induce local rupture have been studied extensively. The natural history of symptomatic plaque healing following an ischemic event is relatively unexplored.

Methods and Results Atherosclerotic carotid plaques were obtained from 804 symptomatic (stroke=204 and TIA=426) and asymptomatic (n=174) patients undergoing carotid endarterectomy. Plaques were analyzed for the presence of macrophages, smooth muscle cells (SMC), collagen, calcification and lipid core size. At protein level, mediators of inflammation (IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, IFN-γ, TNF-α), matrix degrading proteinases (MMPs) and apoptosis (caspase) were determined. We associated plaque characteristics with time elapsed between the latest event and surgery. After stroke and TIA, plaques revealed an unstable phenotype at histological and at protein level compared to plaques obtained from asymptomatic patients. Following stroke, plaques demonstrated a significant decrease of macrophage content ($p=0.02$) after 180 days, whereas SMC content tended to increase over time. At protein level, IL-6, IL-8 expression levels and caspase 3-7 activity strongly decreased within 90 days. Plaque phenotypic changes were less evident following TIA.

Conclusions Symptomatic carotid lesions are associated with an unstable plaque phenotype and remodel into more stable plaques over time following stroke. The changes in IL-6 and IL-8 and caspase preceded the decrease in macrophages following stroke. These temporal phenotypic plaque alterations should be taken into account for diagnostic and therapeutic biomarker validation studies using human atherosclerotic plaques for diagnostic and therapeutic purposes.

Arteriosclerosis Thrombosis and Vascular Biology. accepted

Introduction

Thrombosis of the vulnerable atherosclerotic plaque is the predominant pathological substrate of acute cerebrovascular and cardiovascular events such as stroke and myocardial infarction (MI). Characteristics of the so-called unstable atherosclerotic plaque, which are supposed to contribute to the initial event of plaque rupture, have been described extensively.[1-5] Current concepts, describing the histological features of the unstable atherosclerotic plaque, mainly originate from cross-sectional post mortem studies. The natural history of plaque progression and destabilization is unknown, but it has been suggested that progression of atherosclerosis is a summation of sequential repetitive events resulting in plaque stabilization and plaque destabilization. Most research on plaque progression has been focused on the cellular and molecular structure of the plaque that may precede local rupture.[2, 4-6] The alterations in plaque phenotype following a clinical event are relatively unexplored. To identify the natural history of plaque remodeling following a thrombotic event, we examined the structure of carotid endarterectomy specimen at histological and protein level in relation to the time elapsed between the most recent cerebrovascular event and surgery. For this purpose we used plaque samples and medical data from the multi center study "Athero-Express", including symptomatic and asymptomatic patients who had undergone carotid endarterectomy. Besides histological characteristics we assessed mediators that have been associated with the unstable plaque like cytokines, matrix metalloproteinases (MMP) and an apoptosis marker in 804 protein samples.[7-10] The search for therapeutic options to prevent plaque destabilization is hampered by the lack of surrogate markers of disease progression. Therapeutic and diagnostic molecular targets often require validation of expression levels in human atherosclerotic plaques. In this study we assessed to what extent the outcome of validation studies may be influenced by the time that elapsed between the clinical event and the dissection of the atherosclerotic plaque. We report that following stroke, plaques remodel into a non-inflammatory stable phenotype. The outcome of this study supports the concept that plaque stabilization and destabilization are sequential events in the progression of atherosclerotic disease.

Methods

Athero-Express Biobank

Athero-Express is an ongoing longitudinal vascular biobank study with the main objective to study the predictive value of local plaque markers as determinants for future cardiovascular events.[10] All patients undergoing carotid endarterectomy (CEA) in two participating Dutch hospitals are asked to participate in the study. The Medical Ethics Committee of both hospitals approved the study and participants

provided written informed consent. For the current research questions we have studied the atherosclerotic plaques from consecutive patients who were included between April 2002 and June 2007.

Patient inclusion

In this study 804 plaques from symptomatic (n=630) and asymptomatic (n=174) consecutive patients undergoing carotid endarterectomy (CEA) were included **(Table I)**. The indication for CEA for asymptomatic patients was based on the recommendations published by the Asymptomatic Carotid Surgery Trail (ACST) and for symptomatic patients the indication was based on recommendations based on the European Carotid Surgery Trail (ECST) and the North American Symptomatic Carotid Endarterectomy Trail (NASCET).[11-14] All patients were reviewed by the vascular surgeon or neurologist before CEA to assess the nature and timing of clinical symptoms.

Baseline characteristics

From all patients, baseline data were obtained by extensive questionnaires including history of vascular disease, cardiovascular risk factors (age, diabetes, gender, hypertension, smoking) and medication use **(Table I)**. Symptoms were categorized as; a) asymptomatic, defined as no carotid territory related ischemic symptoms, b) cerebral transient ischemic attack (TIA), defined as ipsilateral focal neurologic deficit of acute onset lasting less than 24 hours. Patients who suffered from amaurosis fugax were also included in this category, c) stroke, defined as ipsilateral acute neurologic deficit lasting more than 24 hours. Results from asymptomatic patients served as control values in comparison with data obtained from patients suffering from TIA or stroke.

Time scale

To assess changes in plaque characteristics at histological and protein levels (plaque remodeling) in relation to time following the clinical event, plaques were categorized by the time episode that elapsed between the latest event and the surgical intervention. Timing of the latest event was determined and documented by the neurologist or vascular surgeon at the outpatient clinics **(Table I)**. From 92% (n=630) of all 685 symptomatic patients who presented in one of the participating hospitals since the initiation of the Athero-Express study, the date of the last event was documented. Symptomatic patients were categorized into 4 different groups according to the delay between event and surgery (<30 days, 30 - 90 days, 91 - 180 days and >180 days). At the start of this study in 2002, symptomatic patients, presenting with TIA or stroke, could undergo CEA after more than 6 months following the initial event, due to the policies of the neurology and surgery departments at that time. All data are provided for stroke and TIA patients separately.

Plaque processing

The atherosclerotic lesions were dissected into 5 millimeter segments. The segment having the greatest plaque area was defined as the culprit lesion. This segment was fixed in formaldehyde 4%, decalcified for 1 week in ethylenediaminetetraacetic acid and embedded in paraffin. Segments adjacent to the culprit lesion were grinded in liquid nitrogen with pre-cleaned and sterilized instruments. The grinded tissue samples had been dissolved in Tripure to separate RNA from the protein samples. The samples were carefully washed out several times to release the Tripure and to dissolve the samples subsequently in 1% Sodium-Dodecyl-Sulfate. Before storage at -80°C, the total protein concentration of every sample was carefully quantified. Expressions were corrected for total amount of protein. The segment of the culprit lesion was cut into sections of 5 microns and served for histological staining. Sections were studied semiquantitatively for macrophages (CD68) and smooth muscle cells (SMC) (α-Actin), collagen (Picro-sirius red), the lipid-core size, calcification and thrombus formation (hematoxylin), which were subsequently classified as no/minor or moderately/heavily as described previously.[10] The criteria for classification were defined as follows: macrophages: **(Figure I)** (1) absent or minor CD68 staining with negative or few scattered cells; (2) moderate or heavy staining, clusters of cells with >10 cells present; smooth muscle cells (SMC): (1) minor α-Actin staining over the entire circumference with absent staining at parts of the circumference of the arterial wall; (2) positive cells along the circumference of the luminal border, with locally at least minor staining with few scattering cells; Collagen staining: (1) no or minor staining along part of the luminal border of the plaque; (2) moderate or heavy staining along the entire luminal border. Three overall phenotypes were considered according to the estimation of the percentage of lipid core size of the total

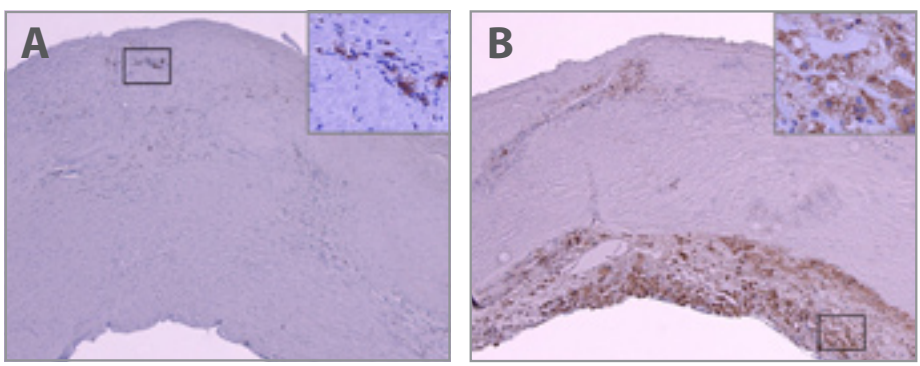

Figure I CD68 staining

A: representative histological appearance of a plaque with minor macrophage infiltration (brown). **B:** CD68 staining, representative histological appearance of a plaque with (moderate/heavy) macrophage infiltration.

plaque area: fibrous plaques containing <10% fat, fibro-atheromatous 10-40% and atheromatous >40%. Recently, we have demonstrated that the segments adjacent to the culprit lesion showed good correlations with histological characteristics, that the histological analyses were also well reproducible and revealed an acceptable inter observer agreement.[15]

For measuring changes in plaque composition at protein level, expression of proteins playing a role in inflammatory pathways or plaque destabilization, such as cytokines, matrix metalloproteinases and caspases, were determined in 568 plaques from symptomatic and asymptomatic patients (stroke, n=139; TIA, n=303 and asymptomatic, n=126). Protein level measurements were performed in a lower number of plaques compared to the histological assessments, due to a consistent delay in protein isolation following surgery, whereas histological analyses were performed immediately following the dissection. Inclusion is still ongoing and seventy percent of the protein isolations (n=568) was full filled at the time of this study. Cytokines are mediators of inflammatory pathways and contribute to lesion progression. Levels of Interleukin-2 (IL-2), IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, Interferon (IFN)-γ and Tumor Necrosis Factor(TNF)-α) were quantified in the atherosclerotic plaque by Fluorescent Bead Immunoassay (Bendermed Systems, Vienna, Austria). Activity of MMP-2, MMP-8 and MMP-9 were measured by Biotrak assays (Amersham Biosciences, Buckinghamshire, UK).[16] It has been demonstrated in multiple studies that programmed cell death or apoptosis contributes to cell death in atherosclerotic lesions. To assess the role of apoptosis in atherosclerotic plaque healing, we determined the activity of the execution protein Caspase-3 with luminescent assay (Promega Corporation, Madison, Wisconsin, USA).

Statistical Analysis

Equal distribution of baseline characteristics in relation to the time episodes between the latest event and surgery was determined by the Kruskal-Wallis test for continuous variables and by the Chi Square test for categorical variables. Protein levels were expressed as mean with the confidence interval as a measure of variance. To determine protein level differences between the different types of events within 30 days following the event and asymptomatic patients, the non-parametric Mann-Whitney U test was used. Histological plaque characteristics were categorized in two groups (no/minor and moderate/heavy) to assess regression following the event over time by using the Chi Square test (P-trend). Findings were expressed as the proportion of plaques representing a specific histological plaque feature at certain time episodes. Protein expression levels were compared for the different time episodes by the Kruskal-Wallis test (P-het). Bivariate Spearman's Correlation analysis was used for linear associations (P-trend). Data analysis was performed by using the statistical software SPSS 15.0 (SPSS Inc. Chicago, Ill).

Table I Baseline characteristics of symptomatic patients regarding episode after operation and asymptomatic patients

	Symptomatic					Asym
	<30 days	30-90 days	90-180 days	>180 days	P	
N	178	202	194	56		174
Clinical Presentation						
Stroke	21.9% (39)	31.2% (63)	42.8% (83)	33.9% (19)		
TIA	78.1% (139)	68.8% (139)	57.2% (111)	66.1% (37)		
Age, years (sd)	70.0 (9.9)	70.7 (9.0)	71.0 (9.4)	71.4 (9.4)	0.61	69.4 (8.5)
Male gender	67.4% (120)	62.4% (126)	69.5% (135)	78.6% (44)	0.11	73.0% (124)
Hypertension	70.3% (109)	77.7% (139)	73.0% (135)	61.1% (33)	0.10	78.0% (133)
Diabetes	25.9% (41)	21.4% (41)	20.9% (39)	11.6% (6)	0.14	19.0% (33)
Smoking	24.0% (35)	26.8% (46)	25.1% (45)	25.5% (14)	0.95	28.0% (47)
Hypercholester-olemia	66.0% (101)	63.3% (112)	64.8% (118)	52.8% (28)	0.37	67.0% (113)
History ipsilateral carotid intervention	3.9% (7)	1.0% (2)	2.1% (4)	5.4% (3)	0.15	9.3% (16)
History Vascular Intervention	32.9% (53)	25.3% (48)	27.7% (52)	25.5% (14)	0.42	50.6% (87)
Statin	71.9% (115)	71.7% (132)	70.2% (132)	60.4% (32)	0.41	76.0% (130)
Aspirin	88.7% (134)	89.8% (159)	90.9% (170)	90.4% (47)	0.93	37.0% (64)
Oral anticoagulants	14.6% (22)	9.0% (16)	14.4% (27)	13.5% (7)	0.37	13.5% (22)
NSAID	7.3% (11)	6.2% (11)	3.7% (7)	3.8% (2)	0.47	7.4% (12)
ACE-inhibitor	37.1% (56)	37.9% (67)	40.1% (75)	21.2% (11)	0.09	40.0% (68)

Clinical presentation and baseline table: Presence of different cardiovascular risk factors or drug use with respect to different time intervals following the cerebrovascular event. Data represent as the proportion of patients. P-values represent the differences between the different time episodes with respect to the baseline characteristics, which are determined by the Kruskal-Wallis test for continuous variables and by the Chi Square test for categorical variables.

Results

Baseline characteristics

The time interval between the latest ipsilateral event and surgery was clearly documented for all patients. Baseline characteristics for the different groups are documented in **Table I**. Plaques from patients who suffered from a TIA represented the majority of symptomatic plaques (68%, n=426) compared with stroke (32%, n=204). No differences in cardiovascular risk factors or drug use between the time categories were observed **(Table I)**. In addition, we included 174 plaques from asymptomatic patients, which served as control values.

Histological plaque characteristics early (<30 days) after stroke or TIA

Tables II and **III** show the histological plaque characteristics for stroke and TIA, in relation to the time following the ischemic events. In carotid plaques obtained from patients operated early after stroke, macrophage infiltration tended to be higher compared with asymptomatic patients (stroke 71.1% versus asymptomatic 59.8%) **(Table II, Figure II-A)**. Early after stroke (<30 days), the SMC content

Table II Interval between stroke and operation in relation to plaque morphology

	Stroke					Asym
	<30 days Proportion (number)	30-90 days Proportion (number)	90-180 days Proportion (number)	>180 days Proportion (number)	P	Proportion (number)
Lipid core >40%	39.5% (15)	31.7% (20)	38.6% (32)	26.3% (5)	0.40	24.9%* (42)
Collagen	78.9% (30)	87.3% (55)	86.6% (71)	78.9% (15)	0.18	86.3% (145)
Calcification	63.2% (24)	57.1% (36)	53.5% (44)	63.2% (12)	0.60	63.9% (108)
SMC	63.2% (24)	61.9% (39)	68.3% (56)	78.9% (15)	0.17	78%* (131)
Macrophages	71.1% (27)	68.3% (43)	63.4% (52)	36.8% (7)	0.02	59.8% (101)
Statin using patients						
Macrophages	82.1% (24/28)	70.8% (34/48)	69.8% (44/63)	33.3% (3/9)	0.03	59.8% (101)

Proportion of plaques demonstrating histological plaque characteristics in relation to different time intervals following stroke. With respect to statin using patients, the association of macrophage infiltration and the different time episodes has been incorporated. All morphological characteristics, except for lipid core, comprise moderate/heavy stainings. Statistical significance expressed by P-value is calculated by Chi Square.

* Significant difference for a certain feature early after stroke (<30 days) compared to asymptomatic patients.

was significantly lower in comparison with the asymptomatic group (63.2% vs. 78%; p=0.03) **(Table II, Figure II-B)**. Moreover, after stroke a significantly higher proportion of plaques revealed a large lipid core (39.5% vs 24.9%, p=0.03). These differences were not observed in the TIA group compared to the asymptomatic group.

Protein expression levels and activity early (<30 days) after stroke or TIA

In comparison with asymptomatic patients, plaques from stroke patients demonstrated significantly elevated levels of pro-inflammatory cytokines IL-6 (132.4 [56.1-208.8] vs. 82.9 [59.8-106.2], p=0.03) and IL-8 (838.5 [118.9-1557.9] vs 158.4 [63.5-253.3], p<0.001) when operated within 30 days after the event **(Table IV, Figure III-A,B)**. In these plaques the increased levels of IL-6 positively correlated with the expression levels of IL-2 (p=0.009 R=.558) and TNF-α (p=0.04 R=.447). Interleukin-8 did not show correlations with other pro-inflammatory cytokines.
Activity of MMP-8 (10.1[4.9-15.3] vs. 4.4 [3.6-5.1], p=0.005), MMP-9 (0.92 [0.66-1.18] vs. 0.72 [0.58-0.87], p=0.04) and caspase (12844 [8257-17430] vs. 8566 [7392-9739],

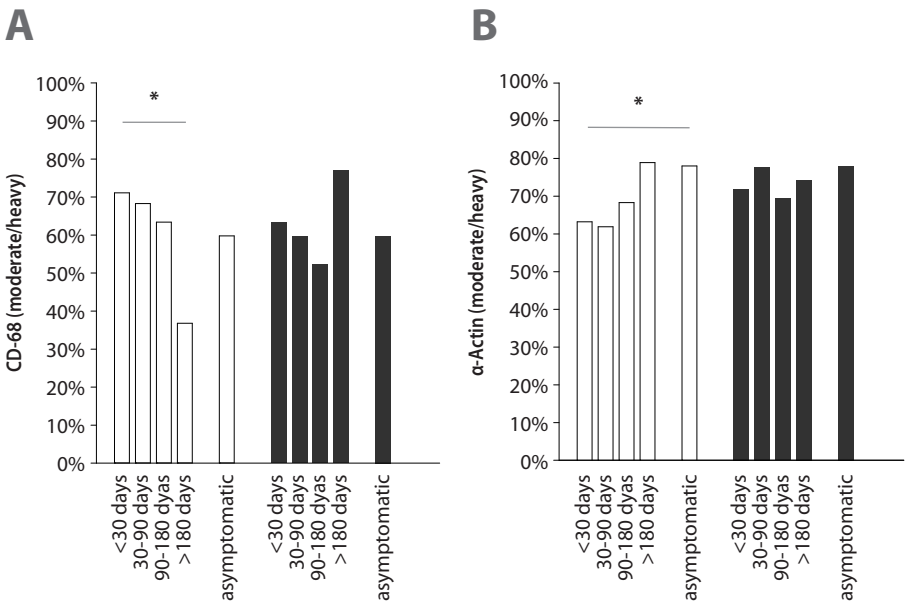

Figure II Time since event and plaque histology

White bars, TIA; Black bars, Stroke **A:** moderate/heavy macrophage (CD68) staining or **B:** moderate/heavy smooth muscle cell (α-Actin) staining in plaques following stroke or Transient Ischemic Attack (TIA) in relation to different time episodes and from asymptomatic patients (control values).
*P<0.05

p=0.02) were also significantly elevated in carotid lesions harvested from patients who had suffered from a recent (<30 days) stroke **(Table IV, Figure III-C,E,F)**.
Within 30 days after TIA, Interleukin-8 levels (301.3 [179.4-423.2] vs. 158.4 [63.5-253.3], p<0.001) and MMP-8 activity (6.4 [5.0-7.9] vs. 4.4 [3.6-5.1], p=0.004) were significantly increased compared to plaques from asymptomatic patients **(Table V, Figure III-B,E)**. Interleukin-6 expression levels were higher compared to plaques from asymptomatic patients and correlated positively with IL-2 (p=0.007 R=.297), IL-12p70 (p=0.01, R=.273), IFN-γ (p=0.04, R=.220), TNF-α (p=0.002 R=.337).

Histological plaque characteristics in relation to time following stroke or TIA

The proportion of plaques demonstrating moderate/heavy macrophage staining after stroke decreased nearly two fold over time (71.1% - 36.8%, p=0.02) and was below the control value after 180 days (asymptomatic patients 59.8%) **(Figure II-A)**. Macrophage decrease sustained over time in plaques from stroke patients who used statins pre-operatively (82.1% - 33.3%, p=0.03) **(Table II)**. Ninety days after stroke, the proportion of plaques with moderate/heavy SMC staining increased significantly (p=0.01) to the control values. These observations suggest that unstable atherosclerotic plaques remodel and stabilize over time following stroke **(Figure II-B)**.

Table III Interval between TIA and operation in relation to plaque morphology

	TIA					Asym
	<30 days Proportion (number)	30-90 days Proportion (number)	90-180 days Proportion (number)	>180 days Proportion (number)	P	Proportion (number)
Lipid core >40%	32.8% (45)	32.1% (44)	36.0% (40)	43.2% (16)	0.39	24.9% (42)
Collagen	84.4% (114)	80.7% (109)	78.7% (85)	72.2% (26)	0.04	86.3% (145)
Calcification	57.8% (78)	60.0% (81)	65.1% (71)	52.8% (19)	0.80	63.9% (108)
SMC	71.9% (97)	77.8% (105)	69.4% (75)	74.3% (26)	1.00	78% (131)
Macrophages	63.4% (85)	59.7% (80)	52.3% (57)	77.1% (27)	0.73	59.8% (101)
Statin using patients						
Macrophages	66.3% (69/104)	61.2% (63/103)	52.5% (42/80)	81.5% (22/27)	0.08	59.8% (101)

Proportion of plaques demonstrating histological plaque characteristics in relation to different time intervals following TIA. With respect to statin using patients, the association of macrophage infiltration and the different time episodes has been incorporated. All morphologic characteristics, except for lipid core, comprise moderate/heavy stainings. Statistical significance expressed by P-value is calculated by chi-square.

Table IV Interval between stroke and operation in relation to protein expression

	Stroke						Asym
	<30 days Mean [95% CI]	30-90 days Mean [95% CI]	90-180 days Mean [95% CI]	>180 days Mean [95% CI]	P-het	P-trend	
IL-2	337.4 [94.7-580.1]	238.2 [123.5-353.0]	209.6 [140.0-279.2]	231.5 [114.3-348.8]	0.96	0.63	345.6 [220.3-470.9]
IL-4	380.2 [50.3-710.2]	175.5 [94.1-256.9]	216.3 [131.4-301.2]	168.2 [52.8-283.6]	0.87	0.53	308.0 [173.8-442.1]
IL-5	484.4 [32.4-936.4]	182.3 [90.0-274.6]	214.9 [153.0-276.8]	260.2 [50.5-469.9]	0.61	0.37	338.2 [205.6-470.7]
IL-6	132.4 [56.1-208.8]	79.0 [35.1-125.2]	49.4[A] [34.3-64.5]	97.2 [23.1-171.4]	0.08	0.11	82.9 * [59.8-106.2]
IL-8	838.5 [118.9-1557.9]	146.26 [61.0-231.6]	145.44 [97.3-193.6]	75.4 [25.9-124.8]	0.001	0.001	158.4 * [63.5-253.3]
IL-10	62.1 [11.2-113.0]	37.6 [10.5-64.8]	32.8 [21.9-43.7]	39.1 [10.1-68.0]	0.90	0.68	47.8 [29.2-66.4]
IL-12p70	289.5 [50.3-528.6]	120.9 [53.4-188.5]	123.0 [82.1-163.9]	148.1 [44.7-251.5]	0.96	0.81	208.3 [135.3-281.3]
IFN-γ	248.3 [61.3-435.3]	146.2 [57.6-234.8]	132.4 [88.4-176.4]	149.4 [46.2-252.6]	0.94	0.71	167.6 [110.7-224.5]
TNF-α	51.3 [2.8-99.8]	29.6 [5.2-53.9]	21.1 [14.1-29.5]	20.8 [6.8-34.8]	0.91	0.58	33.0 [19.2-46.7]
MMP-2	4.1 [2.7-5.4]	4.1 [2.7-5.5]	3.9 [3.0-4.7]	4.2 [2.9-5.5]	0.77	0.65	4.1 [3.2-5.0]
MMP-8	10.1 [4.9-15.3]	5.3 [3.0-7.5]	6.3 [4.3-8.3]	6.4 [1.8-10.9]	0.53	0.12	4.4 * [3.6-5.1]
MMP-9	0.92 [0.66-1.18]	0.88 [0.64-1.11]	0.81 [0.64-0.97]	0.91 [0.34-1.48]	0.22	0.16	0.72 * [0.58-0.87]
Caspase 3-7	12844 [8257-17430]	9907 [7247-12568]	9482 [7757-11207]	8366 [5357-11374]	0.34	0.04	8566 * [7392-9739]

Protein levels, expressed as mean with confidence interval, related to the time intervals following stroke. Regression analysis is described by P-trend (Bivariate Spearman's Correlation) and P-het represents the correlation between different time intervals (Kruskal-Wallis). MMP and Caspase measurments are expressed as arbitrary units, other measurements are pg/µg.

[A]represents the significant decrease of IL-6 within the first 180 days (p=0.02).

* Significant difference between protein levels early after stroke (<30 days) compared to asymptomatic patients.

Table V Interval between TIA and operation in relation to protein expression

	TIA						Asym
	<30 days Mean [95% CI]	30-90 days Mean [95% CI]	90-180 days Mean [95% CI]	>180 days Mean [95% CI]	P-het	P-trend	
IL-2	278.6 [176.0-381.3]	200.7 [141.3-260.0]	333.1 [247.3-418.9]	160.2 [96.3-224.1]	0.01	0.19	345.6 [220.3-470.9]
IL-4	198.5 [117.8-279.2]	140.7 [94.6-186.8]	252.6 [188.5-316.7]	111.8 [61.3-162.4]	0.002	0.18	308.0 [173.8-442.1]
IL-5	225.3 [123.7-326.9]	180.0 [120.9-239.1]	275.0 [201.5-348.5]	123.7 [61.4-186]	0.01	0.18	338.2 [205.6-470.7]
IL-6	134.4 [70.1-198.6]	78.4 [51.1-105.7]	112.5 [35.8-189.2]	38.0 [16.0-59.9]	0.09	0.12	82.9 [59.8-106.2]
IL-8	301.3 [179.4-423.2]	218.9 [131.2-306.7]	169.6 [86.1-253.0]	163.5 [61.7-265.3]	0.05	0.03	158.4 * [63.5-253.3]
IL-10	40.5 [18.9-62.1]	20.0 [13.6-26.5]	42.0 [30.0-53.9]	18.1 [8.5-27.6]	0.01	0.28	47.8 [29.2-66.4]
IL-12p70	174.8 [87.4-262.2]	94.8 [64.2-125.4]	197.7 [138.4-257.0]	90.7 [49.0-132.4]	0.01	0.23	208.3 [135.3-281.3]
IFN-γ	165.0 [90.9-239.1]	88.3 [62.9-113.8]	181.7 [133.6-229.8]	98.5 [55.2-141.7]	0.01	0.21	167.6 [110.7-224.5]
TNF-α	22.4 [9.3-35.5]	15.9 [10.7-21.1]	23.3 [16.4-30.2]	11.7 [5.1-18.3]	0.05	0.37	33.0 [19.2-46.7]
MMP-2	3.9 [3.2-4.7]	4.8 [2.6-7.1]	3.8 [3.0-4.7]	3.2 [2.4-4.0]	0.67	0.50	4.1 [3.2-5.0]
MMP-8	6.4 [5.0-7.9]	6.0 [4.1-8.0]	5.2 [3.9-6.4]	4.8 [3.5-6.2]	1	0.14	4.4 * [3.6-5.1]
MMP-9	0.81 [0.65-0.97]	0.84 [0.66-1.03]	0.80 [0.65-0.96]	0.84 [0.55-1.14]	0.4	0.96	0.72 [0.58-0.87]
Caspase 3-7	7748 [6745-8752]	8806 [7629-9984]	7849 [6717-8981]	9019 [5975-12062]	0.43	0.90	8566 [7392-9739]

Protein levels, expressed as mean with confidence interval, related to the time intervals following TIA. Regression analysis is described by P-trend (Bivariate Spearman's Correlation) and P-het describes the correlation between different time intervals (Kruskal-Wallis). MMP and Caspase measurments are expressed as arbitrary units, other measurements are pg/µg.

* Significant difference between protein levels early after TIA (<30 days) compared to levels in asymptomatic patients.

After TIA, no decrease in macrophage content was observed over time. In the TIA group the proportion of plaques containing macrophages tended to increase after more than 180 days (52.3% vs. 77.1%, p=0.07) **(Figure II-A)**. The increase was also evident in the group of patients who used statins pre-operatively (52.5% vs. 81.5%) **(Table III)**. The proportion of plaques containing macrophages also tended to increase after more than 180 days in this group (52.5% vs. 81.5%, p=0.02). Furthermore, in the TIA group collagen content reduced over time (84.4% vs. 72.2%, p=0.04) **(Table III)**.

Protein expression levels and activity in relation to time following stroke or TIA

Following stroke, IL-6 levels strongly decreased within 90 to 180 days (132.4 [56.1-208.8] vs. 49.4 [34.3-64.5], p=0.02) **(Table IV, Figure III-A)**. IL-8 levels demonstrated an early decrease and a ten fold reduction at later time points (>180 days) (838.5 [118.9-1557.9] vs. 75.4 [25.9-124.8], p<0.001) reaching levels compared to control values of asymptomatic patients **(Table IV, Figure III-B)**. Caspase activity reduced significantly (12844 [8257-17430] vs. 8366 [5357-11374], p=0.04) **(Figure III-C)** over 180 days following stroke, and resulted in nearly similar activity in comparison with asymptomatic patients (8566 [7392-9739]). Surprisingly, matrix metalloproteinase activity did not show significant alterations over time, although MMP-8 activity revealed a trend towards reduction within the first 180 days following stroke (10.1 [4.9-15.3] vs. 6.3 [4.3-8.3], p=0.06).

In plaques from patients who suffered from TIA, IL-8 levels decreased over 180 days following TIA (301.3 [70.1-198.6] vs. 163.5 [61.7-265.3], p=0.03) **(Table V, Figure III-B)**. Other interleukin levels and MMP- or caspase activity barely demonstrated consistent changes following the event. However, temporal fluctuations were observed in interleukin expression levels (IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, IFN-γ and TNF-α) in the TIA group. These changes were not consistent over time, but elevated levels were mainly observed 90-180 days following the event **(Table V)**.

Discussion

Progression of atherosclerosis is a process with repetitive events including cellular and molecular alterations leading to plaque stabilization or plaque destabilization, which may eventually result in acute cerebrovascular and cardiovascular events. The natural history of atherosclerotic plaque stabilization and destabilization is not completely understood. We studied the temporal sequence of carotid plaque remodeling at histological and protein level after ischemic cerebrovascular events.

In this study, we demonstrate that the atherosclerotic carotid plaques from symptomatic patients reveal an unstable phenotype early after the acute ischemic event, which is in line with previous observations.[1, 3, 17] To determine the natural history

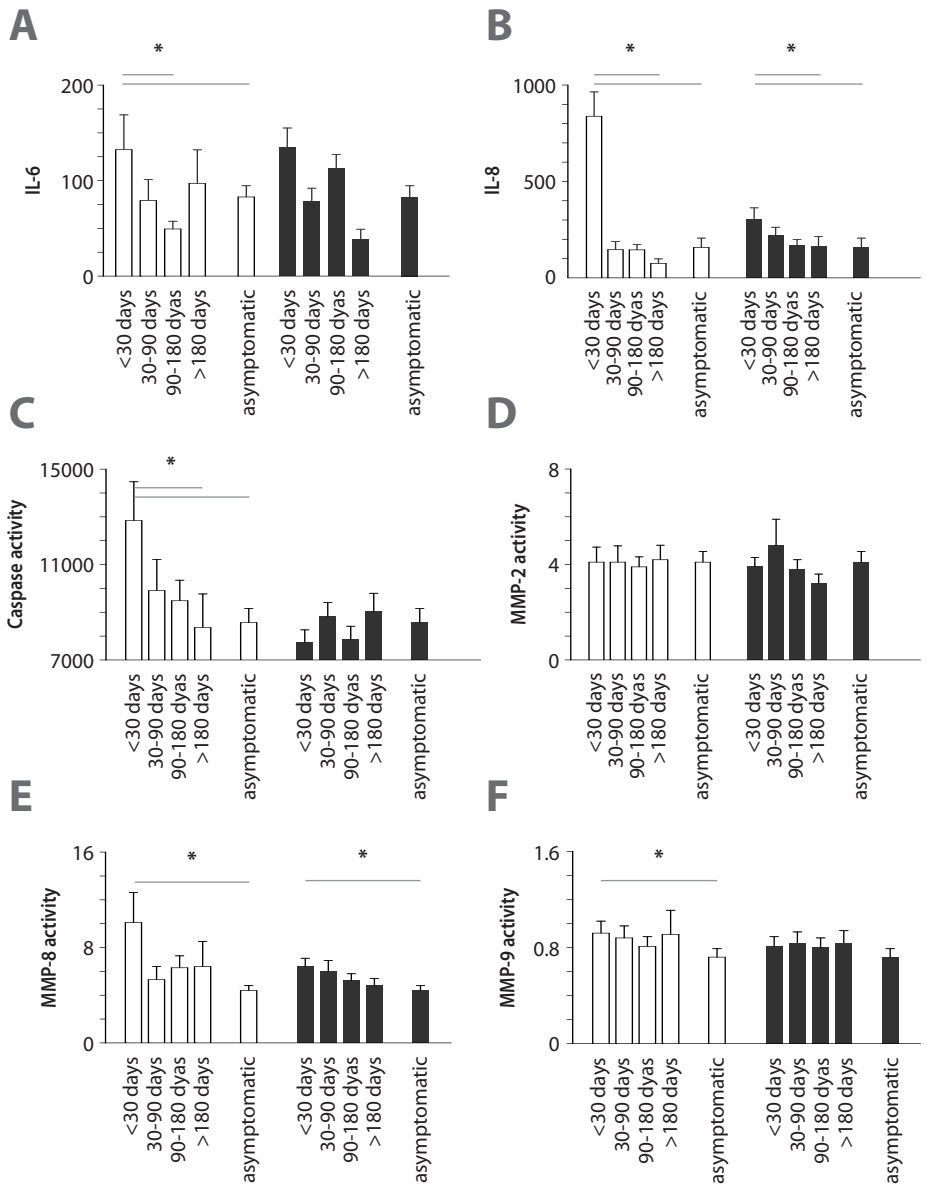

Figure III Protein expression levels in relation to time since event

White bars, TIA; Black bars, Stroke **A:** IL-6 expression levels **B:** IL-8 expression levels and **C:** Caspase-3 activity **D:** MMP-2 activity **E:** MMP-8 activity and **F:** MMP-9 activity in relation to time following stroke or TIA. Protein levels and activity are expressed as mean values (+/- SEM). *P<0.05

of plaque progression following a cerebrovascular event, the plaque phenotype has been associated with different time episodes between the latest ischemic event and surgery. Following stroke, plaques reveal a significant reduction of macrophage content in relation to time and a clear trend of increasing numbers of smooth muscle cells. Plaques from patients who suffered from a TIA did not demonstrate pathohistological changes with increasing time intervals. These findings support previous observations from Redgrave and colleagues, who also suggested significantly reduced macrophage infiltration in relation to time following stroke.[18] In the aforementioned study of Redgrave et al. conclusions regarding the first time episode were drawn on a limited number of plaques. However, this study comprised a large number of samples, which were equally distributed over the different episodes resulting in a sufficiently powered study.

In the current study we also determined plaque protein expressions as a fingerprint of the inflammatory status in relation to time following an event. We showed that IL-6 and IL-8 levels and caspase activity were significantly increased in lesion harvested early after the initial events compared to asymptomatic lesions, which indicates that symptomatic lesions demonstrate an increased vulnerable plaque phenotype. These cytokine expression levels and caspase activity altered subsequently over time in atherosclerotic carotid plaques following stroke or TIA. IL-6 and IL-8 are expressed in atherosclerotic tissue and have pro-inflammatory properties by activating and recruiting inflammatory cells and stimulating matrix degrading enzymes, which contribute to plaque instability and lead to cardiovascular events.[19-24] Decrease of these interleukin levels occurred early following stroke, suggesting that plaque composition alters very early after the initial event towards a more stable plaque phenotype. The remarkable increase of cytokine levels after more than 90 days following TIA, may point to a different pathophysiology compared to stroke and reflects the clinical recurrent pattern of cerebrovascular events after 90 days as described by Johnston et al.[25]

Apoptosis of macrophages and smooth muscle cells is another prominent feature in atherosclerotic tissue and is associated with plaque destabilization.[7, 9, 26-28] Macrophages contribute to the majority of apoptotic cells within the plaque.[27] Caspase-3 can be considered as an apoptosis marker since it plays a key role in the apoptotic pathways.[29] The results of this study indicate that the activity of the execution caspase-3 strongly reduced after stroke, which also points to alterations towards a more stable plaque phenotype.

The mechanisms for plaque stabilization following acute stroke or TIA remain to be investigated. The observed phenotypic changes may reflect a normal response to injury, where inflammation decrease precedes smooth muscle cell migration, matrix deposition and tissue remodeling.

However in conditions of high oxidative stress like atherosclerosis, Nitric Oxide (NO) and peroxynitrite induce apoptotic cell death. Apoptosis in atherosclerotic plaques

is mainly present in advanced atherosclerotic tissue containing numerous foam cells and macrophages.[30, 31] Macrophages contribute to the majority of apoptotic cells and express high levels of inducible Nitric Oxide Synthase (iNOS) and nitrotyrosine as a footprint of peroxynitrite in hypoxic conditions. Under these circumstances, NO targets DNA and induces oxidative damage resulting in oxidative modification and deamination of DNA.[32] DNA damage may lead subsequently to apoptotic cell death and decreased macrophage concentrations. The decrease in caspase activity following stroke may be a result from decreased apoptosis over time.

Besides inflammation markers and mediators of apoptosis, the current study has examined the course of MMP activity following ischemic events within the atherosclerotic plaque. Matrix metalloproteinases are associated with degradation of the extracellular matrix and thereby with plaque destabilization.[8] Macrophages in the atherosclerotic plaque are a major source of matrix metalloproteinases. We assessed the MMP-2, MMP-8 and MMP-9 activity in the atherosclerotic plaque in relation to time. Although macrophage infiltration decreased over time, MMP-levels did not change following stroke. A reasonable explanation is that MMPs can also be produced by other cell types like SMC, which tended to increase with time following stroke, thereby balancing the macrophage related effects on MMP levels. Furthermore, it has been suggested that MMPs are associated with SMC migration suggesting that gelatinases play a role in lesion stabilization.[33]

Patient characteristics and drug use were associated with the different time intervals to determine possible confounding effects. No differences between the groups have been observed indicating that the possible confounders such as preoperative drug use could not explain the results.

The pleiotropic immune-modulatory properties of statins have gained much attention in the last decade.[34] Besides their primary lipid lowering effect, statins may exert anti-inflammatory effects in cardiovascular patients. Additional analyses with respect to patients who used statins pre-operatively in the stroke and TIA group, demonstrated that the decrease of macrophage infiltration over time was still present. It has already been described that biomarkers, such as plasma derived IL-6 and IL-8, have predictive value for future cardiovascular events.[21, 22, 35, 36] Researchers and pharmaceutical companies use human atherosclerotic plaques to validate expression levels of proteins of interest. Carotid plaques are of specific interest since these are plaques originating from patent vessels and regularly obtained in patients who are still alive. The Athero-Express study has been initiated to examine local plaque biomarkers that are predictive for local and systemic progression of atherosclerotic disease. Recently, we have shown that local inflammatory lipid rich plaques are associated with lower restenosis rates following endarterectomy.[37] The current study indicates that inflammatory proteins (IL-6 and IL-8) are associated with clinical presentation and reveals expression patterns that rapidly change over time in atherosclerotic plaques following an event. Insight in temporal changes in protein

expression, in plaques from patients who suffered from stroke or TIA, is relevant for therapeutic and diagnostic research. For instance, levels of diagnostic biomarkers for prediction of secondary manifestations of cardiovascular disease should ideally remain stable over time following an acute event.

Limitations of the current study

Our observations may have been influenced by cardiovascular risk factors or drug use. Patients in the >180 days-group were included in the first phase of the Athero-Express study. We have looked at variations in drug use and risk factors over time and did not observe differences between patient groups. Therefore we may conclude that drug use is not a confounding factor.

The current study examined protein levels in a lower number of atherosclerotic plaques compared with histology. However, the selection of patients for protein expression has been unbiased. Therefore, we feel that this large sample can be considered representative for the total cohort. Histological stainings of inflammatory cytokines IL-6 and IL-8 were not successful. We were not able to correlate macrophage presence with cytokine expression within the atherosclerotic plaque. Therefore, we cannot make inferences regarding the association between the decrease of cytokine expression and the decreased macrophage presence. The retrospective aspect of this study weakens the strength of our observations. Ideally prospective studies would be needed to reproduce these observations, for instance by monitoring the unstable plaque with MRI or CT-Pet fusion techniques. Nowadays, it would be complicated to execute such a study, since symptomatic patients are being operated much faster following the event.[38]

Conclusion

This study demonstrates that the delay between the latest onset of stroke and surgery is significantly associated with the macrophage content of the plaque and a decrease of IL-6 and IL-8 levels and caspase activity. These observations suggest that plaque stabilization and destabilization are sequential events in atherosclerotic disease progression. Carotid plaques are frequently used for drug and biomarker target validation. Based on these observations we suggest that these validation studies merit careful consideration if the temporal aspect of plaque remodeling has not been taken into account.

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PART 3

THE CAROTID ATHEROSCLEROTIC PLAQUE AND RESTENOSIS

CHAPTER 7

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ATHEROSCLEROTIC PLAQUE COMPOSITION AND OCCURENCE OF RESTENOSIS AFTER CAROTID ENDARTERECTOMY

Context Previous studies have assessed the predictive value of clinical and angiographic parameters for development of restenosis after vascular interventions. The composition of the atherosclerotic plaque at the intervention site has not been evaluated as a marker for restenosis.

Objective To investigate the relationship between atherosclerotic plaque histology and the occurrence of restenosis after carotid endarterectomy.

Design, Setting, and Patients The Athero-Express study is a longitudinal vascular biobank study that includes the collection of atherosclerotic plaques of patients undergoing primary carotid endarterectomy. Five hundred patients were prospectively followed up between April 1, 2002, and March 14, 2006, to assess carotid artery restenosis measured by duplex ultrasound 1 year after the intervention.

Main Outcome Measures Risk of carotid restenosis in relation to predefined histological characteristics (macrophage and smooth muscle cell infiltration, collagen, calcifications, intraplaque bleeding, luminal thrombus, and lipid core size), adjusted for clinical characteristics (multivariate logistic regression analysis).

Results At 1 year, 85 patients (17%) developed 50% or greater restenosis, including 40 patients (8%) who developed 70% or greater restenosis of the target vessel. Patients whose histological examination of the plaque revealed marked macrophage infiltration (n = 286) had a lower risk than those with none or minor macrophage infiltration (n = 214) of developing 50% or greater restenosis (risk difference, 11.5% vs 24.3%; adjusted odds ratio [OR], 0.43; 95% confidence interval [CI], 0.26-0.72) and a lower risk of developing 70% or greater restenosis (risk difference, 4.5% vs 12.6%; adjusted OR, 0.36; 95% CI, 0.17-0.74). Patients (n = 177) with a plaque having a large lipid core size (>40%) had a lower risk than those (n = 94) with a plaque having a lipid core size of less than 10% of developing 50% or greater restenosis (risk difference, 11.3% vs 25.5%; adjusted OR, 0.40; 95% CI, 0.19-0.81) and a lower risk of developing 70% or greater restenosis (risk difference, 5.6% vs 14.9%; adjusted OR, 0.42; 95% CI, 0.17-1.04), independent of clinical characteristics.

Conclusions Plaque composition is an independent predictor of restenosis after carotid endarterectomy. The dissection of a lipid-rich, inflammatory plaque is associated with reduced risk of restenosis.

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Introduction

Restenosis is a drawback of catheter-based and surgical interventions in different vascular territories. In coronary and peripheral artery disease, diabetes has been associated with increased development of restenosis.[1-4] In addition, following carotid artery interventions, smoking, age, and female sex have been reported as independent predictors of luminal renarrowing.[5-8] Furthermore, angiographic lesion characteristics, such as lesion length and decreased vessel diameter, are associated with increased risk of restenosis.[9,10]

In contrast with established risk factors and angiographic parameters, the relationship between local composition of the vessel wall and development of restenosis has not been explored. Among many other factors, restenosis largely depends on local smooth muscle cell proliferation and migration, protease activity, and matrix deposition occurring at the lesion site after the intervention has been performed. The mechanisms underlying formation of neointima and geometrical vascular remodeling are influenced by the local homeostatic milieu.[11,12] Therefore, it is reasonable to assume that factors related to the composition of the atherosclerotic lesion at the intervention site may be associated with restenosis development. Studies on atherosclerotic plaque composition in relation to restenosis are limited by their retrospective nature, because histology of the restenotic lesions was investigated rather than the characteristics of the primary target lesion in relation to future restenosis.[13-16]

The lack of prospective evidence relating plaque characteristics with restenosis rate led to the design of the Athero-Express study, which is a longitudinal vascular biobank study coupled with clinical follow-up and duplex follow-up for restenosis.[17] Our primary goal in this study was to investigate the relationship between plaque histology at baseline and restenosis during follow-up among patients who were undergoing primary carotid endarterectomy and longitudinally studied for restenosis development.

Methods

Athero-Express biobank

The design of the Athero-Express study has been reported previously.[17] Briefly, carotid plaques of patients undergoing primary carotid endarterectomy were collected and subjected to histological examination. Patients underwent duplex follow-up to assess patency of the target vessel at 1 year and clinical follow-up at 1 to 3 years after surgery. All patients who underwent primary carotid endarterectomy at 1 of the 2 participating hospitals were asked to participate. The medical ethics boards of both participating hospitals approved the study and all patients

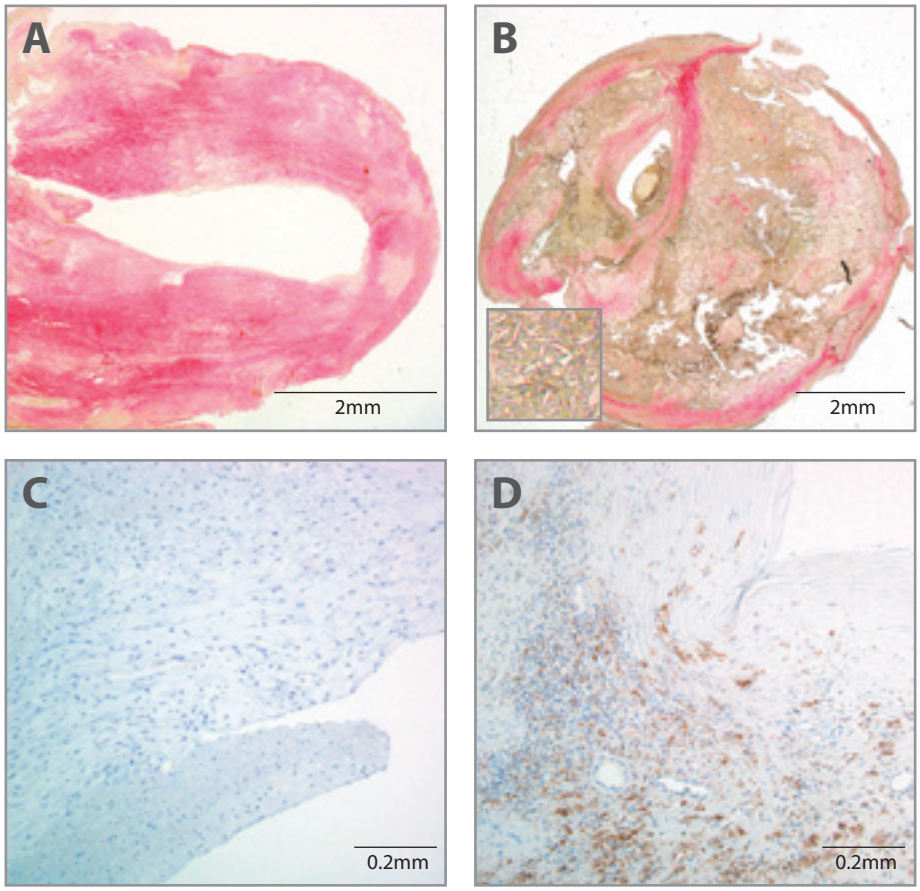

Figure Representative Histology

A: Picro-Sirius Red collagen staining, original magnification 20x. Representative histological appearance of a fibrous plaque with no lipid core. **B:** Picro-Sirius Red collagen staining, original magnification 20x. Representative histological appearance of a plaque with a large lipid core (>40% of plaque area). The inset shows a higher magnification of the same plaque, which reveals the cholesterol crystals in the lipid core. **C:** CD-68 staining, original magnification 100x. Representative histological appearance of a plaque with minor macrophage infiltration. **D:** CD-68 staining, original magnification 100x. Representative histological appearance of a plaque with substantial (moderate/heavy) macrophage infiltration (brown). CD-68 staining was performed using diaminobenzidine (DAB) substrate as chromagen and hematoxylin as counterstaining.

provided written informed consent. The study was initiated on April 1, 2002. Based on a power calculation, restenosis incidence of 10% vs 20%, 80% power, and α = .05,[17] we estimated that a sample size of 500 patients would be sufficient to investi-

Table I Clinical Characteristics of the study population

Characteristics [A]	All patients		Luminal Renarrowing			
	(n=500)		<50% (n=415)		≥50% (n=85)	
Age, mean (range), y	67.7	(40.1-87.8)	67.9	(40.1-87.8)	66.5	(44.9-83.3)
Sex						
female	140	(28)	115	(28)	25	(29)
male	360	(72)	300	(72)	60	(71)
Current smoker	126	(26)	108	(27)	18	(22)
Diabetes	82	(16)	68	(16)	14	(17)
Hypertension	309	(62)	259	(63)	50	(59)
Hypercholesterolemia	284	(57)	229	(55)	55	(66)
Body mass index, mean (SD)	26.5	(4.11)	26.6	(4.20)	26.4	(3.52)
Serum creatinine, mean (SD)	1.10	(0.37)	1.10	(0.39)	1.12	(0.28)
Statin use	352	(72)	288	(70)	64	(77)
Aspirin use	444	(90)	370	(91)	74	(89)
Oral anticoagulant use	66	(11)	56	(14)	10	(12)
Bilateral carotid stenosis	207	(42)	170	(41)	37	(45)
Clinical presentation						
Asymptomatic	110	(22)	84	(20)	26	(30)
Transient ischemic attack	278	(56)	234	(57)	44	(52)
Stroke	112	(22)	97	(23)	15	(18)

SI conversion factor: To convert serum creatinine (mg/dL) to µmol/L, multiply by 88.4.

[A] Data are presented as No. (%) unless otherwise indicated. Body mass index was calculated as weight in kilograms divided by height in meters squared.

gate the relationship between plaque histology and the occurrence of restenosis. The target of 500 one-year duplex follow-ups was reached on April 9, 2007.

Patient population

All patients undergoing primary carotid endarterectomy between April 1, 2002, and March 14, 2006, at 1 of the 2 participating hospitals were considered for inclusion in the Athero-Express study. The criteria to perform carotid endarterectomy were based on the recommendations by the Asymptomatic Carotid Atherosclerosis Study and Asymptomatic Carotid Surgery Trial studies for asymptomatic patients and the North American Symptomatic Carotid Endarterectomy Trial and European Carotid Surgery Trial studies for symptomatic patients.[18-20] All patients were evaluated by a neurologist before primary carotid endarterectomy to document the cerebrovascular symptom status.[21] At baseline, clinical parameters including cardiovascular risk

factors and medication use were recorded. Peripheral vascular disease was defined as intermittent claudication as defined by the Edinburgh questionnaire.[22] Coronary artery disease was defined as angina pectoris as defined by the Rose questionnaire, or history of myocardial infarction or coronary intervention.[23]

Carotid endarterectomy

Before cross-clamping of the carotid artery, 5000 units of heparin was administered intravenously. The choice of closure method of the arteriotomy was at the discretion of the surgeon and could be either primary closure or patch closure with a Dacron or vein patch.

Atherosclerotic plaque characterization

Directly after excision, the atherosclerotic plaque specimen was taken to the laboratory. The plaque was divided in segments of 5-mm thickness along the longitudinal axis. The segment with the greatest plaque burden was subjected to histological examination.[17] Macrophage infiltration (CD-68), smooth muscle cell infiltration (alpha-actin), the amount of collagen (Picro-sirius Red) and calcification (hematoxylin and eosin) were semiquantitatively scored as (1) none or minor or (2) moderate or heavy staining. The criteria for classification were defined as follows: for macrophages: (1) absent or minor CD-68 staining with negative or few scattered cells or (2) moderate or heavy staining, clusters of cells with more than 10 cells present; for smooth muscle cells: (1) minor alpha-actin staining over the entire circumference with absent staining at parts of the circumference of the arterial wall or (2) positive cells along the circumference of the luminal border, with locally at least minor staining with few scattering cells; and for collagen staining: (1) none or minor staining along part of the luminal border of the plaque or (2) moderate or heavy staining along the entire luminal border. Luminal thrombus and intraplaque bleeding were examined in hematoxylin and eosin and Elastin von Gieson stainings and rated as being absent or present.

The size of the lipid core was visually estimated as a percentage of total plaque area using hematoxylin and eosin and Picro-sirius Red stains, with a division in 3 categories of less than 10%, 10% to 40%, and more than 40%. The histological examination was performed by 2 independent observers, who were blinded for clinical data. The interrater and intrarater reproducibility was assessed in 100 specimens. Briefly, 100 specimens were assessed by 2 independent observers and the ratings of both observers were compared with κ statistics. To assess intraobserver reproducibility, the second observer reassessed the specimens 2 months afterwards with blinding for the previous assessments of the plaques. Both interobserver and intraobserver reproducibility were found to be excellent ($\kappa = 0.6\text{-}0.9$).[24]

In protein isolated from adjacent plaque segments, interleukin (IL-8) levels were quantified by Fluorescent Bead Immunoassay (Bendermed, Austria).

Follow-up

All patients underwent follow-up with duplex ultrasound (Philips Medical Systems, Eindhoven, the Netherlands) 1 year after carotid endarterectomy. The occurrence of 50% or greater restenosis at the ipsilateral bifurcation was defined as a peak systolic velocity of at least 125 cm/s. Restenosis of 70% or greater was defined as a peak systolic velocity of more than 230 cm/s.[25] Reocclusion was defined as the absence of flow with duplex, which was confirmed by another imaging modality (magnetic resonance angiography or angiography). Restenosis was assessed 1 year after the index procedure.[26] Duplex measurements were performed by investigators who were blinded for data regarding plaque phenotype and baseline characteristics.
Clinical end points at 1 year included incidence of stroke, myocardial infarction, and all-cause mortality. Transient ischemic attack was not included as a clinical end point.

Ultrasound substudy to investigate remodeling and neointima formation during follow-up

We also performed a substudy in which carotid intima-media thickness was assessed in 50 patients who were included in the Athero-Express study between June 3, 2004, and January 1, 2006, and who underwent follow-up in 1 of the 2 centers where the carotid endarterectomy had been performed. The caliber of the vessel and the thickness of the intima-media complex were measured with B-mode ultrasound (7.5 MHz linear array transducer, Philips Medical Systems) preoperatively and 1 year after carotid endarterectomy. The carotid bifurcation was insonated such that optimal images were obtained along the longitudinal axis of the vessel. Still images were captured and calibrated for measurement of carotid intima-media thickness and vessel caliber (AnalySIS version 3.2, Soft Imaging GmbH, Münster, Germany).
The carotid intima-media thickness was measured at the near wall and far wall and averaged.[27] The vessel diameter was defined as the distance from the adventitial edge of the intima-media complex of the near wall to the adventitial edge of the intima-media complex at the far wall. Three measurement sites were chosen as a representation of the endarterectomy area: the carotid bifurcation, the proximal internal carotid artery exactly 1 cm distal to the bifurcation, and the distal common carotid artery exactly 1 cm proximal to the bifurcation. The resulting measurements of carotid intima-media thickness and vessel caliber at the 3 sites were then averaged.

Data analysis

SPSS version 15.0 (SPSS Inc, Chicago, Illinois) was used for all statistical analyses. The discrete variable clinical presentation was rearranged as nominal variables to

obtain 2 groups (symptomatic [transient ischemic attack and stroke] vs asymptomatic). Cross tables (2 x 2) were constructed to calculate the relative risk (RR) and risk difference with 95% confidence intervals (CIs) for the development of restenosis (≥50% and ≥70% separately) for each clinical and plaque characteristic. The accompanying P-value was calculated with the Chi-square statistic. Continuous variables were additionally compared between patients with and without restenosis during follow-up with the Mann-Whitney U test, to confirm the result of the Chi square test on the dichotomized variables.

The univariate analysis including baseline parameters served as the basis for a multivariate logistic regression model. Two models were constructed: 1 model with 50% or greater restenosis as the dependent variable (model 1) and 1 model with 70% or greater restenosis, which is a subgroup of the group with 50% or greater stenosis, as the dependent variable (model 2). Variables showing association ($P < .10$) with restenosis (either ≥50% or ≥70%) in univariate analysis were included in the multivariate analysis. Age, sex, and clinical presentation (symptomatic vs asymptomatic) were included and retained in all multivariate models. The other variables were retained in the multivariate model based on the likelihood ratio (threshold: $P = .10$), with stepwise removal of nonsignificant variables. The adjusted odds ratios with 95% CIs are given for all variables in the final models. The models were characterized by the area under the receiver operating characteristic curve, with 95% CIs (0.5 = no predictive value; 1.0 = perfect prediction).[28] The substudy patient group (n = 50) was divided in 2 groups based on the absence or presence of histological features (macrophages, lipid). Between these groups, differences in vessel diameter and carotid intima-media thickness with 95% CIs were calculated.

Results

Among 685 patients considered for inclusion in the study, 81 were excluded due to malignant disease, permanent extramural care, residence outside the Netherlands, and follow-up conducted at another hospital. An additional 39 patients did not undergo follow-up because of all-cause mortality, cerebral bleeding, or stroke within 1 year. Sixty-five patients were considered lost to follow-up (9.4%). The baseline characteristics of the study population (n = 500) are shown in **Table I**. The patients who did not have follow-up were older than the patients included in the study (mean age: 69.2 vs 67.2 years; 95% CI of the difference, 0.30-3.34) and more frequently demonstrated intraplaque bleeding (66% vs 58%; $P = .04$), whereas the other baseline and plaque characteristics did not differ.

The overall incidence of 50% or greater restenosis of the target vessel 1 year after carotid endarterectomy was 17%, including 8% with 70% or greater restenosis, of whom 3% had occlusion of the target vessel. Two clinical parameters associated with

Table II Clinical characteristics at baseline vs risk of restenosis development development during follow-up

Patient characteristics	Restenosis (≥50%)		Restenosis (≥70%)	
	RD [95% CI] / AR [A]	P	RD [95% CI] / AR [A]	P
Age, y	-5.3% [-11.8%, +1.3%]	0.12	-1.8% [-6.5%, +3.0%]	0.47
< 70	57/298 (19.1%)		26/298 (8.7%)	
≥ 70	28/202 (13.9%)		14/202 (6.9%)	
Sex	-1.2% [-8.6%, +6.2%]	0.75	0.2% [-5.1%, +5.5%]	0.94
Female	25/140 (17.9%)		11/140 (7.9%)	
Male	60/360 (16.7%)		29/360 (8.1%)	
Current smoker	-3.8% [-11.1%,+3.5%]	0.33	-4.4% [-9.2%, +0.3%]	0.11
No	65/359 (18.1%)		33/359 (9.2%)	
Yes	18/126 (14.3%)		6/126 (4.8%)	
Diabetes	0.2% [-8.7%, - 9.1%]	0.97	2.0% [-4.9%, 8.9%]	0.54
No	70/414 (16.9%)		32/414 (7.7%)	
Yes	14/82 (17.1%)		8/82 (9.8%)	
Hypertension	-1.8% [-8.7%, - 5.0%]	0.50	1.0% [-3.8%, 5.9%]	0.69
No	35/189 (18.5%)		14/189 (7.4%)	
Yes	50/309 (16.2%)		26/309 (8.4%)	
Hypercholesterolemia	5.8% [-0.7%, +12.3%]	0.09	3.4% [-1.2%, +8.1%]	0.16
No	29/214 (13.6%)		13/214 (6.1%)	
Yes	55/284 (19.4%)		27/284 (9.5%)	
Body mass index	0.4% [-7.0%,+7.8%]	0.92	1.5% [-3.9%, +6.8%]	0.60
< 25	29/164 (17.7%)		13/164 (7.9%)	
≥ 25	50/277 (18.1%)		26/277 (9.4%)	
Serum creatinin, mg/dl	3.0% [-3.7%,+9.6%]	0.38	1.4% [-3.4%, +6.2%]	0.58
< 1.0	34/222 (15.3%)		16/222 (7.2%)	
≥ 1.0	49/268 (18.3%)		23/268 (8.6%)	
Statin use	4.6% [-2.3%, +11.6%]	0.22	2.1% [-2.9%, +7.1%]	0.44
No	19/140 (13.6%)		9/140 (6.4%)	
Yes	64/352 (18.2%)		30/352 (8.5%)	
Aspirin use	-2.1% [-13.7%, +9.5%]	0.71	-2.8% [-11.7%,+6.2%]	0.50
No	9/48 (18.8%)		5/48 (10.4%)	
Yes	74/444 (16.7%)		34/444 (7.7%)	

Patient characteristics	Restenosis (≥50%)		Restenosis (≥70%)	
	RD [95% CI] / AR [A]	P	RD [95% CI] / AR [A]	P
Oral anticoagulant use	-2.2% [-11.6%,+7.1%]	0.69	1.3% [-6.0%, +8.7%]	0.71
No	74/426 (17.4%)		33/426 (7.7%)	
Yes	10/66 (15.2%)		6/66 (9.1%)	
Bilateral carotid stenosis	1.9% [-4.8%, +8.6%]	0.58	0.9% [-3.9%, +5.7%]	0.70
No	46/288 (16.0%)		21/288 (7.3%)	
Yes	37/207 (17.9%)		17/207 (8.2%)	
Clinical presentation[B]	-8.5% [-17.2%, -0.2%]	0.04	-6.1% [-12.8%, -0.6%]	0.04
asymptomatic	26/110 (23.6%)		14/110 (12.7%)	
TIA	44/278 (15.8%)		19/278 (6.8%)	
stroke	15/112 (13.4%)		7/112 (6.3%)	

Abbreviations: AR, absolute risk; CI, confidence interval; RD, risk difference. TIA, transient ischemic attack

[A]Absolute risk (AR) is given as restenosis rate/number at risk for the separate entities.

[B]Comparison of TIA and stroke vs. asymptomatic

restenosis were identified in univariate analysis: hypercholesterolemia and asymptomatic clinical presentation (**Table II**). Patch closure was associated with decreased incidence of restenosis during follow-up: the risk of 50% or greater restenosis for venous patch (n = 269), Dacron patch (n = 131), and primary closure (n = 100) were 12%, 21%, and 26%, respectively (patch vs no patch: RR, 0.56; 95% CI, 0.37-0.84; P = .006). Age, sex, and current smoking were not associated with restenosis during follow-up. There were no clinical parameters at baseline that were associated with occlusion of the target vessel during follow-up.

In univariate analysis, marked presence of plaque macrophages (moderate or heavy) (see **Figure** for representative image) was associated with reduced risk of restenosis during follow-up (**Table III**). Patients whose histological examination of the plaque revealed moderate or heavy macrophage infiltration had a lower risk of developing 50% or greater restenosis (RR, 0.47; 95% CI, 0.32-0.71) and a lower risk of developing 70% or greater restenosis (RR, 0.36; 95% CI, 0.19-0.68) compared with patients whose plaque histology showed none or minor macrophage infiltration. Carotid arteries with macrophage-rich plaques were less prone to occlusion during follow-up (1.1% vs 5.2%; RR, 0.20; 95% CI, 0.06-0.72; P = .006). In addition, in univariate analysis, a large lipid core size was associated with lower restenosis rate during follow-up. Patients with a large lipid core size (>40% of plaque area) in the dissected carotid plaques had a lower risk of 50% or greater restenosis (RR, 0.44; 95% CI, 0.26-0.76) and a lower risk of 70% or greater restenosis (RR, 0.38; 95% CI, 0.18-0.82) compared with patients with a very small or absent lipid core (<10%). We observed no association between lipid core size and occlusion. Other histological

Table III Plaque histology versus risk of restenosis development during follow-up

Plaque characteristics	Restenosis (≥50%)		Restenosis (≥70%)	
	RD [95% CI] / AR [A]	P	RD [95% CI] / AR [A]	P
Macrophage infiltration	-12.8% [-19.6%, -5.9%]	<0.001	-8.1% [-13.1%, -3.0%]	0.001
no or minor	52/214 (24.3%)		27/214 (12.6%)	
moderate or heavy	33/286 (11.5%)		13/286 (4.5%)	
Smooth muscle cell infiltration	3.5% [-3.4%, +10.4%]	0.34	0.1% [-5.1%, +5.2%]	0.97
no or minor	22/151 (14.6%)		12/151 (7.9%)	
moderate or heavy	63/349 (18.1%)		28/349 (8.0%)	
Collagen	4.2% [-3.7%, +12.2%]	0.33	4.2% [-0.8%, +9.3%]	0.18
no or minor	12/90 (13.3%)		4/90 (4.4%)	
moderate or heavy	71/404 (17.6%)		35/404 (8.7%)	
Calcifications	-4.0% [-10.7%, +2.8%]	0.24	-2.8% [-7.8%, +2.1%]	0.25
no or minor	40/207 (19.3%)		20/207 (9.7%)	
moderate or heavy	45/293 (15.4%)		20/293 (6.8%)	
Luminal thrombus	0.7% [-6.7%, +8.0%]	0.86	-0.4% [-5.7%, +4.8%]	0.87
no	60/354 (16.9%)		29/354 (8.2%)	
yes	25/142 (17.6%)		11/142 (7.7%)	
Intraplaque bleeding	0.6% [-6.4%, +7.5%]	0.88	3.1% [-1.6%, +7.9%]	0.23
no	28/167 (16.8%)		10/167 (6.0%)	
yes	57/329 (17.3%)		30/329 (9.1%)	
Lipid core[B]	-14.2% [-24.2%, -4.3%]	<0.001	-9.2% [-17.2%, -1.3%]	0.001
<10%	24/94 (25.5%)		14/94 (14.9%)	
10% - 40%	41/229 (17.9%)		16/229 (7.0%)	
>40%	20/177 (11.3%)		10/177 (5.6%)	

Abbreviations: AR, absolute risk; CI, confidence interval;RD, risk difference.

[A]Absolute risk is given as restenosis rate/number at risk for the separate entities.

[B] >40% vs. <10%

plaque characteristics showed no relationship with either restenosis or occlusion. The relationship of macrophages and lipid core size with restenosis persisted when occlusion was excluded from the definition of restenosis.

The IL-8 plaque levels were lower when restenosis was observed, further supporting the concept that features of unstable plaques are related with a lower incidence of restenosis (50%: 78 pg/L [95% CI, 32-125] vs 247 pg/L [95% CI, 189-305]; P < .001; and 70%: 114 pg/L [95% CI, 40-188] vs 258 pg/L [95% CI, 195-321]; P = .006, respectively).

Table IV Multivariate logistic regression analysis

Characteristics	Restenosis (≥50%) Model 1		Restenosis (≥70%) Model 2	
	Odds ratio [95% CI]	P-value	Odds ratio [95% CI]	P-value
Age, per 10 y	0.76 [0.57 - 1.01]	0.06	0.76 [0.52 - 1.10]	0.16
Male sex	0.83 [0.47 - 1.45]	0.51	1.04 [0.48 - 2.23]	0.92
Symptomatic clinical presentation	0.65 [0.38 - 1.14]	0.13	0.53 [0.26 - 1.07	0.09
Hypercholesterolemia	1.62 [0.96 – 2.73]	0.07	NS	NS
Closure Method				
Dacron patch[A]	0.62 [0.32 – 1.20]	0.16	1.07 [0.46 – 2.54]	0.92
Vein Patch[A]	0.27 [0.14 – 0.51]	<0.001	0.34 [0.14 – 0.83]	0.02
Macrophage infiltration	0.43 [0.26 – 0.72]	0.001	0.36 [0.17 – 0.74]	0.006
Lipid Core				
10% - 40% [B]	0.64 [0.34 – 1.17]	0.15	0.44 [0.19 - 0.96]	0.04
>40% [B]	0.40 [0.19 – 0.81]	0.01	0.42 [0.17 - 1.04]	0.06
Model area under the receiver operator characteristic curve	0.73 [0.67 - 0.79]		0.75 [0.69 - 0.82]	

Abbreviations: CI, confidence interval; NS, not significant (variable was excluded from model based on likelihood ratio test).

[A]vs. primary closure.

[B]vs. <10%.

In multivariate logistic regression analysis, macrophage infiltration and lipid core size were independently associated with decreased risk of developing 50% restenosis. The initial variable set of the models consisted of age, sex, clinical presentation (symptomatic vs asymptomatic), hypercholesterolemia, patch closure (primary, venous, Dacron), macrophage infiltration, and lipid core size. The adjusted odds ratios with 95% CIs derived from the resulting models are shown in **Table IV**. The clinical parameter that remained significant in multivariate logistic regression analysis was patch closure. The area under the receiver operating characteristic curve for predicting restenosis was 0.73 (95% CI, 0.67-0.79) for model 1 and 0.75 (95% CI, 0.69-0.82) for model 2.

Within 1 year, the incidence of stroke, myocardial infarction, and death was 9 of 482 patients (1.9%), 5 of 482 patients (1.0%), and 0 of 482 patients (0%) who underwent duplex follow-up (n = 500), and 13 of 164 patients (7.9%), 7 of 164 patients (4.3%), and 17 of 164 patients (10%) who were not included in the study (n = 185). In Kaplan-Meier survival analysis, infiltration of macrophages and lipid core size were not related with clinical outcome in the first year after carotid endarterectomy (oc-

currence of stroke, myocardial infarction, or death), neither in the patients with duplex follow-up nor in the patients without duplex follow-up.

In the exploratory analysis to examine potential mechanisms of protection from restenosis in vessels with macrophage infiltration and lipid-rich plaques, 2 determinants were considered that may contribute to luminal renarrowing: increased carotid intima-media thickness and geometrical remodelling (e.g., constrictive or adaptive remodeling[12]). The B-mode ultrasound substudy (n = 50) revealed that at 1-year follow-up no single determinant was associated with this restenosis protection. Carotid intima-media thickness tended to be lower and vessel diameter larger in vessels with macrophage-rich plaques (n = 32), as observed before the endarterectomy procedure, compared with macrophage-poor plaques (n = 18) (carotid intima-media thickness at 1 year: 1.22 mm [95% CI, 1.07-1.38] vs 1.40 mm [95% CI, 1.18-1.62]; vessel diameter at 1 year: 10.64 mm [95% CI, 10.01-11.27] vs 10.13 mm [95% CI, 9.28-10.98]).

Discussion

This is the first study to our knowledge that provides prospective evidence that the composition of the atherosclerotic plaque, low macrophage infiltration, and small or absent lipid core is associated with risk of restenosis after a vascular intervention.

Theoretically, assessment of atherosclerotic plaque composition using noninvasive imaging modalities may play a role in treatment stratification to help tailor treatment for the patient with carotid artery stenosis (carotid endarterectomy, carotid stenting, or medical treatment). Previously, it was shown that symptomatic clinical presentation is related to plaques with a large lipid core size and strong macrophage infiltration, and it was suggested that benefit of carotid endarterectomy might be less in plaques without these histopathological features.[29] Our study shows that the vessels with these nonvulnerable plaques (low macrophage and lipid content) are more prone to develop restenosis after endarterectomy.

Stenting and endarterectomy of lipid-rich, echolucent plaques have previously been associated with an increased restenosis rate.[13, 30] Stenting of a lipid-rich vulnerable lesion elicits an inflammatory response that results in increased neointima formation.[13] In our study, we examined restenosis when plaques had been dissected, which makes comparison of these studies difficult. In another study,[30] echolucent-rich plaques were associated with more restenosis following endarterectomy. However, in that study, ultrasound parameters were used and histological validation was not performed.

Our exploratory substudy suggests that geometric vascular remodeling may partly explain the reduced occurrence of restenosis in vessels with plaques with high

macrophage and lipid content. Hypothetically, stenting could be an alternative treatment for vessels with plaques with low macrophage and lipid content, because the mechanism of stenting is based on the inhibition of constrictive remodeling.[31] Present imaging techniques may be used to assess specific plaque characteristics preoperatively. The size of the lipid core can be determined with magnetic resonance imaging, whereas in vivo imaging of plaque macrophages may be possible in the future.[32,33] Our results could be helpful for developing strategies for incorporating imaging of atherosclerotic plaques into clinical trials.

Our results also implicate that the use of a patch for closure of the arteriotomy could be adjusted to the composition of the atherosclerotic plaque. Randomized evidence has shown that patch closure decreased the incidence of restenosis, especially when vein patches are used.[34] Restricting patch closure to patients who are more likely to benefit might improve overall outcome by eliminating patch-related complications in patients who may not require patch closure (patients with plaques having high macrophage or lipid content). In our patient cohort, the choice of patch closure was not randomized, which may have biased these results. Studies that incorporate randomization of closure method coupled with imaging and plaque characterization may help elucidate if and how patch closure needs to be adjusted to the composition of the atherosclerotic lesion.

After surgical removal of an atherosclerotic lesion, the composition of the plaque is related to local restenosis. This relationship might be mediated via remainders of plaque at the endarterectomy site. However, we consider this explanation unlikely because medial layers were visible in the majority of plaque specimens. The presence of an inflammatory plaque goes hand in hand with inflammation and protease activity in the media and adventitia,[35,36] the layers remaining after endarterectomy. Leukocytes are highly infiltrated into the media and adventitia of inflammatory plaques, giving rise to production of matrix metalloproteinases, which leads to thinning of the media and expansive remodeling.[37] Our substudy on geometric vascular remodeling and neointima formation suggests that vessels with inflammatory plaques had larger vessel diameters at follow-up, even though vessel diameters were similar at baseline. It could be hypothesized that the plaque composition at baseline is related to a dynamic vascular remodeling process after the endarterectomy procedure.

Our study has several limitations. First, duplex ultrasound is not the gold standard for determining the degree of carotid stenosis. However, duplex ultrasound has been extensively validated against angiography and pathology,[38,39] and we standardized our data according to internationally accepted criteria. Second, we did not study plaque rupture. Assessment of plaque rupture was not always reliable in carotid endarterectomy specimens. The study by Lovett et al[40] showed that plaque rupture in carotid endarterectomy specimens had relatively low reproducibility. Third, postprocedure angiography was not performed to determine residual steno-

sis after carotid endarterectomy. Therefore, the stenosis grade measured at 1 year might be due to residual stenosis in some patients. Finally, our findings are based on a small group of patients and our data need confirmation in larger studies in other populations at risk.

In conclusion, atherosclerotic plaque composition is associated with risk of restenosis after carotid endarterectomy. Macrophage infiltration and large lipid core size are associated with less restenosis.

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CHAPTER 8

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HISTOLOGICAL CHARACTERIZATION OF RESTENOTIC CAROTID PLAQUES IN RELATION TO RECURRENCE INTERVAL AND CLINICAL PRESENTATION

A COHORT STUDY

Backgrounds and purpose Restenosis is an important complication after carotid endarterectomy, but little is known about plaque composition in early versus late restenosis and which plaque characteristics are associated with symptomatic clinical presentation of restenotic lesions.

Methods Endarterectomy specimens of 822 consecutive patients undergoing carotid endarterectomy (33 restenotic; 789 primary) were subjected to histological examination for the presence of macrophages, smooth muscle cells, collagen, calcifications, luminal thrombus, intraplaque bleeding and lipid core size.

Results Early restenotic plaques showed marked accumulation of smooth muscle cells and fibrous tissue, whereas late restenotic plaques demonstrated increased macrophage infiltration, calcification and lipid core (P trend <0.05), resembling primary plaques. Patients with symptomatic restenosis had plaques with higher macrophage infiltration (P=0.01) and a larger lipid core (P=0.02) than asymptomatic patients, independent of recurrence interval.

Conclusions Restenosis occurring >5 years after primary carotid endarterectomy resembles primary plaques. Symptomatic presentation of restenotic lesions is independently associated with an unstable plaque phenotype.

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Introduction

Restenosis remains an important problem challenging the results of carotid endarterectomy (CEA). Restenosis occurs in about 10% of patients in the first year after CEA and in 20% renarrowing of the lumen occurs during longer follow-up.[1]
Recent histopathologic studies revealed that primary carotid artery stenotic lesions that give rise to local thromboembolic events share a vulnerable plaque phenotype, characterized by a large lipid core and infiltration of inflammatory cells.[2–4] However, it has not been studied if the restenotic plaque that is prone to cause a cerebrovascular event shares the same histological characteristics. Furthermore, it is unclear if early and late restenosis could be considered comparable or different processes. The objective of the present study was to investigate the characteristics of restenotic plaques in relation to recurrence interval and clinical presentation.

Methods

Consecutive patients who underwent CEA were included (April 2002 to February 2007) in the Athero-Express study.[5] Written informed consent was obtained from all patients.

Table I Comparison of plaque histology between primary and restenotic plaques

	Restenosis				Primary	P	
	All	Early	Inter-mediate	Late		Restenosis vs. Primary	Recurrence interval (trend)
N	33	6	11	16	789		
Macrophages[A]	66%	33%	73%	73%	60%	0.83	0.13
Smooth muscle cells[A]	62%	100%	64%	50%	71%	0.87	0.02 *
Collagen[A]	75%	100%	73%	66%	83%	0.86	0.14
Calcifications[A]	12%	0%	0%	25%	62%	<0.001 *	0.05 *
Luminal thrombus	65%	50%	82%	57%	26%	<0.001 *	0.45
Intra-plaque Bleeding	55%	50%	46%	64%	64%	0.30	0.94
Overall phenotype						0.008 *	0.01 *
Fibrous	58%	83%	77%	35%	32%		
Fibro-atheromatous	21%	17%	18%	25%	35%		
Atheromatous	21%	0%	9%	38%	33%		

[A]percentages of plaques rated as "moderate" or "heavy" (e.g. moderate or heavy infiltration of macrophages)
*p <0.05

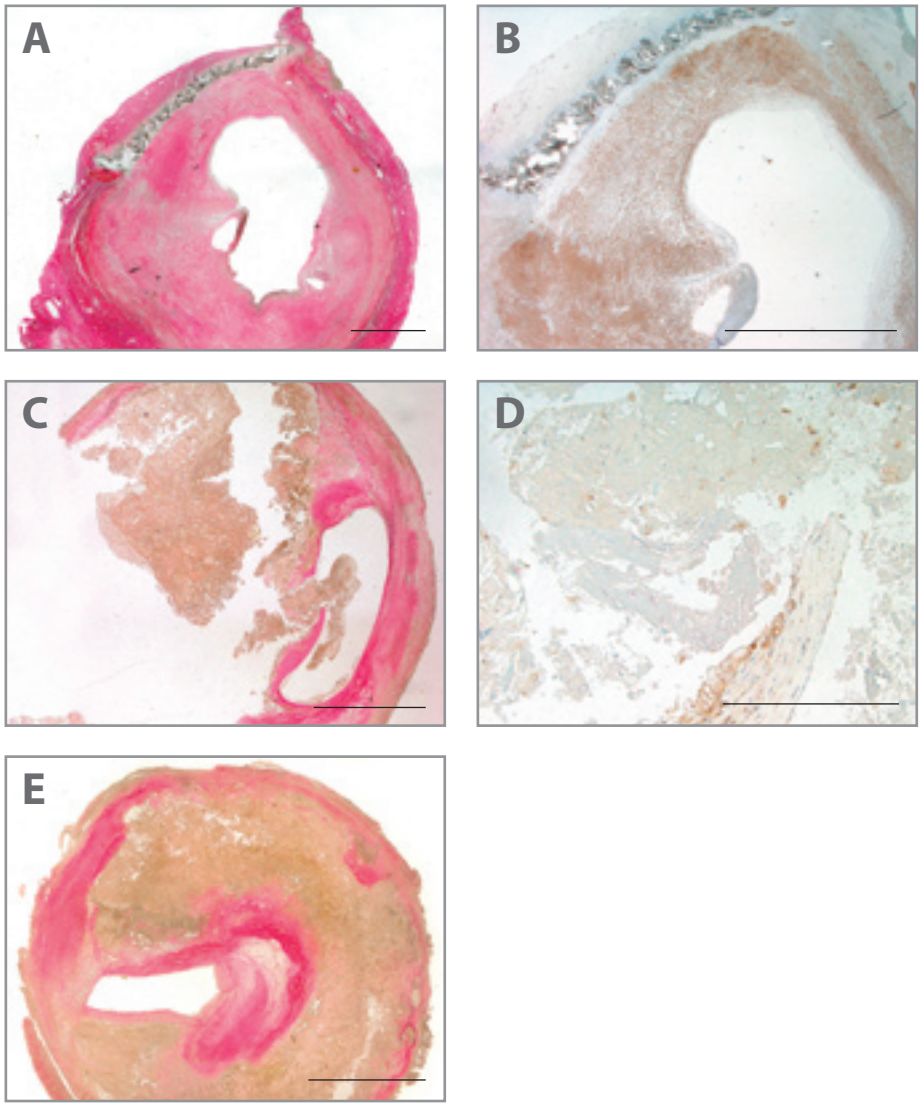

Figure I Plaque histology

Scalebars: 2 mm (A,B,C,E); 0.5 mm (D). **A:** Early restenotic plaque (Picro-Sirius staining). The plaque consists of fibrous tissue with massive accumulation of smooth muscle cells (see panel B). Note the patch at the top of the specimen. **B:** Smooth muscle cell accumulation in early restenotic plaque (Alpha-actin staining; brown). A large accumulation of smooth muscle cells is observed, which was present throughout the plaque. **C:** Late restenotic plaque (Picro-Sirius staining). Symptomatic restenosis 6 years after primary CEA. A large lipid core (left) is observed. Note the comparison with the primary plaque in panel E. **D:** Macrophage infiltration in the fibrous cap of a late restenotic plaque (CD68 staining; brown). **E:** Primary plaque (Picro-Sirius staining).

Definition of restenosis

Restenosis was defined as a recurrent luminal narrowing of at least 50% (peak systolic velocity >=125 cm/s; duplex ultrasound) in patients who had undergone prior carotid endarterectomy of the ipsilateral carotid artery. The indications for performing CEA in restenotic patients were cerebrovascular symptoms or a progressive asymptomatic lesion (>=70%; except near-occlusions). Based on recurrence interval we distinguished between early (<2 years), intermediate (2 to 5 years) and late restenosis (>5 years).

Plaque characterization

Carotid endarterectomy was performed using selective shunting and selective patching. The excised plaques were directly transferred to the laboratory and processed and examined as described previously.[5] Macrophages (CD68), smooth muscle cells (Alpha-Actin), collagen (Picro-sirius) and calcifications (hematoxylin and eosin [HE]) were rated as no/minor or moderate/heavy. Luminal thrombus and intraplaque bleeding (HE, Elastin von Gieson) were rated as absent or present. Overall phenotype was based on lipid core size: fibrous (<10% of plaque area), fibroatheromatous (10% to 40%), and atheromatous (>40%).

Statistical Analysis

Histological characteristics were compared between groups using the Chi square test. For each plaque characteristic associated with symptomatic presentation of restenosis, a logistic regression model was constructed to adjust for recurrence interval. The result was expressed as odds ratio (OR) with 95% CI. Probability values <0.05 were considered statistically significant.

Results

The patient group with recurrent stenosis encompassed more female (48% vs. 30%; p=0.01) and asymptomatic patients (49% vs. 22%; p=0.001) than the primary group. The prevalence of other risk factors and medication use did not differ. Histological examination revealed that restenotic plaques contained less calcification, more luminal thrombus and a smaller lipid core compared to primary plaques **(Table I)**. These characteristics were not related to patch use during the original CEA. Plaque composition was dependent on recurrence interval: early restenotic plaques showed a fibrous phenotype whereas late restenotic plaques appeared like primary lesions **(Table I; Figure I)**.

We explored if differences in clinical presentation of restenotic plaques could be based on differences in plaque composition **(Figure II)**. Symptomatic plaques had

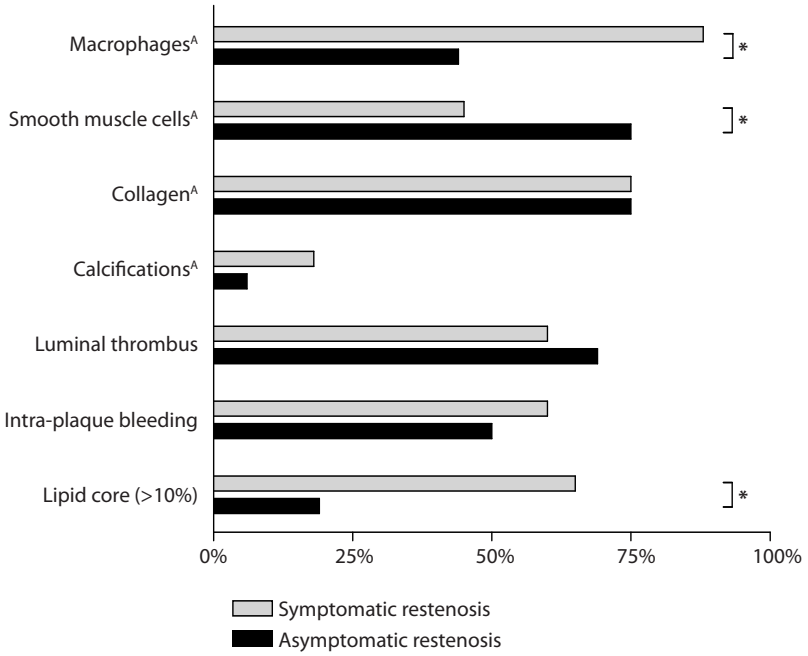

Figure II Comparison of plaque histology between symptomatic and asymptomatic restenosis

[A]percentages of plaques rated as "moderate" or "heavy" (e.g. moderate or heavy infiltration of macrophages). *p <0.05

higher infiltration of macrophages, a smaller smooth muscle cell component and larger lipid pools.

Symptomatic presentation was more frequently observed in the group with late restenosis compared to early restenosis, which matched well with the plaque characteristics associated with symptomatic recurrent disease. This could imply that the associations between symptomatic presentation of restenosis and plaque characteristics might be biased by the recurrence interval, which led us to adjust for recurrence interval. A paucity of smooth muscle cells was no longer associated in the adjusted analysis (OR=0.63[0.12 to 3.3]). However, large lipid core showed a trend (OR=2.1[0.67 to 6.24]) and macrophage infiltration was associated with symptomatic presentation of restenosis, independent of recurrence interval (OR=8.3[1.2 to 59.4]; p=0.03).

Discussion

The present study defines histological characteristics of the vulnerable restenotic plaque. We show that in restenotic plaques, macrophage infiltration and large lipid core are associated with symptomatic presentation, comparable to primary plaques. These associations are independent of the recurrence interval.

The implication of these findings is that macrophage infiltration and lipid core size are the plaque characteristics that should be targeted with noninvasive plaque imaging to assess the risk of patients with recurrent carotid stenosis to become symptomatic. With present plaque imaging techniques, lipid core and macrophage infiltration can be detected quite reliably.[6,7] Therefore, imaging studies to validate our observations are warranted.

The present study also clarifies that late restenosis (>5 years) is not clearly different from primary carotid artery stenosis, with marked macrophage infiltration, calcifications and lipid core. In contrast, early restenotic plaques are characterized by the presence of smooth muscle cells with absence of typical features of atherosclerotic plaques such as calcifications and a large lipid core. Because carotid plaque composition may influence peri-interventional complication rate and incidence of restenosis after the intervention,[8,9] our results indicate that differentiating between early and late restenotic lesions could be important for clinical studies investigating treatment of restenosis.

In conclusion, the present study shows that restenosis occurring >5 years after primary carotid endarterectomy resembles primary plaques, and symptomatic presentation of early and late restenotic plaques is associated with high macrophage infiltration and large lipid core, independent of recurrence interval.

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PART 4

THE CAROTID ATHEROSCLEROTIC PLAQUE AND CLINICAL OUTCOME

CHAPTER 9

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THE CAROTID ATHEROSCLEROTIC PLAQUE AND MICROEMBOLISATION DURING CAROTID STENTING

Microembolisation is an important issue in carotid artery stenting. During different phases in the stenting process, numerous emboli are dislodged from the atherosclerotic plaque. Embolisation can be measured as microembolic signals detected by transcranial Doppler (TCD) monitoring during the procedure or as new ischemic areas determined by magnetic resonance imaging. This article gives an overview of the principles of emboli detection methods, their clinical relevance, and risk factors associated with microembolisation. In addition, protection devices are discussed in relation to embolisation. Although they potentially protect the brain, particularly filter devices increase the amount of TCD-detected cerebral microemboli. Special attention is paid to the carotid artery plaque, which is subject to ongoing research that may yield important implications for clinical practice in the near future. Evidence is accumulating that unstable, vulnerable plaques are associated with increased microembolisation during carotid interventions. This knowledge of the vulnerable plaque can be translated to the clinical setting by plaque imaging. A first approach has been made by duplex imaging of carotid plaque morphology. More advanced methods such as molecular magnetic resonance imaging and optical coherence tomography could aid in optimal treatment selection based on plaque characteristics thus reducing microembolisation and associated cerebral adverse events.

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Introduction

Carotid artery stenting (CAS) is an evolving method to treat carotid stenosis. Currently, different trials are investigating the outcome of CAS compared to carotid endarterectomy (CEA), the standard of treatment for haemodynamically significant carotid stenosis.[1-4] Advantages of CAS are avoidance of general anaesthesia, smaller surgical trauma and no need to access hostile necks. The main drawback of CAS however, is the high incidence of emboli shed into the distal vascular bed. These emboli are the result of manipulation of the plaque with guide wires, balloon inflation and stent placement. A fragile plaque, hiding large atheroma and thrombus, can be severely disturbed in its integrity during a stenting procedure, which can give rise to distal embolisation and subsequent cerebral adverse events. Differences in plaque type, assessed in-vivo by advancing imaging techniques may therefore be relevant to predict embolisation during carotid interventions. In the current study we give an overview of emboli detection methods, studies on CAS related microembolisation and current knowledge on plaque characteristics in relation to microembolisation.

Literature search

To identify relevant literature, we performed a search on PubMed using a wide spectrum of search terms describing the terms carotid artery stenting en microembolisation. For carotid artery stenting, we used the words “carotid angioplasty”, “carotid stenting”, “cas”, “carotid artery stenting”, and the combination of MeSH terms “Carotid Stenosis”, “Angioplasty”, and “Stents”. For embolisation and embolisation techniques we used the words “emboli”, “embolization”, “embolisation”, “microemboli”, “microembolization”, “microembolisation”, “transcranial Doppler”, “tcd”, “DWI” and the MeSH terms “Embolism”, “Ultrasonography, Doppler, Transcranial” and “Diffusion Magnetic Resonance Imaging”. We combined these keywords into a PubMed search such that the results matched at least one term describing carotid stenting and one term describing embolisation. We selected all relevant articles and related articles for this review with (Oxford) level of evidence 4 or higher. In addition, relevant literature known to the authors and most recent data from our institutions were used for the current work.

Emboli detection methods

There are two methods that can be used to quantify the embolic burden induced by carotid artery interventions. Transcranial Doppler (TCD) can be used to measure emboli during and after the procedure directly. Magnetic resonance imaging (MRI)

can be used to detect ischaemic brain lesions after the stenting procedure, which are an indirect measure of the embolic load.

Transcranial Doppler monitoring

The middle cerebral artery (MCA) is insonated by a 2 MHz pulsed Doppler probe, which is fixed to the head by a frame during the carotid intervention. The ultrasound signal passes through the acoustic window of the temporal bone, which is present in 85% of patients. Emboli are readily detected as very intense, short signals clearly distinct from the normal flow signal. These microembolic signals have high specificity and can not be produced by flow turbulence or artifacts.[5] Criteria for determination of emboli have been described by consensus committees.[6, 7] Embolic signals can represent plaque debris or thrombus fragments dislodged from the plaque, but also air emboli, which are regularly observed during plain angiography of the carotid arteries.[8]

Optimally, the TCD signal is continuously monitored by a technician who is present in the angiography suite. Continuous online monitoring of the Doppler signal of the MCA during CAS provides the following possibilities: 1) any cerebral malperfusion can immediately be detected and 2) cerebral microembolisation can be detected and 3) post-interventional hyperperfusion syndrome can be predicted.

The following case underlines the ability of TCD to immediately detect clinically relevant embolisation during carotid artery stenting **(Figure I-A)**. The normal TCD signal was suddenly disturbed by an embolus, which caused a subtotal occlusion of the vessel, confirmed by angiography **(Figure I-B)**. In this specific case, the embolus dissolved spontaneously resulting in restoration of flow. The patient suffered a transient ischaemic attack with paresis of the contralateral hand.

Magnetic resonance imaging

The emboli, which are dislodged during the carotid stenting procedure, proceed distally into the intracranial arteries causing occlusion of these arteries. For research purposes, the ischaemic areas resulting from this process can be sensitively imaged by magnetic resonance imaging (MRI). Diffusion weighted MRI (DWI) is an enhancement of the MRI technique which can detect diffusion abnormalities present in brain areas with acute ischaemia, thus making it the most sensitive technique for detection of ischaemic areas currently available.[9]

Clinical relevance of cerebral emboli

Tables I and II give an overview of studies that investigated cerebral microembolisation during or after CAS. Studies investigating TCD during CAS show a median of 40 or more embolic signals per intervention.[10-16] In this study, the majority of emboli

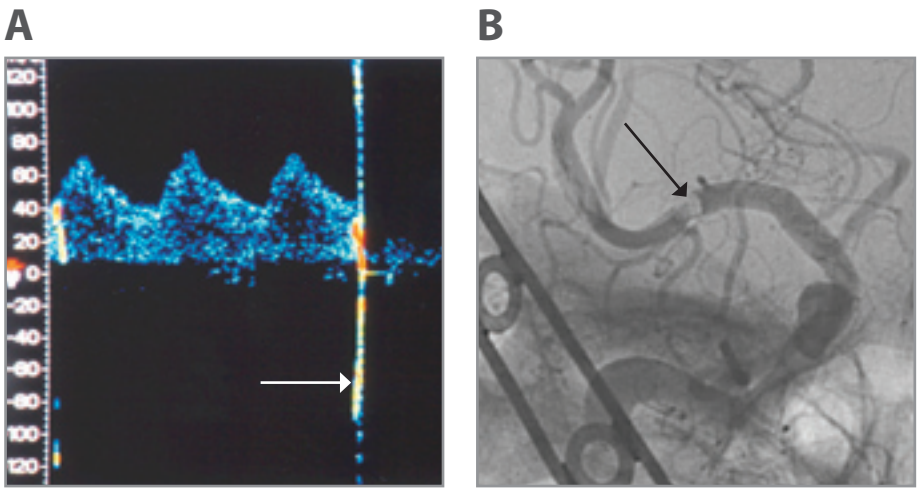

Figure I TCD case

A: A normal TCD signal is followed by a macroembolus (arrow), resulting in a decrease of flow. An angiogram confirmed the presence of an embolus (Panel B). **B:** Angiogram performed after detection of macroembolus by Transcranial Doppler (Panel A). The embolus is located at the bifurcation of the main stem of the middle cerebral artery (arrow).

occur during stent deployment. Our series of 550 patients shows a median of 52 emboli during stent deployment, 14 during predilation and 17 during postdilation.[10] The number of embolic showers (the number of heartbeats filled with embolic signals) are distributed between the different phases in the same fashion as individual embolus counts: the majority occurs during stent deployment, with a median of 17, compared to 3 during predilation and 7 during postdilation.

The number of embolic showers during postdilation is the most powerful predictor of clinical outcome. Patients having > 5 embolic showers during postdilation had a 2.6 fold greater risk of an adverse cerebral event during or within 7 days of the procedure. During postdilation, high pressure is applied to the vessel while the stent is in place. This could lead to dislodgment of plaque fragments and thrombi, or provoke plaque rupture with subsequent in-stent thrombosis, resulting in cerebral microembolisation. The association between microemboli and cerebral adverse events is also found in carotid endarterectomy patients.[17] Usually, the occurrence of a stroke during a carotid intervention is caused by multiple microemboli instead of one macroembolus. This is underlined by the rare occurrence of macroemboli in our series.

Besides the relation between embolic showers during postdilation and stroke, there are certain embolisation patterns that are evidently related to adverse events. Particulate macroembolisation, which is defined as detection of an embolic signal

immediately followed by marked attenuation of MCA flow, is directly associated with adverse events.[18] The same is true for rupture of a balloon resulting in massive air embolism.[10]

The high number of TCD-detected microemboli during carotid artery stenting is paralleled by a high number of new ischaemic brain lesions detected by magnetic resonance imaging. Using conventional MRI, new ischaemic lesions were found in 11% of patients after carotid stenting.[19] The more sensitive diffusion weighted MRI (DWI) detects new lesions in 23% - 57% of patients.[20-27] These lesions are mostly small (<1 mL) and asymptomatic. While DWI imaging gives an interesting insight in pathophysiology, conventional MRI findings correlate better with the clinical outcome. The positive predictive value of MRI detected abnormalities for clinical ischaemic events is 45% compared to around 10% for DWI **(Table I)**. The high load of asymptomatic lesions found by DWI raises questions about the influence of carotid artery stenting on more subtle neurological deficits like decline of cognitive function.[28] For carotid angioplasty without stenting it has been shown that the intervention does not result in worse or better cognitive function, although a larger study would be required to firmly establish these findings.[29] Haugh and coauthors followed-up the early DWI lesions at 6 months with DWI and MRI. They noted that most lesions had disappeared, except for the lesions initially detectable by MRI, and suggested that the DWI detected ischaemia might be reversible.[30]

Determinants of cerebral microembolisation

The amount of microembolisation is influenced by different factors. First, technical aspects of the CAS procedure could have an important influence. The study of Hammer and coworkers described a relation between embolic load as determined by DWI and difficult anatomy (common carotid artery(CCA) -arch angle < 45 degrees or tortuosity of the CCA).[23] Emboli were observed in the ipsilateral anterior circulation in 36% of patients with difficult anatomy compared to 5% of the other patients. The authors suggested that most emboli occur during manipulation of the guide wire for selective catheterization of the CCA. However, this is not consistent with our TCD findings, which suggest that most clinically relevant embolisation occurs during stent deployment and postdilation. Secondly, cardiovascular risk factors and other clinical characteristics are related to microembolisation. Jordan and coworkers describe more TCD detected emboli in symptomatic patients, which is paralleled by more new MRI/DWI lesions in the studies of Van Heesewijk and Hammer, although study sizes are insufficient to reach statistical significance.[13, 19, 23] Different studies consistently show that advanced age is a risk factor for occurrence of new lesions, although the difference did not reach statistical significance.[23, 24, 26] These findings are consistent with the observation that older patients have a higher

Table I Magnetic Resonance Imaging studies

Study	Patients (no prot/ prot)	Tech-nique	Medication	Peri-inter-ventional stroke/tia	% with emboli (no prot)	% with emboli (prot)	Relation with adverse events	Determinants of emboli
Cossottini 2005	52 (22/30)	DW 1.5-T	ASA and ticlopidine	1/n.r.	8/22 (36%)	8/30 (26%)	no relation	Protection device (negative relation)
Hammer 2005	53 (0/53)	DWI 1.5T	ASA and clopidogrel	2/n.r.		21/53 (40%)	ppv 2/21 npv 32/32	difficult aortic arch implantation 36% vs. 5%; p=0.04 Symptomatic vs. asymptomatic 50% vs. 34%; p=n.s. age >75 vs. age < 75 52% vs. 31%; p=n.s.
Krapf 2005	74 (74/0)	DWI 1.5-T	ASA and clopidogrel	3/n.r.	42/74 (57%)		ppv 3/42 npv 32/32	stenosis length positive relation; p=0.01 echolucent vs. -genic plaque 60% vs. 42%; p= n.s.
Roh 2005	22 (22/0)	DWI 1.5-T	ASA and ticlopidine	2/1	8/22 (36%)		ppv 2/8 npv 13/14	n.r.
Flach 2004	21 (0/21)	DWI 1.5/T	Clopidogrel	1/0		9/21 (43%)	Bigger lesion volume in symptomatic patients; P=0.03	n.r.
Poppert 2004	41 (41/0)	DWI 1.5-T	ASA and clopidogrel	1/n.r.	22/41 (54%)		no relation	Diabetes vs. no diabetes 50% vs. 11%; p=0.01
Schluter 2003	42 (0/42)	DWI 1.5-T	ASA and clopidogrel	1/1		9/42 (23%)	n.r.	n.r.
Heesewijk 2002	72 (72/0)	MRI 0.5-T	ASA and ticlopidine or clopidogrel	3/4	11/72 (15%)		ppv 5/11 npv 60/61	symptomatic vs. asymptomatic 23% vs. 12%; p=n.s.
Jaeger 2002	70 (70/0)	DWI 1.5-T	ASA and clopidogrel	1/n.r.	20/70 (29%)		ppv 1/20 npv 50/50	positive vs. negative DWI: mean age 70 vs. 66; p=0.08

Abbreviations: Prot, cerebral protection device; n.r., not reported; ppv, positive predictive value for clinical events; ppv, negative predictive value for clinical events.

Table II Transcranial Doppler studies

Study	Patients (no prot/ prot)	Medication	Peri-inter-ventional stroke/tia	Emboli median (no prot)	Emboli median (prot)	Relation with adverse events	Determinants of emboli
Orlandi 2005	57 (57/0)	ASA and ticlopi-dine	2/7	74 9 showers		events: lower starting blood flow (32 vs. 48 cm/s); p<0.001 emboli: no relation with events	n.r.
Ackerstaff 2005	550 (369/181)	ASA and clopi-dogrel	21/36	83 13 showers	42 3 showers extra (filter)	>5 embolic showers at postdilatation; OR=2.6 asystole; OR=6.7 particulate macroembolus; OR=27.4 massive air embolus; OR=75.7 symptomatic patients; OR=5.0 p<0.001; ROC area 0.80	n.r.
Aranow 2005	43 (36/7)	ASA and Ticlopidine or clopidogrel GPIIa/IIIb inhibi-tor (77%)	2/0	303[B,C]	190 less (not specified)	Death, myocardial infarction or stroke vs. No event: 379 vs. 278 emboli (p=0.12)	Leukocyte count (p=0.02) COPD (p=0.05) Protection device (negative relation, p=0.02)
Schmidt 2004	42 (0/42)	ASA and clopi-dogrel	0/1		196[A] (filter) 57[A] (occlusion)	n.r.	Distal occlusion vs. filter (<0.001)
Al Mubarak 2000	76 (39/37)	ASA and clopi-dogrel	2/0	164 [A]	68 [A](occlusion)	n.r.	Distal occlusion vs. no protection (p=0.002)
Jordan 1999	40 (40/0)	n.r.	2/2	40 [C]		patients with events did not have higher emboli counts	TIA/stroke vs. asymptomatic: 115/91 vs. 60 emboli (p=NS)
McCleary 1998	9 (9/0)	ASA heparin (72h) enoxiparin	1/0	128		n.r.	n.r.

Abbreviations: prot, cerebral protection device; n.r., not reported. [A] mean number of emboli for all interventions [B] median number of emboli for all interventions
[C] Showers are counted as 10 microemboli

probability of CAS related stroke.[2, 31, 32] Diabetes mellitus and chronic obstructive pulmonary disease were both mentioned by a single study as determinant of cerebral microembolisation, but this was not confirmed by any of the other studies.[12, 26] Third, platelet aggregation inhibitors could reduce embolisation during carotid artery stenting. A regimen of clopidogrel and acetylsalicylic acid (ASA) is widely prescribed in patients experiencing carotid stenosis. Recently, a randomized trial showed reduction of TCD detected microemboli in symptomatic carotid patients after 7 days of treatment with a combined regiment of clopidogrel and aspirin compared to aspirin alone.[33] To our knowledge, there are no randomized studies investigating the influence of clopidogrel on CAS related embolisation.

Protection devices and cerebral microembolisation

Cerebral protection devices are currently being investigated as an extension to carotid artery stenting. There are two main concepts: distal filtering and endovascular occlusion. Interestingly, application of filter devices does not result in a reduction of microembolic load, but instead results in an increased number of TCD-detected emboli.[34] This may be caused by extra manipulation during placement and retrieval of the device or insufficient alignment to the vessel wall. The apparent success of filtering devices in reduction of cerebral adverse events may be the result of the ability to block the passage of bigger emboli. Particulate macroemboli were indeed less prevalent in procedures with protection devices in our series (0% vs. 2.2%).
Protection devices based on endovascular occlusion establish a significant reduction of microembolisation as measured by TCD. Schmidt and coworkers showed a fivefold reduction of micro-embolic signals by use of the MO.MA device compared to a filter device.[16] The MO.MA device uses an endovascular balloon in the proximal common carotid and external carotid arteries, with a suction device which establishes flow reversal in the carotid artery. Use of this device does not totally prevent microembolisation, which could be explained by collateral flow or by suction of blood from the circle of Willis.[35] A similar reduction in microembolisation has been shown for a distal balloon protection system, which has the disadvantage of possible microembolisation via the external carotid artery.[11] Findings in DWI studies support the effectiveness of protection devices: less new lesions were found in protected procedures.[20] An inherent flaw of the flow reversal systems is the interruption of antegrade blood flow through the carotid artery, which is not tolerated in patients with insufficient collateral circulation.

Post-interventional monitoring of emboli

Post-interventional monitoring following carotid endarterectomy in patients who develop cerebral infarction after a symptom free interval demonstrated either sustained TCD detected emboli from the endarterectomy area or a thrombotic occlusion of the carotid artery. Although post-operative monitoring has been studied to some extent in CEA,[36-39] there is only one study which investigated post-interventional TCD monitoring for CAS.[40] Two out of the ten study subjects showed late embolisation after CAS. The value of post-interventional emboli monitoring after CAS still has to be determined.

CAS embolisation in comparison to CEA

In comparison to CEA, CAS produces a multitude of microembolic signals during the procedure.[13] In line with this finding, the number of new lesions detected by DWI is significantly higher after carotid artery stenting.[21, 22] Interestingly, there is no substantial difference in clinical outcome of CAS compared to CEA.[41] This may indicate that the emboli during carotid artery stenting have a different composition or size than CEA related emboli. However, a high load of embolisation in a short time frame gives rise to adverse clinical events, as is observed in the postdilation phase of CAS.[10]

The atherosclerotic plaque and microembolisation

The frequently observed embolisation during CAS raises the objective to predict embolisation and clinical events. The atherosclerotic plaque itself, which can be characterized by plaque imaging in-vivo, may be a strong predictor of embolisation during carotid interventions. We will introduce the key concepts in the current knowledge of atherosclerotic plaque in relation to embolisation during and after CAS.

The vulnerable plaque: current concepts

Atherosclerosis is an inflammatory process which is slowly progressive throughout life.[42, 43] At a certain stage the plaques have developed to such extent that the disease reaches the clinical horizon. These advanced plaques found in clinically manifest atherosclerosis can be classified according to their morphology and cell content.[44, 45] Stable plaques, also called fibrous plaques, posses a well developed matrix with a great amount of smooth muscle cells and collagen, and a small lipid pool **(Figure II-A)**. The fibrous component of the plaque is beneficial because it protects the plaque from disruption. Unstable plaques, also called atheromatous plaques,

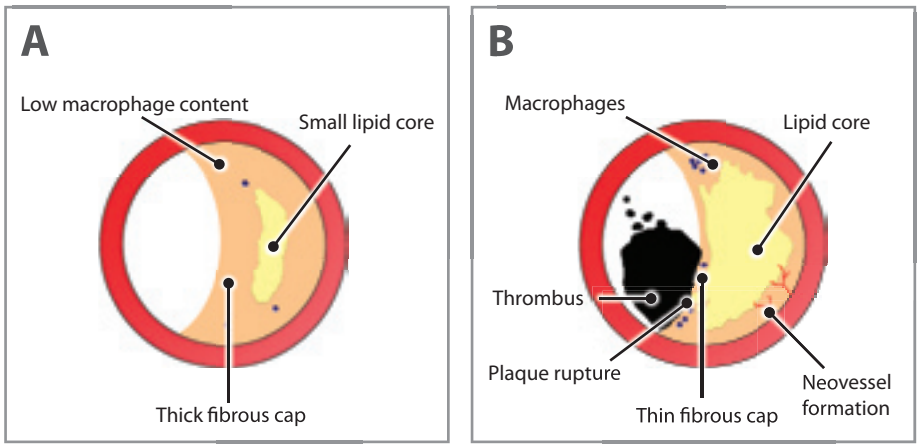

Figure II Schematic drawing of atherosclerotic plaque phenotypes
A. Stable plaque **B.** Unstable plaque

vulnerable plaques, or thin cap fibro-atheromas, posses a large lipid pool covered by a thin fibrous cap **(Figure II-B)**. These unstable plaques are at risk for disruption because of the small fibrotic component and high content of macrophages, which produce pro-inflammatory cytokines and proteases responsible for degradation of the fibrous cap. Unstable plaques are further characterized by neovessel formation, predisposing to intraplaque bleedings. These intraplaque bleedings add to plaque formation and may provoke acute plaque disruption.[46, 47] When the plaque is disrupted and a plaque rupture occurs, the bloodstream comes into contact with the highly thrombogenic lipid pool resulting in acute thrombus formation. The thrombus may occlude the lumen, for instance in myocardial infarction, or give rise to distal embolisation, as is the case in symptomatic carotid disease.[48, 49]

Plaque imaging

To translate plaque characteristics into the clinical decision making process, information on plaque composition, e.g. the amount of fat, must be acquired in vivo. Different approaches are made for this purpose. A simple method is plaque imaging with B-mode echography. Fatty plaques are less echogenic than plaques with low fat content.[50] Most studies express plaque echogenicity as grey scale median (GSM); lower GSM means less echogenicity. Since unstable plaques contain more fat than stable plaques, this method allows differentiation between those phenotypes. Inherent problems are signal to noise ratio, artefacts caused by calcifications

and observer dependency. Nevertheless, a low GSM has been shown to correlate with the history of stroke in patients with high grade stenosis.[51-53]
High resolution MRI provides more detailed plaque images than echography. Contrast enhanced MRI in a 1.5-T scanner allows reliable distinction of different plaque components. Necrotic core size, matrix components and fibrous cap imaged before carotid endarterectomy showed good correlation with the histological appearance of the endarterectomy specimen.[54-56] Plaque images obtained with MRI can be reliably interpreted by computer aided analysis.[57] The use of 3.0-T or higher MRI scanners will boost resolution further. Echography and MRI both have the limitation that they can only image structures and not biological function. The most promising application that is currently being developed is molecular imaging. Specific antibodies can be coupled to contrast agents or radiotracers. Thus, specific molecular targets can be visualized by MRI or SPECT, such as macrophage or other specific antigens.[58-61] Another promising technique is optical coherence tomography, an optical analogue of ultrasound. Recently, two studies have shown that acute coronary syndrome was related to macrophage infiltration and cap rupture as determined by this technique.[62, 63] Clinical applications of these imaging techniques are currently evolving.

Embolisation in relation to plaque characteristics determined by histopathology

A study in 200 carotid endarterectomy specimens showed that plaque phenotype correlated with embolisation during carotid endarterectomy.[64] Vulnerable, atheromatous plaques were more prone to cause microembolisation due to manipulation of the carotid artery in the dissection phase compared to fibrous plaques. The presence of an atheromatous plaque was associated with adverse events during carotid surgery. This confirms the hypothesis that especially vulnerable plaques are prone to disruption during vascular interventions, causing distal embolisation and subsequent ischaemic events. These findings could likely be extrapolated to CAS, because CAS related emboli are also a result of manipulation of the carotid plaque, although the manipulation is endovascular instead of extravascular. In this study another phenomenon was noticed. Surprisingly, the more stable fibrous plaques were associated with increased microembolisation after plaque removal, i.e. at clamp release and during wound closure. This is possibly a result of increased collagen exposure after removal of fibrous plaques. Since in CAS the plaque is not removed these associations do not apply to CAS. The relation between unstable plaques and microembolisation in the dissection phase of CEA was also reported in a smaller set of patients as part of a study investigating the relation between Chlamydia pneumoniae and microembolisation during carotid endarterectomy.[65]

Interestingly, patient groups suffering from increased embolisation during CAS (e.g. older patients and symptomatic patients) have more unstable plaques as shown by endarterectomy studies.[66, 67] The association between atheromatous plaques and embolisation is further confirmed by histopathological analysis of debris from distal filter protection devices. The debris consists mainly of the constituents of atheromatous plaques: cholesterol clefts, lipid laden macrophages and fibrin entrapped platelets.[68, 69] A study of Aranow and colleagues provided a link between inflammation, a key element of atherosclerosis, and microembolic signals during carotid stenting.[12] It showed that preprocedural leukocyte count is associated with increased microembolisation during CAS. This relation may be based on inflammation causing plaque instability, but the increased leukocyte count may also be a response that is provoked by the emboli.

Embolisation in relation to plaque characteristics determined by plaque imaging

Two studies investigated the relation between plaque morphology determined by B-mode echography to embolisation and stroke during CAS. Krapf and coworkers found that echolucent plaques were associated with new lesions on DWI.[25] New lesions were found on 60% of patients with echolucent plaques and 42% of echogenic plaques. However, study size was insufficient to establish if this difference was more than a matter of chance. In contrast to the negative findings on plaque morphology, the authors do report a relation between duplex determined carotid lesion length and new lesions on DWI. Carotid lesions with a length > 7 mm pose an 80% risk of new DWI lesions, whereas lesions with a length of 0.4 mm are associated with a 40% chance of new DWI lesions. The relation between lesion length and embolisation was confirmed other authors, and in addition it has been shown that larger lesion length is associated with increased chance of stroke.[31, 70] Remarkably, the reported lesion lengths (2-12mm) reported by Krapf were much shorter than the size of carotid plaques removed in carotid endarterectomy studies or lesion sizes as determined by angiography.[11, 16, 31]

The ICAROS study compared plaque morphology, expressed as GSM, to outcome after CAS in a multi-centre setting.[71] This study reports a strong association between stroke and GSM, with an optimal GSM cut-off value of 25. Of the patients with GSM <25, 7.1% suffered a CAS related stroke in comparison to 1.5% with GSM > 25. Since lower GSM correlates with atheromatous plaques, and these atheromatous plaques are probably associated with increased microembolisation and intervention related adverse events, it would be expected that protection devices would be especially effective in patients with a low GSM. Surprisingly, use of protection devices was unfavourable in patients with low GSM. In this patient group, cerebral

complications occurred in 12.5% of protected procedures in contrast to 5.2% of unprotected procedures (p=0.15). This could be explained by the fact that the utilized protection devices were mostly distal occlusion balloons which requires passage of the lesions with inherent risk of embolisation. Patients with GSM >25 benefited from cerebral protection, with a statistically significant reduction of strokes to 0% in the protected group.

Experimental studies on plaques and embolisation

Ex-vivo carotid stenting models have been used to gain insight into the process of embolisation during CAS. A model which used an endarterectomy specimen enclosed in a PTFE tube showed that distal filtering devices trap 88% of emboli.[72, 73] All emboli greater than 360 micrometer were caught. The debris found in the effluent fluid was comparable to material observed in distal filter devices in in-vivo studies. Use of statins decreased emboli count and maximum emboli size.[74] Smaller emboli may be less harmful to the brain, which was confirmed by Rapp and coworkers in a rat model.[75] The protective influence of statins on experimental embolisation is consistent with the positive effect of statins on plaque stability.[76-79]
Muller-Hulsbeck and colleagues pointed out that movement of a protection device along the vascular wall can cause embolisation.[80] Adverse movement of different filter devices was introduced and debris was captured in the effluent using a 100 micrometer filter. The study showed that protection devices themselves can be a source of embolisation, with several mg of debris caused by their placement and retrieval.

Approaches to minimize plaque related embolisation

Patient selection based on plaque imaging may determine clinical decision making in the near future. Since in-vivo and experimental studies show that embolisation is related to vulnerable plaques, plaque composition as determined by in vivo plaque imaging may be used for treatment allocation. Patients with more vulnerable plaques could preferably undergo CEA or CAS with endovascular occlusion, because plaque related embolisation forms a smaller problem in these procedures.
Apart from protection devices, which are discussed elsewhere, there are other mechanical approaches to minimize plaque related embolisation.[81] Covered stents may prevent embolisation during stent deployment and postdilation, which is the most critical embolisation phase.[82, 83] Disadvantage is a bigger diameter when undeployed, which can lead to difficulty in passing the stenosis with associated embolisation.

Yet another approach to minimize embolisation could be more extensive use of statins. Statins have a protective effect on stroke, although there is no relation between cholesterol levels and stroke.[84, 85] The mechanisms through which statins exert these effects are antithrombotic effects and anti-inflammatory effects on the plaque.[86, 87] In addition, statin use improves outcome after stroke and after carotid endarterectomy.[88-90] In combination with the protective effects of statin on ex-vivo embolisation, these findings suggest that statin use could be associated with less and smaller emboli resulting in better clinical outcome after CAS.

Conclusion

CAS related embolisation is very frequent compared to embolisation during CEA. A multitude of embolic signals is detected by transcranial Doppler. Diffusion weighted MRI shows that these multiple emboli cause new ischemic lesions in 23-57% of patients. Interestingly, these lesions are asymptomatic in over 90% of patients. Thus, the concept arises that although a high embolic load is induced by CAS, the brain has sufficient resistance to clear these emboli. The resistance of the brain is broken by high embolic loads within a short time (subsequent showers of emboli), which are associated with adverse outcome. Embolisation can be effectively counteracted by endovascular occlusion devices. In contrast, distal filter devices increase embolic load in some studies. Older age and symptomatic clinical presentation are associated with a higher microembolic load across studies. This is consistent with the finding that these patients have higher chance of immediate adverse outcome and more unstable carotid plaques. As shown in carotid endarterectomy series and ex vivo stenting models, unstable plaques shed more emboli then stable plaques. These plaque characteristics can be integrated into the clinical process by plaque imaging. Low plaque echolucency is associated with higher chance of peri-interventional stroke. The authors of the ICAROS study suggest that plaque echolucency should become part of treatment allocation. However, determination of plaque morphology based on echography has not been widely applied. Evolving imaging techniques such as molecular MRI and optical coherence tomography will be able to provide a link from vascular biology to the patient. Fine-tuning of treatment based on the plaque may hold a great promise for the future to reduce microembolisation and adverse clinical events associated with CAS.

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CHAPTER 10

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ATHEROSCLEROTIC PLAQUE HISTOLOGY AND CLINICAL OUTCOME AFTER CAROTID ENDARTERECTOMY

Background It is a major challenge to identify patients at high risk for primary and secondary manifestations of cardiovascular disease. The composition of atherosclerotic plaques is thought to be an important risk factor for future cardiovascular events. However, prospective studies have not been performed to test if plaque composition is associated with the occurrence of vascular events during follow-up.

Methods The Athero-Express study includes patients who undergo carotid endarterectomy. Endarterectomy specimens obtained between April 1, 2002 and March 11, 2008 were subjected to histological examination. Patients underwent clinical follow-up yearly, up to 3 years after carotid endarterectomy. The primary outcome was defined as the composite of vascular event (vascular death, non-fatal stroke, non-fatal myocardial infarction) and vascular intervention.

Findings During a mean follow-up of 2.3 years, 196 of 818 patients (24%) reached the primary outcome. Patients whose excised carotid plaque revealed presence of a thrombus demonstrated 30.6% risk of the primary outcome compared to 17.2% in patients without plaque thrombus (hazard ratio [HR] with 95% confidence interval = 1.7 [1.2-2.5]). Local macrophage infiltration (HR=1.1 [0.8-1.5]), large lipid core (HR=1.1 [0.7-1.6]), calcifications (HR=1.1 [0.8-1.5]), collagen (HR=0.9 [0.7-1.3]) and smooth muscle cell infiltration (HR=1.3[0.9-1.8]) were not associated with outcome. The relation between local plaque thrombus and clinical outcome was independent of recognized clinical risk factors and medication use.

Interpretation Presence of thrombus in a dissected carotid plaque is independently associated with an increased risk of future cardiovascular events. This is the first study demonstrating that the histological composition of a single atherosclerotic plaque contains information about systemic cardiovascular outcome.

Introduction

The clinical manifestations of atherosclerosis still form the number one cause of mortality in Western society. It has been recognized for some time now complications of atherosclerotic disease such as myocardial infarction and stroke are caused by acute thrombosis which is triggered by atherosclerotic plaque instability, rather than by gradually progressive luminal narrowing.[1-5] Furthermore, from cross-sectional pathology studies, evidence has accumulated that atherosclerotic plaques destabilization is strongly related to certain plaque characteristics, like a large lipid core, thin fibrous cap, and marked inflammation.[6]

However, current knowledge on predictive value of plaque characteristics for future events is severely hampered by the fact that no prospective studies have been performed, that link local plaque composition to clinical outcome during follow-up.[7] Evidence originating from previously conducted cross-sectional studies suggesting that vulnerable plaque characteristics portray an increased risk of future cardiovascular events can be considered circumstantial. Prospective studies are needed to provide insight into the natural process of atherosclerotic disease progression and to deliver clinically applicable markers to identify patients at risk for future atherosclerosis related events.

Since atherosclerosis is a systemic disease,[8-12] and plaque composition correlates between different arterial segments within individuals,[13, 14] we hypothesized that plaque composition of a single atherosclerotic plaque could provide information on systemic cardiovascular outcome. Therefore, we conducted a prospective study to investigate the relation between the composition of the atherosclerotic plaque in the carotid artery and cardiovascular events originating from all vascular territories during follow-up. For this purpose, we included a consecutive cohort of patients undergoing carotid endarterectomy (CEA). In this patient cohort, we related histological characteristics of the plaque excised at baseline to clinical outcome during 3-year follow-up.

Methods

Athero-Express biobank

Athero-Express is an ongoing longitudinal study which includes patients undergoing carotid endarterectomy (CEA). The study design has been reported earlier.[15] In short, plaques obtained during carotid endarterectomy are collected and processed following a standardized protocol. After the operation, the patients undergo follow-up. All patients undergoing carotid endarterectomy in the participating centers (St. Antonius Hospital Nieuwegein and University Medical Center Utrecht) were asked to participate in the study. The medical ethics board of the participat-

ing hospitals approved the study and all participants of the study provided written informed consent. For this study, the inclusion started on April 1, 2002 and ended on March 11, 2008.

Patient population

The indication to perform CEA was guided by the outcomes of the ACAS and ACST trials for asymptomatic patients and the NASCET and ECST trials for symptomatic patients.[16-20] All indications for surgery were reviewed in a multidisciplinary vascular team. The patients filled out questionnaires considering cardiovascular risk factors, medical history and medication use. Further clinical data were obtained from patient charts. The definitions of hypertension, hypercholesterolemia and diabetes were restricted to those cases requiring medical treatment. All patients were ex-

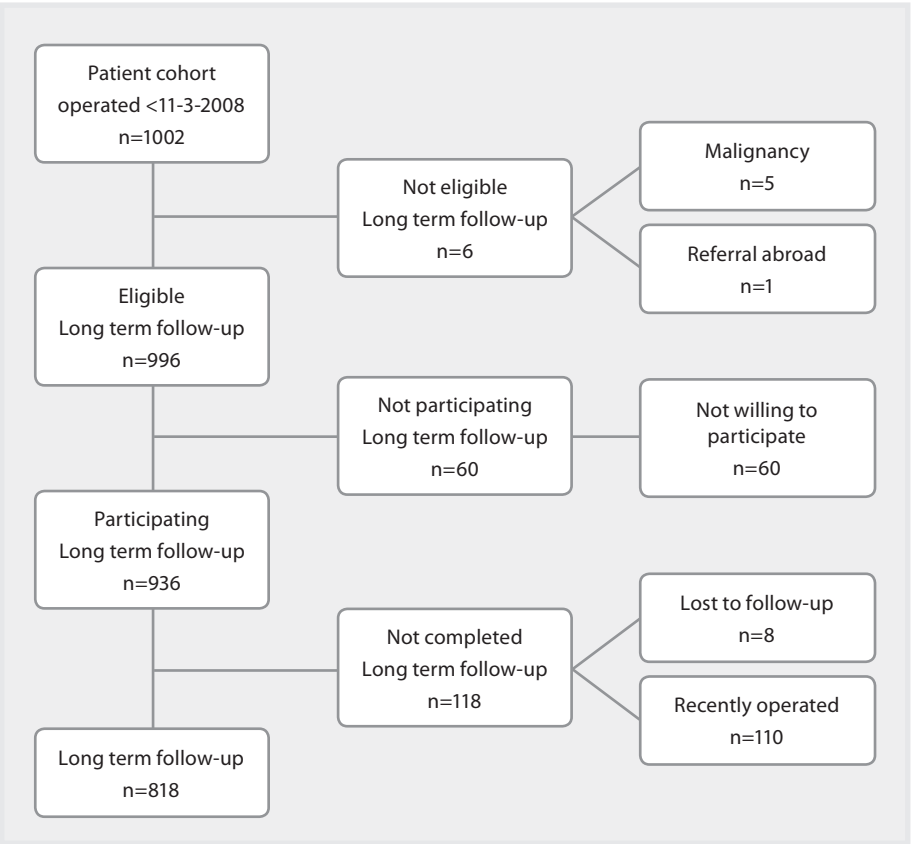

Figure I Flow chart describing flow of patients included in the study

amined by a neurologist pre-operatively and post-operatively to document the cerebrovascular symptom status and to record any new neurologic deficits after CEA. Percentage stenosis of both carotid arteries was recorded with duplex ultrasound pre-operatively following internationally accepted guidelines.[21]

Carotid endarterectomy

Pre-operatively, patients were started on aspirin, except patients taking oral anticoagulants for other indications and patients with contra-indications for aspirin use. Before exposure of the carotid artery, patients received 5000 units of heparin intravenously. Using a non-eversion technique, the plaque was carefully dissected and removed in toto. Immediately after dissection, the plaque was transferred to the laboratory.

Histological examination

According to a standardized protocol, the plaque was divided in segments of 5 mm thickness along the longitudinal axis. The segment with the greatest plaque burden was subjected to histological examination as described previously.[15] Macrophage infiltration (CD68), smooth muscle cell infiltration (alpha-actin), the amount of collagen (Picro-sirius Red (PS)) and calcification (Hematoxylin and eosin (H&E)) were semi-quantitatively scored as (1) "no or minor" or (2) "moderate or heavy" staining. The criteria for classification were defined as follows: Macrophages: (1) absent or minor CD-68 staining with negative or few scattered cells; (2) moderate or heavy staining, clusters of cells with >10 cells present; Smooth muscle cells: (1) minor alpha-actin staining over the entire circumference with absent staining at parts of the circumference of the arterial wall; (2) positive cells along the circumference of the luminal border, with locally at least few scattering cells; Collagen staining: (1) no or minor staining along part of the luminal border of the plaque; (2) moderate or heavy staining along the entire luminal border. Thrombus, the definition of which included both luminal thrombus and intra-plaque hematoma, were examined in H&E and Elastin von Gieson (EvG) stainings and rated as being "absent" or "present".[22] The size of the lipid core was visually estimated as a percentage of total plaque area using H&E and PS stains, with a division in three categories: <10%, 10 - 40% and >40%. The histological examination showed good to excellent intra-observer and inter-observer reproducibility on the different items (κ = 0.6 - 0.9).[23] In addition, the stainings for macrophages and smooth muscle cells were scored quantitatively using computer-based analyses. The semi-quantitative and quantitative scorings revealed an excellent correlation.[23]

Follow-up and outcome

Definition of outcome

The primary outcome was defined as any vascular event or intervention. This composite endpoint included any death of presumed vascular origin (fatal stroke, fatal myocardial infarction, sudden death, other vascular death), non-fatal stroke, non-fatal myocardial infarction, and any arterial vascular intervention (e.g. carotid surgery or angioplasty / stenting, coronary bypass, percutaneous coronary intervention, peripheral vascular surgery or angioplasty / stenting). For additional analyses, the primary outcome was divided into the outcome events in separate vascular territories: stroke, coronary event (myocardial infarction or coronary intervention), peripheral vascular intervention (all vascular intervention except coronary interventions), non-stroke vascular event (primary endpoint except stroke). Peri-operative events were defined as outcome events occurring ≤ 30 days after surgery.

Table I Clinical characteristics of patients undergoing carotid endarterectomy

Patient Characteristics		
Mean age, years (range)	68.1	(37 – 91)
Sex		
male, n (%)	571 / 818	(70%)
female, n (%)	247 / 818	(30%)
Current smoker, n (%)	219 / 797	(28%)
Diabetes, n (%)	157 / 816	(19%)
Hypertension, n (%)	542 / 814	(67%)
Hypercholesterolemia, n (%)	513 / 817	(63%)
History: Vascular intervention	272 / 816	(33%)
History: Myocardial infarction	174 / 813	(21%)
Mean body mass index, kg/m^2 (sd)	26.5	(3.7)
Mean serum creatinin, µmol/l (sd)	98.7	(41.0)
Statin use, n (%)	623 / 816	(76%)
Aspirin use, n (%)	728 / 816	(89%)
Oral anticoagulant use, n (%)	117 / 816	(14%)
Dipirydamole use, n (%)	392 / 816	(48%)
Bilateral carotid stenosis, n (%)	349 / 818	(43%)
Clinical presentation		
asymptomatic, n (%)	153 / 818	(19%)
TIA, n (%)	479 / 818	(58%)
stroke, n (%)	186 / 818	(23%)

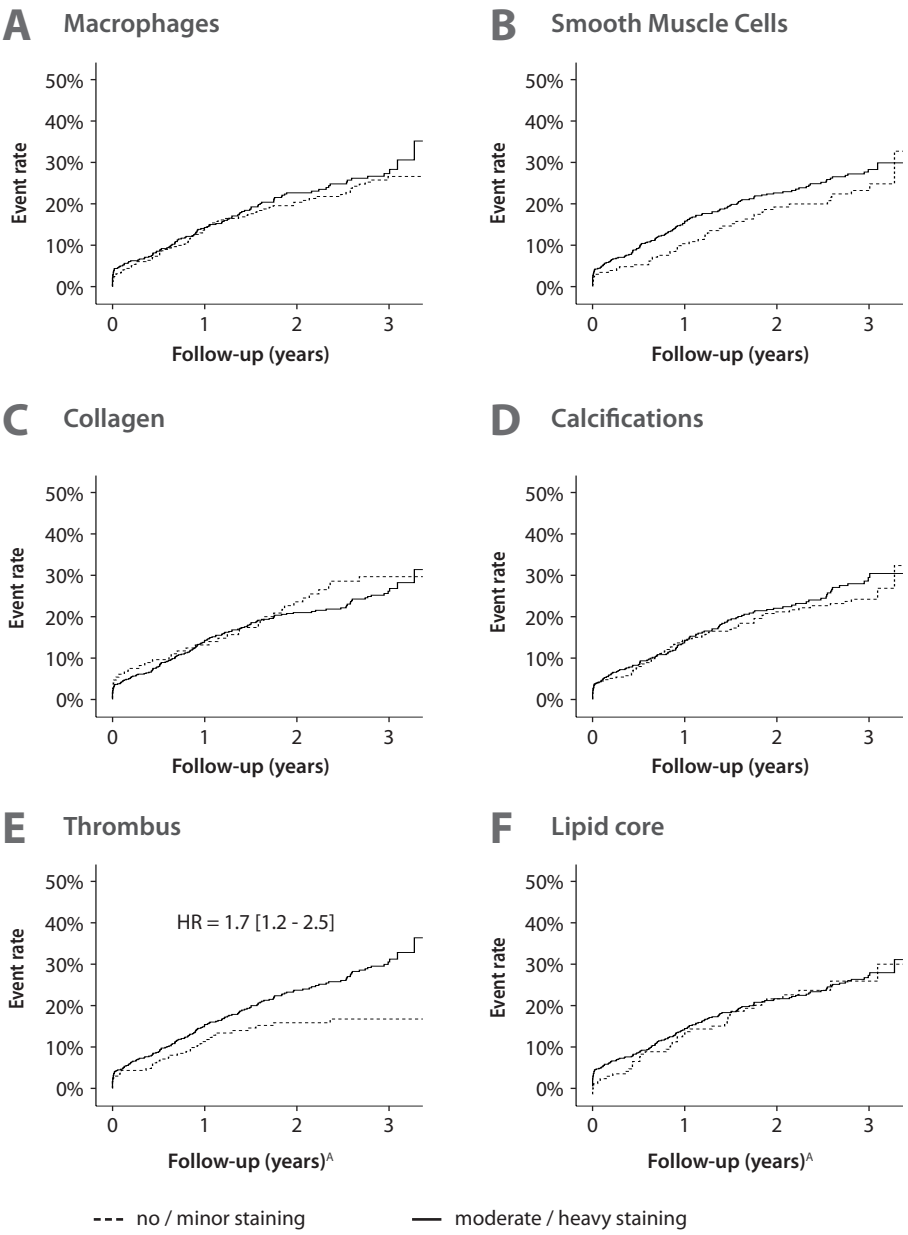

Figure II Kaplan-Meier survival curves of plaque histology versus primary outcome

A: Macrophages **B:** Smooth muscle cells **C:** Collagen **D:** Calcifications **E:** Thrombus **F:** lipid core
[A]Thrombus: absent (dashed line) vs. Present and lipid core: <10% (dashed line) vs. ≥ 10%

Follow-up protocol

After carotid endarterectomy, all patients underwent clinical follow-up. First, all perioperative adverse events were recorded from the patient charts. At 1, 2 and 3 years after the operation patients received a questionnaire if patients had experienced any vascular event and if patients had been hospitalized in the past year. If any of these was answered positively, further research was performed to check the potential outcome event. Following a standard scheme, discharge letters, and if needed laboratory measurements and results of additional studies such as electrocardiograms or imaging studies were collected from the institution where the potential event occurred. If patients did not respond to the follow-up questionnaire, the general practitioner was contacted. Per potential outcome event, all available information was assessed by two members of the outcome assessment committee. If the two members disagreed if the criteria for the outcome event were met, a third opinion was requested.

Statistics and data analysis

SPSS 15.0 was used for all analyses (SPSS Inc, Chicago, Illinois). Kaplan-Meier Survival analysis was used to obtain cumulative event rate. The survival curves were compared between the two groups (no/minor vs. moderate/heavy staining) for each plaque characteristic, or between absent vs. present for thrombus. The Kaplan-Meier estimate of cumulative event rate at 1, 2 and 3 year after CEA, and the corresponding risk difference according to plaque characteristics at baseline were calculated. Univariate Cox regression was used to obtain the hazard ratio with 95% confidence interval [95% CI]. A statistically significant association with clinical outcome was defined as a 95% confidence interval not including 1 or $P<0.05$. Additionally we related perioperative events, defined as primary outcome within 30 days after the index procedure, to baseline plaque characteristics in 2x2 tables with Chi-Square test to assess statistically significant association. Plaque characteristics showing statistically significant association with clinical outcome were further analyzed. Further, in a more detailed analysis these plaque characteristics were also studied in relation to vascular outcomes in the different vascular territories. To adjust for potential confounders, we performed a bivariate analysis to check if the hazard ratio obtained in univariate analysis changed when one of the clinical parameters was added to the model. A change of the hazard ratio > 0.10 was considered a significant change. All parameters causing a change in the hazard ratio >0.10 were included in the multivariate model to obtain the adjusted hazard ratio.

Table II Carotid plaque histology in relation to primary outcome

Plaque characteristics	Number of patients with event [A]	Cumulative risk (KM estimate)			Risk difference (vs. ref)			HR [95% CI]
		1 year	2 years	3 years	1 year	2 years	3 years	
Macrophage infiltration								
No / minor	76 / 316	14.0%	20.5%	26.5%		- ref -		- ref -
Moderate / heavy	119 / 497	15.2%	23.2%	27.6%	+ 0.8%	+ 2.7%	+ 1.1%	1.1 [0.8 – 1.5]
Smooth muscle cell infiltration								
No / minor	47/ 225	10.7%	18.8%	22.7%		- ref -		- ref -
Moderate / heavy	147 / 589	16.2%	23.3%	28.1%	+ 5.5%	+ 4.5%	+ 5.4%	1.3 [0.9 – 1.8]
Collagen								
No / minor	37 / 146	13.8%	23.1%	29.0%		- ref -		- ref -
Moderate / heavy	157 / 669	14.7%	21.6%	26.5%	+ 0.9%	- 1.5%	- 2.5%	0.9 [0.7 – 1.3]
Calcification								
No / minor	77 / 331	15.5%	21.7%	24.6%		- ref -		- ref -
Moderate / heavy	119 / 486	14.3%	22.4%	29.6%	- 0.8%	0.7%	5.0%	1.1 [0.8 – 1.5]
Thrombus								
Absent	36 / 227	12.0%	16.4%	17.2%		- ref -		- ref -
Present	160 / 591	15.8%	24.1%	30.6%	+ 3.8%	+ 7.7%	+ 13.4%	1.7 [1.2 – 2.5] *
Lipid core size								
< 10%	41 / 174	13.4%	21.7%	25.9%		- ref -		- ref -
10% - 40%	90 / 379	15.6%	22.5%	27.9%	+ 2.2%	+ 0.8%	+ 2.0%	1.1 [0.7 – 1.5]
> 40%	65 / 265	14.4%	21.7%	26.9%	+ 1.0%	0.0%	+ 1.0%	1.1 [0.7 – 1.6]

Abbreviations: CI, confidence interval; HR, Hazard ratio; KM, Kaplan Meier; Ref, reference.

[A] primary outcome (vascular event or vascular intervention); *P<0.05

Table III Carotid Plaque thrombus in relation to outcome events in different vascular territories

Endpoint [A]	Number of patients with event	Cumulative risk (KM estimate)			Risk difference (vs. ref)			HR [95% CI]
		1 year	2 years	3 years	1 year	2 years	3 years	
Primary outcome								
No thrombus	36 / 227	12.0%	16.4%	17.2%		- ref -		- ref -
Thrombus	160 / 591	15.8%	24.1%	30.6%	+ 3.8%	+ 7.7%	+ 13.4%	1.7 [1.2 – 2.5] *
Vascular event								
No thrombus	17 / 227	5.3%	7.7%	8.6%		- ref -		- ref -
Thrombus	82 / 591	9.5%	12.8%	15.5%	+ 4.2%	+ 5.1%	+ 6.9%	1.8 [1.1 – 3.1] *
Stroke								
No thrombus	9 / 227	3.1%	4.2%	4.2%		- ref -		- ref -
Thrombus	50 / 591	6.8%	8.3%	9.5%	+ 3.7%	+ 4.1%	+ 5.3%	2.1 [1.1 – 4.4] *
Coronary event								
No thrombus	12 / 227	3.1%	6.1%	6.1%		- ref -		- ref -
Thrombus	51 / 591	3.8%	7.1%	10.3%	+ 0.7%	+ 1.0%	+ 4.2%	1.6 [0.9 – 3.0]
Peripheral intervention								
No thrombus	19 / 227	6.4%	8.6%	9.4%		- ref -		- ref -
Thrombus	69 / 591	6.2%	10.9%	13.7%	- 0.2%	+ 2.3%	+ 4.3%	1.4 [0.8 – 2.3]
Non-stroke vascular event								
No thrombus	31 / 227	9.4%	13.9%	15.6%		- ref -		- ref -
Thrombus	117 / 591	10.1%	17.6%	23.2%	+ 0.7%	+ 3.7%	+ 7.6%	1.4 [1.0 – 2.1] *

Abbreviations: CI, confidence interval; HR, Hazard ratio; KM, Kaplan Meier; Ref, reference.
[A]definitions are given in methods section; *P<0.05

Table IV Multivariable analysis

	Risk of primary outcome	
	HR [95% CI]	HR of Plaque thrombus adjusted for characteristic
Crude Hazard Ratio plaque thrombus		1.7 [1.2 – 2.5]
Age ≥ 70	1.2 [0.9 – 1.6]	1.7 [1.1 – 2.5]
Male gender (%)	1.5 [1.1 – 2.2] *	1.6 [1.1 – 2.4]
Current smoker (%)	1.3 [0.9 – 1.8]	1.7 [1.2 – 2.5]
Diabetes (%)	1.0 [0.7 – 1.5]	1.7 [1.2 – 2.5]
Hypertension (%)	1.2 [0.8 – 1.6]	1.7 [1.1 – 2.5]
Hypercholesterolemia (%)	1.4 [1.0 – 1.9]	1.7 [1.2 – 2.5]
History: Vascular intervention	1.9 [1.4 – 2.6] *	1.6 [1.1 – 2.3] **
History: Myocardial infarction	1.6 [1.1 – 2.2] *	1.7 [1.1 – 2.5]
BMI ≥ 25 kg/m2	0.8 [0.6 – 1.2]	1.7 [1.2 – 2.5]
Statin use	1.7 [1.1 – 2.5] *	1.8 [1.2 – 2.6]
Aspirin use	0.7 [0.4 – 1.1]	1.7 [1.1 – 2.5]
Oral anticoagulant use	1.4 [1.0 – 2.2]	1.7 [1.1 – 2.5]
Dipirydamole use, n (%)	0.9 [0.7 - 1.3]	1.7 [1.2 – 2.6]
Bilateral carotid stenosis	1.8 [1.3 – 2.5] *	1.6 [1.1 – 2.4]
Symptomatic carotid stenosis	1.1 [0.7 – 1.6]	1.7 [1.2 – 2.5]
Adjusted Hazard Ratio plaque thrombus		1.6 [1.1 – 2.3]

This table shows the hazard ratios (HR) of clinical variables and the hazard ratio of plaque thrombus bivariately adjusted for each clinical variable. Variables for which adjustment caused a change in the hazard ratio of plaque thrombus more than 0.10, were included in the multivariable analysis. All analyses were performed in the sub group of patients with no missing values on any variable (n=726)

* $p < 0.05$

** Change of HR > 0.10

Results

In total, 1002 patients underwent carotid endarterectomy, of who 818 completed at least one year follow-up **(Figure I)**. One hundred and ten patients did not undergo follow-up yet because they were operated within one year of the end of the closure of data collection. In those patients, perioperative follow-up was available. The baseline characteristics of the patient population are given in **Table I**. During mean follow-up of 2.3 years (SD = 1.2, maximum 4.7), 204 patients (25%) reached the primary outcome.

Histological examination of the carotid endarterectomy specimen in relation to the clinical follow-up revealed that the presence of thrombus in the endarterectomy specimen was related with a higher event rate during follow-up. The Kaplan-Meier

estimate of 3-year risk was 30.6% in patients whose plaque revealed the presence of thrombus at baseline, versus 17.2% in patients with a plaque not having any thrombus (Hazard ratio = 1.7 [1.2 – 2.5]). The risk difference was not due to difference in perioperative events (n=45): 10/227 (4.4%) vs. 35/591 (5.9%; RR = 1.3 [0.7 - 2.7]), and increased with time: + 3.8% at 1 year, + 7.8% at 2 years and + 13.4% at 3 years.

Local macrophage infiltration and lipid core size in the dissected lesion, the hallmarks of the so-called vulnerable plaque, revealed no association with long-term outcome **(Figure II, Table II)**. Confirming earlier observations,[24] the size of the lipid core was associated with perioperative events: 41/644 (6.4%) for lipid core >=10% vs. 4/174 (2.3%) for lipid core <10% of plaque area (RR = 2.8 [1.1 - 7.6]). Smooth muscle cell and macrophage infiltration, collagen content and extent of calcifications were not associated with long term outcome or perioperative events **(Table II)**.

When we excluded patients with perioperative events from the analysis, the relation between plaque thrombus and the primary outcome remained intact: Kaplan-Meier estimate of cumulative event rate: 14.5% (no thrombus) vs. 26.2% (thrombus; HR = 1.8 [1.2 - 2.8]). Additionally, we performed analyses of plaque thrombus at baseline versus outcome in the different vascular territories: stroke, coronary events, peripheral interventions and non-stroke vascular events. **Table III** shows that the same trend was observed for events originating from all vascular territories with hazard ratios between 1.4 and 2.1.

In 221 plaques, the thrombus was located intraluminal (27%) and in 515 plaques (63%) the thrombus was located in the plaque. Of these plaques, 145 had both luminal and intraplaque thrombus. In 2 plaques, the location of the thrombus could not be determined reliably. The presence of intraplaque thrombus was associated with occurrence of the primary endpoint (HR = 1.5 [1.1 - 2.1]), while the presence of luminal thrombus did not show an equally strong association (HR = 1.2 [0.9 - 1.6]).

Thrombus was more frequently observed in male patients (76% vs. 64%; Δ= +12% [+6% - +19%]), patients with hypertension (75% vs. 66%; Δ= + 9% [+2% - +16%]), patients taking dipyridamole (76% vs. 69%; Δ= +7% [+1% - 13%]) and patients with bilateral carotid stenosis (77% vs. 69%; Δ= +7% [+1% - +14%]). Plaques of patients taking statins revealed a thrombus less frequently (70% vs. 79%; Δ= - 9% [-15% - -2%]). The presence of plaque thrombus was not associated with any of the other baseline characteristics. To investigate potential confounding we adjusted the relation between plaque thrombus and clinical outcome for all clinical parameters. In our patient group, the following parameters were associated with worse clinical outcome: male gender, previous vascular intervention, previous myocardial infarction, statin use and bilateral carotid stenosis. The hazard ratio of plaque thrombus changed > 0.10 when adjusting for previous vascular intervention **(Table IV)**; the adjusted hazard ratio for plaque thrombus = 1.6 [1.1 – 2.3].

Discussion

Athero-Express is the first study to link histological examination of local atherosclerotic plaque composition to clinical follow-up. In this longitudinal study, we found that plaque thrombus at a single plaque excised from the carotid artery is predictive of systemic cardiovascular outcome. The association of local plaque thrombus with clinical outcome could not be explained by traditional risk factors or other potential confounders.

There remains a pressing need for diagnostic tools to identify vulnerable patients at high risk for future cardiovascular events.[25, 26] Epidemiological approaches which link systemic markers to outcome, such as C-reactive protein,[27, 28] or sets of multiple circulating biomarkers,[29] have yielded limited predictive value so far and their clinical application is still being debated. Therefore, research focus has shifted to identify more specific markers of cardiovascular vulnerability. Autopsy series indicated that a ruptured or eroded plaque with superimposed thrombus is the pathological substrate of atherosclerotic events such as myocardial infarction and stroke.[4, 30] Plaque rupture has been associated with specific histological features, such as large lipid core and marked inflammation. Therefore, the presence of these so-called "vulnerable plaque characteristics", is thought to portray an increased risk of plaque rupture and thus clinical events. However, longitudinal imaging studies that are obligatory for validation of this concept are lacking.

In the current study we followed a different approach. We hypothesized that plaque characteristics may show similarities among vascular territories and related local histological plaque characteristics to all clinical events due to progression of atherosclerotic disease. For this purpose, we conducted a longitudinal study to correlate the vulnerable plaque characteristics of a single atherosclerotic lesion to systemic clinical outcome. We show that local plaque macrophage infiltration and large lipid core in local plaques, which are considered hallmark features of the vulnerable plaque, are not predictive of systemic cardiovascular outcome. In contrast, local plaque thrombus was shown to be an independent predictor of outcome. The prevalence of thrombus in our series is in line with the findings from Spagnoli et al, who found thrombi in 74% of carotid plaques from patients presenting with stroke and 35% of patients presenting with TIA.[4] Our hypothesis that the composition of a single atherosclerotic plaque could predict systemic cardiovascular outcome is supported by several findings. Atherosclerosis is a systemic disease,[8-12] and the plaque composition correlates between different vascular beds, both in coronary and peripheral arteries.[13, 14, 31] This study was not designed to link local plaque characteristics to future plaque instability of the local plaque itself, since the plaque is excised. The outcome of this study therefore does not challenge the current concept of plaque destabilization or the histopathological definition of the vulnerable plaque. The current study suggests however, that these local destabiliz-

ing plaque features may not become the biomarkers of first choice to assess risk for future cardiovascular events. Macrophage and lipid accumulation are widely spread throughout the vascular tree in patients with advanced atherosclerosis. The frequent observation of macrophage infiltration and large lipid pools in asymptomatic lesions,[32, 33] may explain the lack of predictive value for systemic cardiovascular outcome. In our opinion, for clinical management the prediction of clinical outcome will become of greater importance than the diagnosis of local plaque vulnerability. However, the latter will be of interest for mechanistic insights in the progression of atherosclerotic disease.

Kolodgie et al. have shown that intraplaque bleeding / thrombus is an important source of plaque progression.[34] Although unproven, a tendency for systemic intraplaque bleedings would explain the association between local (intra)plaque thrombus and clinical outcome in our study. Furthermore, intraplaque bleeding can also cause severe disruption of plaque integrity and thereby accelerate thrombosis with associated clinical events.[35] Our observations support the current idea that intraplaque bleeding may be an important determinant of plaque growth and disease progression. It was specifically not the goal of our study to provide mechanistic explanations, and further research is needed to elaborate on this subject.

To our knowledge, no studies investigating plaque histology in relation to clinical outcome have been conducted previously. One prospective imaging study has attempted to connect local plaque characteristics to systemic cardiovascular outcome. A study investigating plaque irregularities by angiography in relation to clinical outcome revealed that the presence of an irregular carotid plaque was related with increased incidence of cardiovascular death during follow-up.[31] Three years after carotid endarterectomy, there was no difference between the group with smooth and irregular plaques, and the difference only became evident after long term follow-up (10-years). Most imaging studies however, focus on local plaque composition to predict local outcome, e.g. the composition of coronary plaques in relation to future myocardial infarction. The IBIS study performed intravascular ultrasound (IVUS) and IVUS-based elastography and correlated the baseline findings to 6-months follow-up.[36] After six months of follow-up no difference in IVUS detected lumen and plaque dimensions was found in the coronary system, probably due to limited patient number and limited follow-up duration. More advanced plaque imaging techniques and improvement of current techniques, such as high resolution magnetic resonance imaging, as well as large scale follow-up studies with plaque imaging are needed to help to better characterize plaques in vivo and thus enable the evaluation the natural history of plaque progression towards instability and associated clinical manifestations non-invasively.[37]

Our study has potential clinical implications. The results indicate that plaque thrombus could serve as a target for plaque imaging, with the objective to serve as a biomarker for future cardiovascular events. Local carotid thrombus detection could

serve as a surrogate endpoint in clinical studies. Using magnetic resonance direct thrombus imaging (MRDTI), visualization of plaque thrombus is feasible.[38] A pilot study already suggested that MRI detected plaque thrombus was associated with adverse clinical events during follow-up.[39] At present, pathohistological examination of atherosclerotic plaques excised during surgical interventions is not common practice. This study and our previous report [40] clearly demonstrate that thrombotic, inflammatory and lipid components hide prognostic value for future cardiovascular events or restenosis development and therefore strengthen the idea that pathological examination of dissected plaques should be executed after surgery.

Our study has some potential weaknesses. As mentioned previously, the carotid plaque was excised and therefore, by definition, we could not link the carotid plaque composition to local clinical outcome (i.e. stroke) or local plaque progression. Next, we did not investigate plaque rupture as a determinant of outcome. In our and others' experience,[41] plaque rupture is difficult to assess reliably in carotid endarterectomy specimens and therefore it was not included in our study. The present study also has unique strengths. It encompasses the largest cohort of patients presented so far whose carotid plaque was examined using the gold standard: histology. Second, the plaque histology was linked to clinical follow-up, which has been lacking in studies conducted so far.

In conclusion, this is the first study to investigate the histological composition of local atherosclerotic plaques in relation to clinical outcome. We report that the presence of thrombus in the carotid endarterectomy specimen is independently associated with increased risk of future cardiovascular events.

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PART 5

BIOMARKERS IN THE CAROTID PLAQUE PROTEIN

CHAPTER 11

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NGAL AND NGAL/MMP-9 COMPLEX IN RELATION TO ATHEROSCLEROTIC PLAQUE COMPOSITION AND MYOCARDIAL INFARCTION

Aims Neutrophil Gelatinase-associated Lipocalin (NGAL) is an effector molecule of the innate immune system. One of its actions is enhancement of MMP-9 activity by formation of a degradation resistant NGAL/MMP-9 complex. Considering the role of MMP-9 in atherosclerotic plaque instability, we studied the expression of NGAL in relation to plaque phenotype and myocardial infarction.

Methods and Results Increased gelatinolytic activities of the NGAL/MMP-9 complex (zymography) were observed in atherosclerotic plaques obtained during carotid endarterectomy (n=122) compared to control mammary arteries (n=10), and associated with macrophage infiltration and large lipid core. MMP-9 activity (ELISA) was strongly related with NGAL and NGAL/MMP9 (ELISA) levels. In vitro experiments showed that NGAL enhanced MMP-9 activity in a dose-dependent manner. NGAL/MMP-9 complex was locally elevated in the blood, drawn near the carotid atherosclerotic plaque, compared to peripheral blood levels, suggesting release into the systemic circulation. In addition, in patients suffering from acute myocardial infarction (n=23), circulating levels of NGAL and NGAL/MMP-9 complex were increased compared to controls (n=10).

Conclusions The high expression of NGAL and NGAL/MMP-9 complex in unstable plaques, and the increased circulating levels in patients with acute myocardial infarction suggest the involvement of NGAL in unstable atherosclerotic disease.

Introduction

Neutrophil gelatinase-associated lipocalin (NGAL) is a 25 kD glycoprotein which was discovered in human neutrophils.[1,2] It is a member of the family of lipocalins, which are funnel-shaped proteins with a central binding site for lipophilic proteins. NGAL is an effector molecule of the innate immune system through inhibition of bacterial iron uptake by binding bacterial siderophores.[3] It is also thought to mediate inflammatory activity because it can bind to formyl-methionyl-leucyl-phenylalanine (fMLP; a chemotactic peptide), leukotriene B4 and platelet activating factor.[4-6] Production of NGAL is induced via the nuclear factor kappa B (NFκB) pathway, and highly increased levels are found in inflammatory conditions.[5,7] In relation to MMP activity, NGAL is able to form a stable, biologically active complex with matrix metalloproteinase 9 (MMP-9), which prevents MMP-9 degradation, and therefore prolongs activity of MMP-9.[8] In addition, NGAL is involved in the allosteric activation of MMP-8 and MMP-9.[9]

The Matrix Metalloproteinases (MMPs) are key players in atherosclerotic disease. They are capable of degrading a broad spectrum of extracellular matrix components and held responsible for vascular remodeling and breakdown of the fibrous cap of atherosclerotic lesions.[10] Especially MMP-9 is implicated to play a crucial role in atherosclerotic plaque destabilization, both in human studies and experimental models.[11-15]

Hemdahl et al demonstrated that the mouse analogue of NGAL, 24p3, was present in mouse atherosclerotic lesions and that NGAL was present in human atherosclerotic lesions, co-localized with MMP-9.[16] Considering its function in the innate immune system and its effect on MMP-9 activity, NGAL might play an important role in unstable atherosclerotic disease. To investigate this hypothesis, we investigated the presence of NGAL and the complex of NGAL with MMP-9 in atherosclerotic plaques, its association with plaque histology, inflammation and MMP activity, and circulating levels in patients with myocardial infarction.

Methods

Atherosclerotic specimen

Carotid plaques were obtained from a consecutive series of patients undergoing carotid endarterectomy (N=122), participating in the Athero-Express biobank.[17] This ongoing biobank is running in two Dutch Hospitals: The University Medical Center in Utrecht and the St. Antonius Hospital Nieuwegein. These patients all suffered from high grade (>70%) carotid stenosis. Indications for carotid endarterectomy were based on recommendations from the NASCET, ECST, ACAS and ACST trials.[18-21] In a subset of these patients, two blood samples were drawn during carotid endart-

erectomy: one directly from the carotid artery in the proximity of the atherosclerotic plaque and one from a peripheral catheter in the radial artery. Non-atherosclerotic mammary artery segments (n=10), obtained during coronary artery bypass grafting, served as arterial control tissues.

Serum samples from patients with myocardial infarction

Peripheral arterial blood samples were obtained from twenty-three consecutive patients with acute myocardial infarction and 8 consecutive patients with stable coronary artery disease undergoing coronary angiography (University Medical Center Utrecht and St. Antonius Hospital Nieuwegein). The blood samples were drawn from the arterial sheath directly after sheath insertion. The diagnosis of myocardial infarction was confirmed by CK-MB levels > 2 times the normal upper limit.
The study was approved by the institutional review boards of the University Medical Center Utrecht and the St. Antonius Hospital Nieuwegein and all patients provided written informed consent. The investigation conforms to the principles outlined in the Declaration of Helsinki.

Carotid plaque characterization

The carotid endarterectomy specimens were divided in segments of 5 mm thickness along the longitudinal axis of the vessel. The segment with greatest plaque burden was embedded in paraffin for histological characterization. The directly adjacent segment was used for protein extraction by dissolving in 40 mM Tris-HCl (pH=7.5) at 4°C as previously described.[17] Paraffin sections of the plaques were stained and analyzed semi-quantitatively for macrophages (CD68), smooth muscle cells (Alpha-Actin), collagen (Picro-sirius Red), calcifications (Hematoxylin and Eosin (H&E)) and overall phenotype, as described previously.[17] Interleukin-6 (IL-6) and IL-8 were measured in plaque protein extracts with a multiplex suspension array system according to the manufacturer's protocol (Bio-Rad Laboratories, California, USA). MMP-2, -8 and -9 activities were measured by Biotrak RPN2631, RPN2635 and RPN2634, respectively. (Amersham Biosciences, Buckinghamshire, UK). The measurements for MMPs and interleukins were standardized for total protein concentration of the protein extracts. (DC protein assay, Bio-Rad).

Detection of NGAL in carotid plaques

NGAL concentration in protein extracts of the tissue specimens was measured with a NGAL Enzyme-Linked Immunosorbent Assay (ELISA) kit, according to the manufacturer's protocol (Antibodyshop, Gentofte, Denmark). This assay has no cross-reactivity with NGAL/MMP-9 complexes. The measurements were standardized for total protein concentration of the protein extracts.

Detection of NGAL/MMP-9 complex in carotid plaques

Gelatin zymography was performed as described before.[22] An equal amount of total protein extract was loaded for each sample (1 µg). The combined gelatinolytic activity of NGAL/MMP-9 (125 kD) and NGAL-dimer/MMP-9 (150 kD) was measured as the degradation of gelatin at their these molecular sizes and further referred to as NGAL/MMP-9 gelatinolytic activity.[8] Gelatinolytic activity of free MMP-9 (92kD) was measured and is further referred to as MMP-9 gelatinolytic activity.

Western blotting for NGAL was additionally performed using monoclonal rat-anti-human NGAL antibody (5 µg/mL; R&D Systems) followed by biotinylated rabbit-anti-rat IgG (0.675 µg/mL; DAKO, Glostrup, Denmark) and horseradish peroxidase conjugated streptavidin.

Levels of the NGAL/MMP-9 complex were determined by ELISA (R&D Systems, Minneapolis, MN). An equal amount of protein extract was loaded for all samples. However, because the sensitivity of the ELISA was relatively limited (lower detection limit 312 ng/mL for NGAL/MMP-9 ELISA compared to 10 ng/mL for NGAL ELISA), only a limited subset of samples with high protein concentrations could be used for the analysis due to loading limitations.

Interaction of NGAL and MMP-9 in vitro

In vitro reconstruction of the NGAL/MMP-9 complex was performed by incubation of recombinant human MMP-9 (Oncogene sciences, PF024; concentration 0.05 µM) with recombinant human NGAL (R&D Systems, 1757 LC) at 1:1 molar ratio in 50 mM sodium acetate (pH 7.0) for 1 hour in the presence of Dithiobis (sulfosuccinimidyl propionate, DTSSP) cross linker. In order to assess NGAL dependent preservation of MMP-9 activity as shown by Yan et al,[8] MMP-9 was mixed with NGAL at different molar ratios (MMP-9:NGAL 1:0 - 5:1 – 1:5 – 1:25 - 1:125) in gelatinase buffer (50mM Tris, 5mM Calcium Chloride, 1 µM Zinc Chloride; pH 7.0). The mixture was incubated for 30 minutes at 37^{0}C to induce autodegradation of MMP-9, and put on ice for 1 hour with (DTSSP) cross linker. Subsequently, residual MMP-9 activity was measured by gelatin zymography.[22]

NGAL immunohistochemistry

Paraffin sections (5-µm thickness) were rehydrated, blocked for 30 minutes in 1.5% hydrogen peroxide in methanol, and boiled for 12 minutes in 10 mM sodium citrate (pH 6.0). After pre-incubation with 10% normal rabbit serum in 1% PBSA for 1 hour, incubation with primary antibody was performed overnight (monoclonal rat-anti-NGAL, 10ug/mL; R&D Systems,). Biotinylated rabbit-anti-rat IgG (0.4ug/mL; DAKO) was used as the secondary antibody, followed by horseradish peroxidase conjugated streptavidin, AEC substrate and counterstaining with hematoxylin. Specificity of the staining was ascertained by absence of staining in isotype controls and increas-

ing intensity of the staining with increasing concentrations of the primary antibody. Co-localization with macrophages, smooth muscle cells and endothelial cells was assessed by double staining, which was performed by NGAL staining under the same conditions, using Fast Blue substrate, followed by CD68 staining (KP-1, 3.3 µg/mL; DAKO), Alpha-actin (1A4, 1:1500; Sigma, St. Louis, MO) and CD34 (Qbend10, 0.5 µg/mL; Beckman Coulter, Fullerton, CA) with AEC substrate, respectively. Neutrophil infiltration was assessed using myeloperoxidase staining (polyclonal rabbit anti-human, 1:10000; DAKO).

Release of NGAL and NGAL/MMP-9 complex from carotid plaques

To assess release of NGAL or NGAL/MMP-9 complexes from plaques into the systemic circulation, we applied a novel model comparable to the system applied by Maier et al.[23] A blood sample obtained at the site of the plaque was compared to a peripheral blood sample for the concentration of NGAL and NGAL/MMP-9 complex in 9 consecutive patients undergoing carotid endarterectomy (CEA). The difference in concentration between the local and peripheral obtained samples is a measure of secretion of the protein by the plaque. During CEA, the blood sample at the plaque site ("plaque-related blood") was drawn directly from the already exposed common carotid artery **(Figure IV)**. This was done after the blood had been halted for 2 minutes at the plaque site during test-clamping, which is routinely performed in our hospital before incision of the carotid artery. At exactly the same time, a peripheral blood sample was drawn from the indwelling arterial line in the radial artery. NGAL and NGAL/MMP-9 complex were measured by ELISA as described above.

Data analysis

All measurements are presented as mean +/- SEM. Associations between variables were tested by Mann-Whitney U test or Spearman's non-parametric correlation where appropriate. Wilcoxon's signed rank test was used to test significance for the carotid plaque secretion experiments. To adjust the relation between MMP-9 and NGAL for the presence of macrophages, a linear regression models was constructed in which NGAL and macrophage infiltration were entered as determinants and MMP-9 as the predicted variable. P-values < 0.05 were considered statistically significant.

Results

NGAL expression in atherosclerotic carotid plaques

The carotid plaques (n=122) did not show significantly higher NGAL expression levels than the control arteries (n=10; 8.4 ng/g [sem=0.79] and 7.1 ng/g [sem=1.74], respectively). However, in the atherosclerotic carotid plaques, a strong positive correlation was observed between total NGAL content and total MMP-9 activity (**Figure I-A**; R=0.528; p<0.001) and MMP-8 activity (R=0.654; p<0.001) as determined by activity ELISA, but no association with MMP-2 activity could be observed **(Figure I-B)**.

Table I Relation between plaque characterization and expression of NGAL

Plaque characteristics		NGAL/MMP-9 gelatinolytic activity (A.U.)	P	NGAL (ng/mg)	P
n		122		122	
Overall	Fibrous	4.6 +/- 1.0		45 +/- 6	
	F- Atheromatous	7.0 +/- 0.8	0.01 [A]	101 +/- 14	0.007 [A]
	Atheromatous	7.4 +/- 0.9		80 +/- 9	
MO	Minor	6.2 +/- 0.8	0.1	80 +/- 10	0.9
	Heavy	7.2 +/- 0.7		89 +/- 12	
SMC	Minor	7.6 +/- 0.9	0.03	95 +/- 14	0.05
	Heavy	6.1 +/- 0.6		79 +/- 10	
MO/SMC	SMC dominant	6.0 +/- 0.7	0.03	92 +/- 11	0.8
	MO dominant	8.4 +/- 0.3		82 +/- 16	
Collagen	Minor	7.6 +/- 1.0	0.08	86 +/-12	0.2
	Heavy	6.3 +/- 0.6		84 +/- 10	
Calcifications	Minor	7.0 +/- 0.7	0.6	94+/- 14	0.7
	Heavy	6.5 +/- 0.8		74 +/- 8	
IL-6	Low [B]	5.8 +/- 0.8	0.1 [A]	69 +/- 9	0.003 [A]
	High [C]	6.9 +/- 0.8		101 +/- 13	
IL-8	Low [B]	4.5 +/- 0.6	<0.001 [A]	49 +/- 4	<0.001[A]
	High [C]	8.6 +/- 0.8		128 +/- 15	

Abbreviations: A.U., artibrary units; F-Atheromatous, Fibro-Atheromatous; IL, interleukin; MO, macrophage infiltration; SMC, smooth muscle cell infiltration. All values are presented as mean +/- standard error of the mean.

[A] Spearman's correlation

[B] low: <median

[C] high: >= median

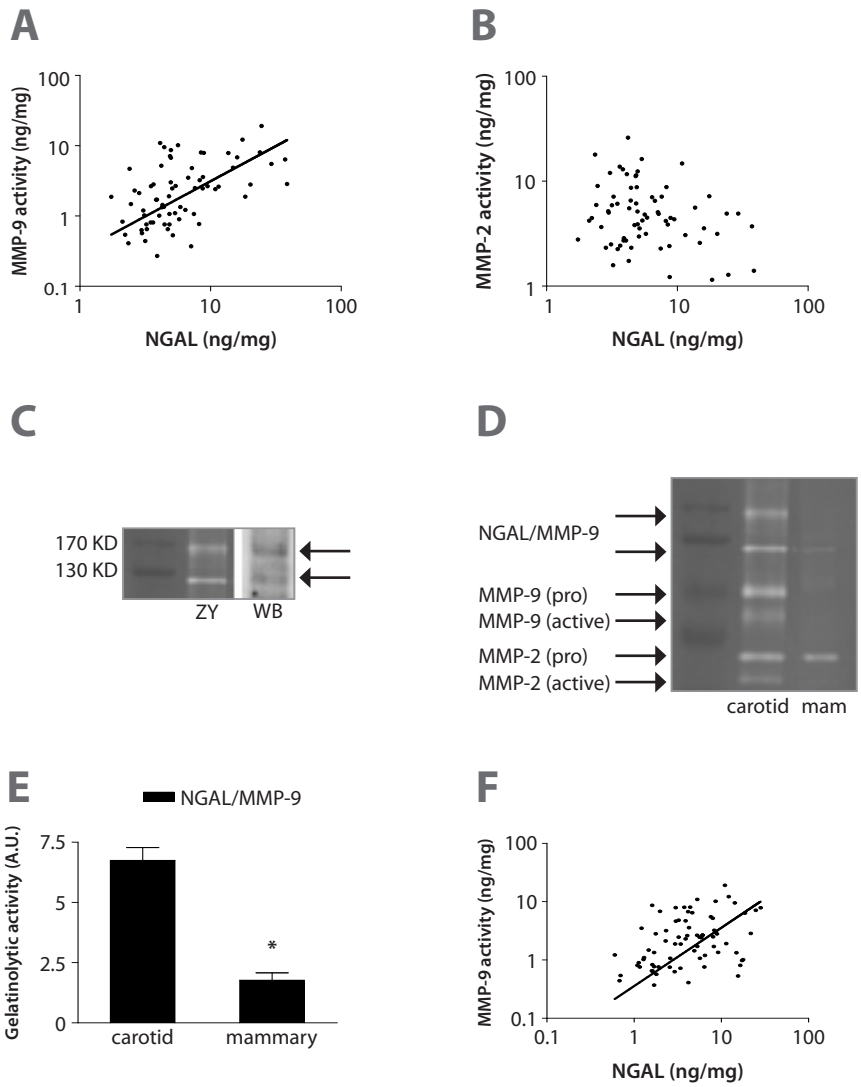

Figure I Association between NGAL and MMP activity

A: Association between plaque NGAL concentration and plaque MMP-9 activity (ELISA) (R=0.528; p<0.001). **B:** Association between plaque NGAL concentration and plaque MMP-2 activity (ELISA) (p=n.s.) **C:** Representative zymogram (ZY) and NGAL Western Blot (WB), demonstrating that the gelatinolytic active bands at 125 and 150kD contain NGAL. **D:** representative zymogram detecting NGAL/MMP-9 complex at 125kD and NGAL-dimer/MMP-9 complex at 150kD. **E:** Quantification of zymography among different tissues demonstrated that NGAL/MMP-9 activity is significantly higher expressed (p<0.01) in carotid plaques than in non-atherosclerotic mammary arteries. **F:** MMP-9 activity (Biotrak) is strongly related with gelatinolytic activity of the NGAL/MMP-9 complex (R=0.315; p=0.005) in carotid plaques.

Interaction of NGAL and MMP-9 in atherosclerotic carotid plaques

Gelatinolytic activity of NGAL/MMP-9 complexes (**Figure I-C**) was detected at 125 kD (NGAL/MMP-9) and 150 kD (NGAL-dimer/MMP9) as previously described by Yan et al.[8] This was confirmed via Western Blotting: specific NGAL bands were detected at 125 kD and 150 kD, corresponding to the two bands observed in zymography (**Figure I-C**). In addition, free NGAL was detected by Western blotting at 25 kD (monomer) and 50 kD (dimer; data not shown). The NGAL/MMP-9 complexes could be reconstructed in vitro by incubation of MMP-9 and NGAL recombinants (**Figure III-A**). Gelatinolytic activity of the NGAL/MMP-9 complex was significantly higher in carotid artery plaques compared to the control mammary arteries (**Figure I-D,E**). Moreover, as can be appreciated in **Figure I-D**, an important part of total gelatinolytic activity in the plaques was attributable to the NGAL/MMP-9 complexes (6.75 A.U.) compared to free MMP-9 (15.3 A.U.), which was confirmed by quantification of the NGAL/MMP-9 complex levels via ELISA in a subset of samples (0.60ng/g vs. 2.51 ng/g, respectively). Gelatinolytic activity of the NGAL/MMP-9 complex was strongly related to NGAL concentration (R=0.310; p=0.001) and MMP-9 activity (**Figure I-F**; R=0.315; p=0.005), whereas NGAL/MMP-9 gelatinolytic activity was not associated with MMP-8 or MMP-2 activity levels (data not shown).

NGAL and NGAL/MMP-9 complex versus plaque composition

Quantitative comparison of NGAL content and gelatinolytic activity of the NGAL/MMP-9 complex with plaque characteristics demonstrated that both were higher in plaques with an unstable phenotype (**Table I**). Atheromatous plaques, with larger necrotic core size, and dominance of macrophages had higher NGAL content and higher NGAL/MMP-9 gelatinolytic activity than stable plaques that are fibrous and have a higher smooth muscle cell content. Moreover, NGAL/MMP-9 gelatinolytic activity was strongly related with IL-8 content and NGAL concentration was strongly related with both IL-6 and IL-8 content. Together, these findings indicate a strong association of NGAL and NGAL/MMP-9 complex with unstable plaque characteristics.

The immunohistochemical stainings for NGAL demonstrated expression of NGAL in smooth muscle cells, macrophages and endothelial cells (**Figure II-A-D**). High NGAL expression was mainly observed in macrophages, and although NGAL expression was observed in smooth muscle cells and endothelial cells in the carotid plaques, their number was limited. **Figure II-B** shows an example of luminal endothelium with absent staining for NGAL. Additionally, neutrophils were examined as a possible source of NGAL within the plaques, but none or very few neutrophils were observed. Therefore, neutrophils are probably not an important source of NGAL in carotid atherosclerotic plaques. The smooth muscle cells in the media of the mam-

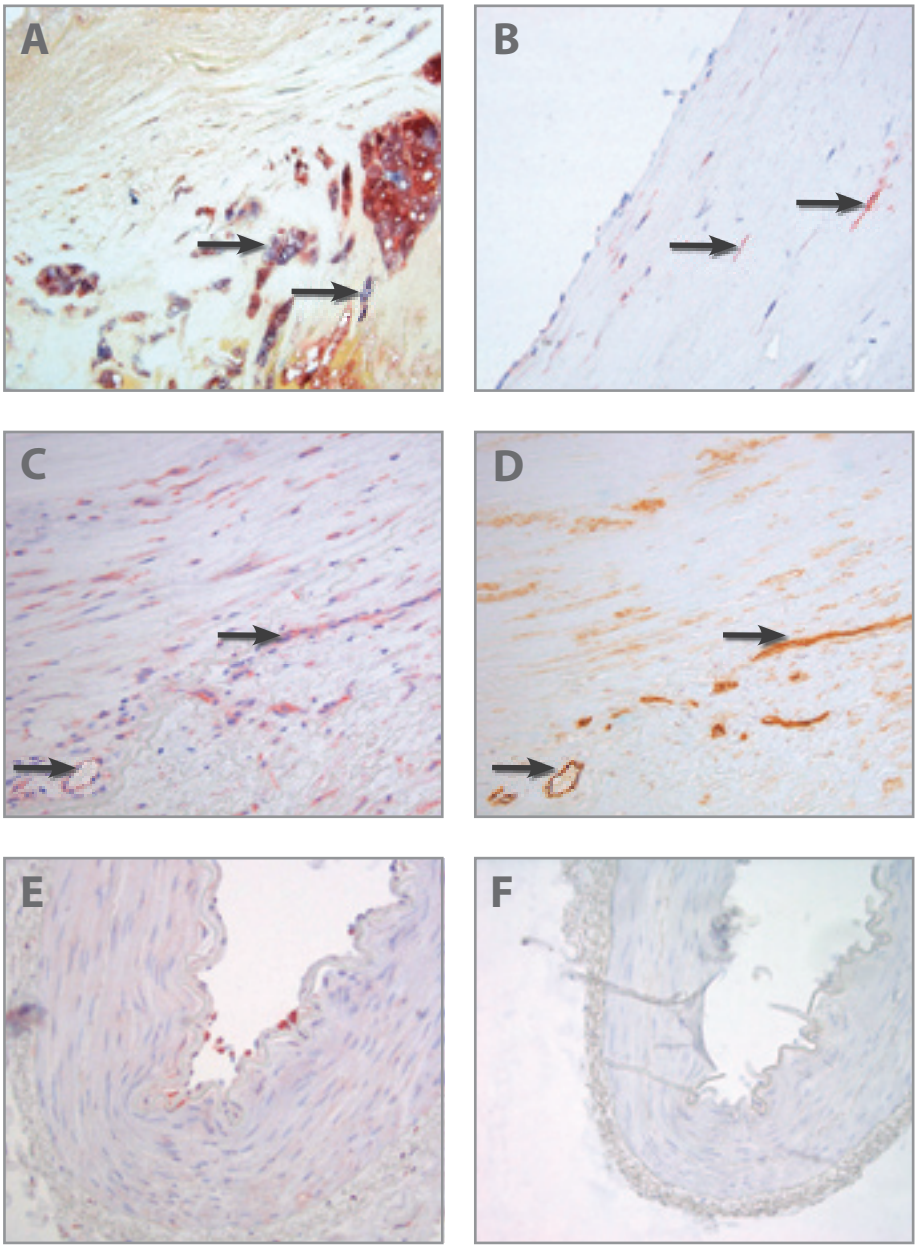

FIGURE II NGAL localization in atherosclerotic plaques

A: Double staining of NGAL (blue) and macrophages (CD68; red) reveals co-localization (purple). **B:** NGAL staining observed in smooth muscle cells (arrows) but not in endothelium. **C, D:** NGAL staining observed in CD34+ endothelial cells aligning neovessels. **E:** Mammary artery: smooth muscle cells reveal slight NGAL staining. **F:** Mammary artery isotype control.

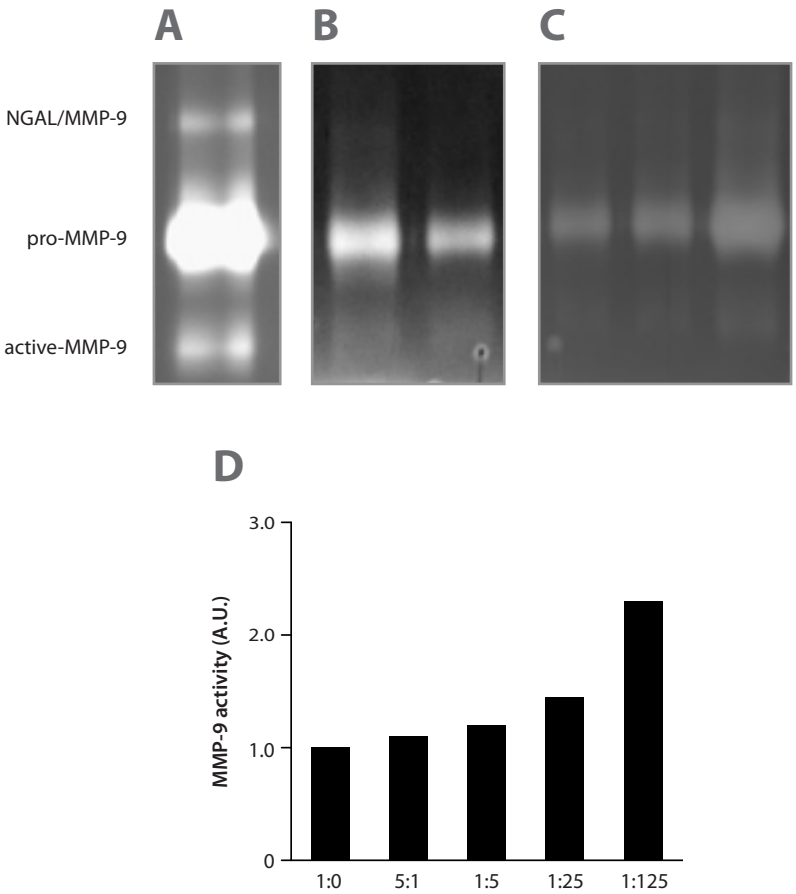

Figure III NGAL preserves MMP-9 activity

Quantification of *in vitro* experiments by gelatin zymography. **A:** *In vitro* reconstruction of NGAL/MMP-9 complexes by combining recombinant NGAL and MMP-9. **B:** MMP-9 activity diminishes after 30 minutes incubation at 37°C (left lane: no incubation, right lane: 30 minutes incubation) **C:** Increasing addition of NGAL prevents the autodegradation of MMP-9. (MMP-9:NGAL molar ratio 1:5 – 1:125) **D:** Quantification of C.

mary artery showed a slight diffuse staining for NGAL, whereas no staining was observed in the isotype control (**Figure II-E,F**).

Independence of the association between NGAL and MMP-9

We further investigated if the association between NGAL and MMP-9 might be caused by the expression of both by macrophages, i.e. that both would be higher

in plaques with a high number of macrophages. Therefore, we adjusted the relation for NGAL and MMP-9 for the extent of macrophage infiltration in a linear regression model. This showed that NGAL was associated with MMP-9 independently of the presence of macrophages (β=0.43; p<0.001).

Preservation of MMP-9 activity by NGAL

To explore a potential function of NGAL, we tested whether MMP-9 autodegradation was affected by addition of NGAL. After 30 minutes incubation at 37^{0}C, MMP-9 autodegradation **(Figure III-A)** was diminished by increasing addition of NGAL **(Figure III-B)**, thereby leading to prolonged MMP-9 activity **(Figure III-C,D)**.

Release of NGAL from atherosclerotic plaques

The local plaque related blood samples (n=9), drawn during carotid endarterectomy directly from the carotid artery holding the atherosclerotic plaque, showed borderline higher concentration of NGAL than peripheral samples drawn at the same time (**Figure IV-B**; p=0.06). Comparison of NGAL/MMP-9 complex levels (ELISA) in the plaque related blood samples vs. peripheral samples revealed a statistically significant local increase of NGAL/MMP-9 complex in the blood in proximity of the atherosclerotic plaque. (**Figure IV-C**, p=0.01).

Circulating levels of NGAL in patients with myocardial infarction versus controls

Since we observed an association of NGAL and NGAL/MMP-9 with unstable plaque characteristics, as well as the release from atherosclerotic plaques, we compared circulating levels of NGAL and NGAL/MMP-9 between patients with myocardial infarction (MI; n=23) and controls with stable coronary artery disease (n=8). The presence of hypercholesterolemia was higher in patients with stable CAD (MI: 42%; CAD: 100%; p=0.02) but the groups showed no statistically significant differences in gender, diabetes, smoking and hypertension. The Serum levels of circulating NGAL (mean increase 193%; p=0.04) and circulating NGAL/MMP-9 complex (mean increase 217%; p=0.02) were significantly increased in patients with MI compared to stable CAD **(Figure IV-D,E)**.

Discussion

The current study reports several new findings regarding the expression of NGAL in atherosclerosis and its interaction with MMP-9. First, a positive association between expression levels of NGAL and MMP-9 activity in plaques. Second, NGAL and the NGAL/MMP-9 complex are associated with unstable plaque characteristics (inflammation, macrophage infiltration, large lipid core). Third, we confirmed that MMP-9 activity is preserved by addition of NGAL in vitro. Finally, the combination

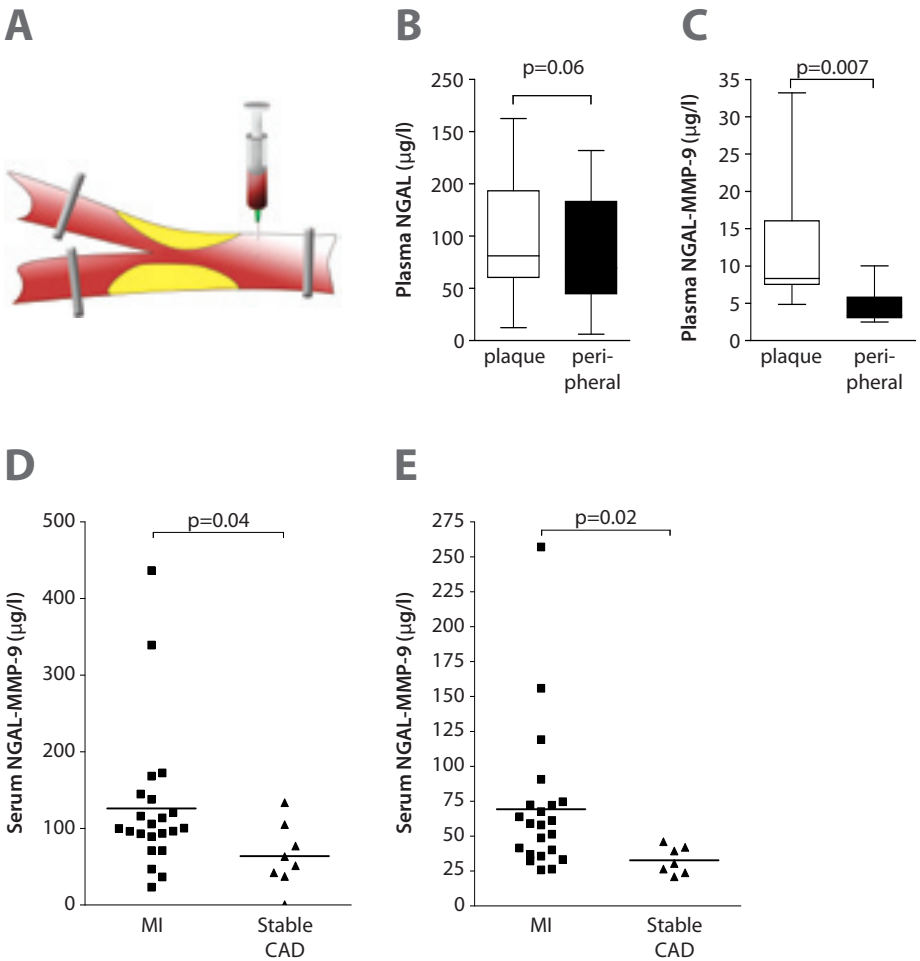

Figure IV Circulating NGAL and NGAL/MMP-9

A: Schematic representation of blood drawn directly from the carotid artery during carotid endarterectomy. **B:** NGAL concentrations: local plaque-related blood vs. peripheral blood. (n=9; p=0.06) **C:** NGAL/MMP-9 concentrations: local plaque-related blood vs. peripheral blood (n=9; p=0.007). **D:** Circulating Levels of NGAL in patients with acute myocardial infarction compared to stable coronary artery disease **E:** Circulating Levels of NGAL/MMP-9 complex in patients with acute myocardial infarction compared to stable coronary artery disease.

of secretion from plaques and elevated circulating levels in patients suffering from myocardial infarction, suggests the involvement of NGAL in unstable atherosclerotic disease.

The role of NGAL in the plaque

Different findings point to the interaction between NGAL and MMP-9 as an important effector mechanism of NGAL in atherosclerotic plaques. In atherosclerotic carotid lesions, gelatinolytic activity of the NGAL/MMP-9 complex was strongly increased in comparison with healthy vessels. The current study is the first to show the coupling of NGAL and MMP-9 in human atherosclerotic plaques. This finding was confirmed by different laboratory techniques: gelatin zymography, western blotting and ELISA. A large proportion of total gelatinolytic activity of MMP-9 is attributable to the NGAL/MMP-9 complex, as determined by zymography. This suggests that the NGAL-MMP-9 interaction could significantly contribute to increased or prolonged protease activity, as was indicated by the in vitro experiments. The association between NGAL/MMP-9 and an unstable plaque phenotype lends further support to the concept that NGAL may lead to plaque destabilization via enhancement of MMP-9 activity. Another interesting finding is the strong association between NGAL levels and MMP-8 activity; NGAL was previously shown to be involved in activation of MMP-8.[9]

Besides the interaction with MMPs, other functions of NGAL could be important in atherosclerotic plaques. NGAL is implicated to regulate inflammation, because it binds fMLP, leukotriene B4 and platelet activating factor.[4-6] In addition, NGAL is proposed to be involved in cell survival, but it is disputed if its function is pro- or anti-apoptotic.[24-26] In kidney ischemia, NGAL protects renal damage by preventing cell death via induction of haem-oxygenase.[27] Since inflammation, cell survival and ischemia are key processes in atherosclerosis, NGAL, not bound to MMP-9, could play a role in atherosclerotic lesions via these functions. These mechanisms were not addressed in the present study and warrant further investigation.

The comparable levels of NGAL in healthy vessels and carotid atherosclerotic plaques seems to conflict with the association between NGAL and plaque instability. A possible explanation for this discrepancy is the low expression of NGAL by smooth muscle cells, abundantly present in healthy vessels. In contrast, the carotid atherosclerotic plaques contain hypocellular intima and in some cases minor parts of media.

NGAL in myocardial infarction

The pathological substrate in the majority of patients with myocardial infarction is an unstable atherosclerotic plaque in the coronary circulation, triggering thrombus formation resulting in (near) occlusion of a coronary artery, which then leads to myocardial ischemia.[28] The current results show that expression of NGAL is

increased in plaques with unstable features, i.e. inflammation, macrophage infiltration and large lipid core. Furthermore, concentrations of NGAL and especially the NGAL/MMP-9 complex are increased in the proximity of the atherosclerotic lesions, which suggests the release from atherosclerotic plaques. Together, these observations suggest that unstable plaques in patients with myocardial infarction release NGAL and NGAL/MMP-9 complex which can be measured in a peripheral blood sample.

Clinical applications

NGAL measurements in sera of patients with myocardial infarction add to pathophysiological insights but do not have direct clinical application. Potentially, NGAL might be a biomarker for screening of sub-clinical unstable atherosclerosis. Alternatively, NGAL could be an interesting target for molecular plaque imaging or pharmacological intervention in order to reduce MMP-9 activity in the plaque.

Relation with previous studies

The modulation of MMP-9 activity by NGAL was extensively investigated by Yan et al.[8] Two animal studies investigated NGAL with regard to atherosclerotic disease. Hemdahl et al. demonstrated that the mouse analogue of NGAL, 24p3, was present in mouse atherosclerotic lesions, co-localized with MMP-9, and found that NGAL was upregulated in vascular tissue from mouse who suffered myocardial infarction.[16] Bu et al showed in a rat model that vascular injury led to production of NGAL in the vascular wall which could be inhibited by interfering with the NFκB pathway.[29] The current data confirms the hypothesis that NGAL interacts with MMP-9 in human atherosclerotic lesions, and further extends the previous findings to human atherosclerosis by showing the relation of NGAL with unstable plaque characteristics and myocardial infarction.

Strengths and weaknesses of the study

The current study encompasses a relatively large series of endarterectomy specimens, which allows investigation of the quantitative rather than qualitative association between NGAL, MMP-9 and plaque instability. The relatively large study size also minimizes the likelihood of chance findings. A possible limitation is that the data on NGAL in the plaque are cross-sectional and therefore it could be argued that the strong association between NGAL and MMP-9 in the plaque is caused by expression of both in macrophages. However, we were able to show that both proteins are not only co-expressed but also interact, and that the association between expression of NGAL and MMP-9 is still observed when we adjust for the extent of macrophage infiltration.

Conclusions

NGAL interacts with MMP-9 in atherosclerotic plaques by formation of a NGAL/MMP-9 complex; moreover NGAL expression is related with MMP-9 activity independent of the extent of macrophage infiltration. Increased levels of NGAL and NGAL/MMP-9 complex are associated with inflammation and unstable plaque characteristics. NGAL and the NGAL/MMP-9 complex are released from atherosclerotic plaques and circulating levels of both are elevated in patients with myocardial infarction. The high expression levels of NGAL in unstable plaques, and the increased circulating levels in patients with acute myocardial infarction suggest the involvement of NGAL in unstable atherosclerotic disease.

Acknowledgements

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CHAPTER 12

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CAVEOLIN-1 INFLUENCES VASCULAR PROTEASE ACTIVITY AND IS A POTENTIAL STABILIZING FACTOR IN HUMAN ATHEROSCLEROTIC DISEASE

Caveolin-1 (Cav-1) is a regulatory protein of the arterial wall, but its role in human atherosclerosis remains unknown. We have studied the relationships between Cav-1 abundance, atherosclerotic plaque characteristics and clinical manifestations of atherosclerotic disease. We determined Cav-1 expression by western blotting in atherosclerotic plaques harvested from 378 subjects that underwent carotid endarterectomy. Cav-1 levels were significantly lower in carotid plaques than non-atherosclerotic vascular specimens. Low Cav-1 expression was associated with features of plaque instability such as large lipid core, thrombus formation, macrophage infiltration, high IL-6, IL-8 levels and elevated MMP-9 activity. Clinically, a down-regulation of Cav-1 was observed in plaques obtained from men, patients with a history of myocardial infarction and restenotic lesions. Cav-1 levels above the median were associated with absence of new vascular events within 30 days after surgery [0% vs. 4%] and a trend towards lower incidence of new cardiovascular events during longer follow-up. Consistent with these clinical data, Cav-1 null mice revealed elevated intimal hyperplasia response following arterial injury that was significantly attenuated after MMP inhibition. Recombinant peptides mimicking Cav-1 scaffolding domain (Cavtratin) reduced gelatinase activity in cultured porcine arteries and impaired MMP-9 activity and COX-2 in LPS-challenged macrophages. Administration of Cavtratin strongly impaired flow-induced expansive remodeling in mice. This is the first study that identifies Cav-1 as a novel potential stabilizing factor in human atherosclerosis. Our findings support the hypothesis that local down-regulation of Cav-1 in atherosclerotic lesions contributes to plaque formation and/or instability accelerating the occurrence of adverse clinical outcomes. Therefore, given the large number of patients studied, we believe that Cav-1 may be considered as a novel target in the prevention of human atherosclerotic disease and the loss of Cav-1 may be a novel biomarker of vulnerable plaque with prognostic value.

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Introduction

Atherosclerotic plaque formation, destabilization and rupture with subsequent thrombus formation give rise to acute coronary syndromes,[1] symptomatic carotid artery disease[2] and sudden cardiac death.[3] Plaque rupture is associated to an elevated inflammatory response,[4] increased proteolytic activity[5,6] and intraplaque bleeding.[7] The current lack of knowledge about the natural history of human atherosclerotic disease and plaque progression restricts the possibility to identify subjects at risk for cardiovascular events. In order to evaluate the relationship between local protein expression in atherosclerotic plaques and future vascular events, the ATHERO-EXPRESS vascular biobank was established.[8] The main goal of this study is to identify the expression patterns of specific proteins expressed within the vascular tree that may make patients prone to suffer cardiovascular events in all vascular territories. These proteins, diffusely expressed in the vasculature, could then be locally detected using local molecular imaging or in endarterectomy specimens, and serve as a surrogate marker to identify the patient at risk for future adverse cardiovascular events, the so-called vulnerable patient.[9] In this context, we have tested the possibility that Caveolin-1 (Cav-1) plaque abundance is related to plaque and patient stability.

Cav-1 is the main coat protein of caveolae and is expressed by different vascular cells.[10] Caveolae and Cav-1 have emerged as novel targets in the control of various important cellular processes involved in the maintenance of cardiovascular homeostasis such as protein trafficking, lipid metabolism and signal transduction.[11] The involvement of Cav-1 in arterial occlusive disease like atherosclerosis still remains controversial and not well understood.[12] Cav-1/ApoE double-knockout mice show less lipid accumulation in the aorta, while the absence of Cav-1 promotes smooth muscle cells proliferation increasing intimal hyperplasia upon carotid injury.[13,14] In addition, the analysis of plaques from hypercholesterolemic rabbits and humans showed reduced Cav-1 levels, suggesting athero-protective actions for Cav-1.[15,16] However; the study of the relationships between Cav-1 abundance, atherosclerotic plaque phenotype and clinical manifestations of atherosclerotic disease has not been undertaken yet.

Therefore, we determined Cav-1 expression in carotid atherosclerotic plaques from a cohort of 378 patients undergoing carotid endarterectomy obtained from the ATHERO-EXPRESS study. We hypothesized that plaque levels of Cav-1 might be related to plaque morphology, inflammation and matrix metalloproteinase (MMP) activity. We also investigated if local Cav-1 expression levels in the carotid plaque were related to clinical characteristics. In addition, the design of the biobank study allowed the execution of a follow up with the objective to study the predictive value of local Cav-1 expression for the future development of cardiovascular adverse events and thus might help identifying patients at risk. Additionally, we hypoth-

esized that potential associations of Cav-1 expression with adverse outcomes could be explained by an inhibitory effect on MMP activity in the atherosclerotic lesion. In order to address this hypothesis we further analyzed the effect of Cav-1 on intimal hyperplasia in Cav-1 null mice and whether an increased intimal hyperplasia response could be attenuated by MMP blockade. In addition, we studied the impact of Cav-1 scaffolding domain (CSD) on gelatinase activity, COX-2 expression and expansive arterial remodeling in vitro and in mice.
This clinical and pre- clinical data provides evidence supporting an important role for Cav-1, and its related peptides, in vascular pathologies such as intimal hyperplasia, expansive remodeling and human atherosclerotic plaque destabilization and rupture.

Methods

Human study - the Athero-Express vascular biobank

Athero-Express is an ongoing vascular biobank project with the goal to investigate locally expressed plaque markers in relation to clinical presentation and clinical outcome.[8] The biobank project is running in two Dutch hospitals and was approved by the ethical committees of the Antonius hospital Nieuwegein, The Netherlands and the UMCU, Utrecht, the Netherlands. Written informed consent is obtained from all patients. Criteria for patient selection for carotid endarterectomy (CEA) were based upon the recommendation of NASCET and ESCT for symptomatic patients and ASCT for asymptomatic patients.[17,18] Stenosis degree was assessed by duplex. At baseline, medication use, cardiovascular risk factors, history of cardiovascular disease, and other baseline characteristics were retrieved from questionnaires. Additional clinical data were recorded from patient charts. Lipid spectra and hs-CRP were measured in blood samples drawn at baseline. For the current study, carotid endarterectomy patients between the start of the study in April 2002 until April 2006 were included.

Follow-up Protocol and Clinical Outcome Events

After carotid surgery, patients were followed yearly up to 3 years (mean: 23 months). The primary outcome was defined as vascular event: the composite of vascular death, non-fatal myocardial infarction, non-fatal stroke, non-fatal rupture of an abdominal aortic aneurysm, and vascular surgical intervention, whichever occurred first. Additional outcomes were: 1) myocardial infarction (fatal and non-fatal) and coronary revascularization and 2) ischemic stroke. Definitions and assessment procedures of the outcome events were described previously.[19]

Tissue sampling

Carotid endarterectomy was performed by an open, non-eversion technique with careful dissection of the atherosclerotic plaque. Following excision, the plaque was immediately transferred to the laboratory to undergo standardized processing. First, it was divided into 5-mm cross-sectional segments. The culprit lesion, defined as the segment with greatest plaque burden, was fixated in 4% formalin for 7 days and then decalcified in EDTA and embedded in paraffin. The other segments were snap frozen in liquid nitrogen and stored at -80°C. Protein extraction was performed on the carotid segments adjacent to the culprit lesion by mechanical crushing followed by 1) protein isolation with Tripure reagent, according to the manufacturer's protocol (Boehringer Mannheim, Germany) and 2) by dissolving in 40 mM Tris-HCl (pH=7.5) at 4°C. Segments of macroscopically non-atherosclerotic mammary arteries (n=9) obtained during coronary artery bypass surgery served as a non-diseased control.

Histological assessment of carotid atherosclerotic plaques

All plaques were characterized as described earlier.[8] The following stainings were applied on serial cross-sections (5-µm) of paraffin embedded tissue and semi-quantitatively analyzed as no, minor, moderate or heavy staining by observers blinded for patient characteristics: macrophages (CD68), smooth muscle cells (alpha-actin), collagen (Picro-sirius red), calcifications (HE) and thrombus (HE and Picro-sirius red). The size of the lipid core was visually estimated as a percentage of plaque area using H&E and Picro-sirius stainings (<10%, 10% - 40%, >40%). In addition, computerized measurements of CD68 and alpha-actin were performed to assess macrophage and smooth muscle content respectively. Representative images of semi-quantitative staining can be found in [20].

Determination of intraplaque interleukin levels, MMP activity and EMMPRIN levels

Interleukin-6 and -8 (IL-6 and IL-8) concentrations were determined with a multiplex suspension array system according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA). MMP-2 and MMP-9 activities and Extracellular Matrix Metalloproteinase Inducer (EMMPRIN) levels were measured in a randomly selected subgroup of 128 patients. MMP-2 and MMP-9 activity measurements were performed with Biotrak activity assays RPN 2631 and RPN 2634, respectively (Amersham Biosciences, Buckinghamshire, UK). EMMPRIN expression levels were determined by Western blotting as described previously 21. The ratio between the two forms of EMMPRIN (58 kD highly glycosylated and a 45 kD less glycosylated forms) was calculated.

Cav-1 immunohistochemistry

Serial cross-sections (5-µm) from carotid endarterectomy specimens and mammary arteries were deparaffinized and rehydrated, boiled in sodium citrate and blocked in 10% normal goat serum. The sections were incubated for 1 hour at room temperature with 0.2 µg/mL polyclonal rabbit-anti human-Cav-1 antibody (610059, BD biosciences, Franklin Lakes, NJ), as determined by titration, followed by biotinylated goat-anti-rabbit antibody (Vector, Burlingame, CA) and horseradish peroxidase (HRP) labeled streptavidin (Vector). Staining was developed with AEC substrate with Mayer's hematoxylin as counterstaining. Negative controls were obtained avoiding the primary antibody. Double labeling for Cav-1 and alpha-actin SMC (Sigma, St. Louis) and CD34 (Dako, Denmark) were also performed.

Animal studies

Animals were housed conformed to the Guide for the care and use of Laboratory Animals (NIH publication No.85-23, 1985) and all experiments were approved by the ethical committee on animal experiments of the University Medical Center, Utrecht. BALB/c mice, Cav-1 null mice (Cav-1^{tm1Mls}) and appropriate Wild type (WT) genetic background controls were purchased by the Jackson laboratories (Bar Harbor, MA).

Induction of intimal hyperplasia and matrix- metalloproteinase (MMP) inhibition

A group of Cav-1 null mice were treated daily with doxycycline (DOX), an orally available MMP inhibitor, in drinking water at the dose of 30 mg/kg/day as used earlier.[22] The treatment started a week before cuff placement and was continued for another 3 weeks. Polyethylene cuffs, to induce intimal hyperplasia, were placed around the right femoral artery in WT (n=11), Cav-1 null (n=12) and Cav-1 null + DOX (n=11) mice. The animals were sacrificed 3 weeks after cuff placement and the femoral arteries were harvested and analyzed as reported before.[23]

Recombinant peptides experiments

Synthetic peptides corresponding to scaffolding domain of human Caveolin-1 (residues 82-101) (Cavtratin) and scrambled version were prepared as previously reported.[24, 25] For in vitro experiments, final concentration of each peptide was achieved by diluting the stock in culture medium. For in vivo experiments, peptides were dissolved in 30% dimethyl sulfoxide (DMSO) solution in 0.9% saline buffer (Braun) and mini-osmotic pumps (Alzet) with an internal volume of 200 µl and infusion rate of 0.5 µL/ hour were filled with a daily dose of 1.5mg/kg of Cavtratin or scrambled peptides. Subsequently, mini-osmotic pumps were attached to a small flexible catheter (Alzet). Catheters were surgically connected to the jugular vein of BALB/c mice and pumps were placed subcutaneously. Thereafter, a right ca-

rotid ligation was performed as a model for expansive remodeling in BALB/c (n=14), BALB/c + scrambled (n=6) and BALB/c + Cavtratin (n=6) as described earlier.[26] Non-ligated arteries (BALB/C, n=10) were used as a reference (0 days). After 4 weeks, the contralateral left carotids were pressure-fixed, harvested and embedded in paraffin. Total circumference area (EEL area), intimal and luminal area were measured after elastin staining and compared to non-ligated carotids (0 days). Expansive remodeling was defined as an increase in EEL area compared with the reference group. Data were analyzed by ANOVA.

Gelatin and in situ zymography

Gelatin zymography was performed as described previously.[27] For in situ zymography, porcine arterial rings were embedded in Tissue-Tek (Sakura), sectioned (5 µm) and incubated at 37°C overnight with a fluorogenic gelatin substrate (DQ gelatin, Molecular Probes) to a final concentration of 25 µg/mL. Proteolytic activity was detected under the microscope as green fluorescence at 530 nm.

Culture of porcine arterial rings

Internal mammary arteries were surgically harvested from 2 adult male pigs and sliced in approximately 0.5 cm rings. After washing with PBS, arterial rings were immediately frozen (0 days) and the remaining fragments were incubated overnight in presence or absence of Cavtratin or scrambled peptides in a serum-free D-MEM (Gibco) and after extensive washing were cultured in 5% FBS D-MEM for 3 days. Thereafter, samples were processed for gelatin and in situ zymography.

Human Cav-1, murine EMMPRIN and COX-2 western blotting

Equal amounts of total protein were denaturized and subjected to a SDS-PAGE in 10% or 12% polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes (Schleier & Schuell, Dasel, Germany) and correct transfer was checked by Ponceau Red S staining. The membranes were incubated with either polyclonal rabbit-anti-human Cav-1 antibody (0.1 µg/mL, BD biosciences, Franklin Lakes, NJ), polyclonal goat anti EMMPRIN (0.4 µg/mL, G-19 Santa Cruz, Biotechnology) or polyclonal rabbit anti murine COX-2 (0.5 µg/mL, Cayman Chemicals, Ann Arbor, Mi) followed by incubation with appropriate HRP conjugated secondary antibodies. Signal detection was performed by enhanced chemiluminescence (Sigma, Saint Louis, MO). For western blotting in CEA samples, a pooled sample of mammary arteries (n=6) was loaded on each gel as a positive control. Accordingly, in every gel, expression levels of Cav-1 in pooled mammary arteries were considered as 100. Cav-1 expression levels in CEA samples were standardized and calculated as percentages relative to standard positive control.

Table I Baseline characteristics of the studied population

Characteristics	n (%)
Patient number	378
Age, years (sd)	67.4 (8.8)
Male	258 (68%)
Hypertension	247 (70%)
Diabetes	70 (20%)
Smoking	88 (26%)
Body mass index, kg/m2 (sd)	26.7 (4.5)
Total cholesterol, mmol/l	5.0 (1.1)
HDL cholesterol, mmol/l	1.2 (0.37)
LDL cholesterol, mmol/l	3.0 (1.0)
Triglycerides, mmol/l	2.1 (1.1)
Hs-CRP, mg/l (IQR)	3.4 (1.6 - 6.9)
Prior ipsilateral CEA	12 (3%)
Prior vascular intervention	139 (35%)
History of myocardial infarction	72 (20%)
Carotid stenosis grade	
50 – 64%	12 (3%)
65 – 89%	133 (35%)
90 – 99%	232 (62%)
Symptoms	
Asymptomatic	87 (23%)
Ocular symptoms	51 (14%)
TIA	130 (35%)
Stroke	102 (28%)

Cell culture

Raw-264.7 murine macrophages were obtained from ATCC (Manassas, VA) and grown and propagated accordingly to manufacture's recommendations.

Data analysis

Comparison of Cav-1 expression levels between different artery types and different patient groups was done by Mann-Whitney U tests. The Mann-Whitney U test was also used to test the association between Cav-1 measurements and semi-quantitatively measured plaque characteristics, comparing no and minor staining to moderate and heavy staining. For survival analysis, Cav-1 levels were dichotomized at the median. The group with low Cav-1 expression levels (<median) was compared with the group high Cav-1 measurements (≥ median) by Kaplan-Meier

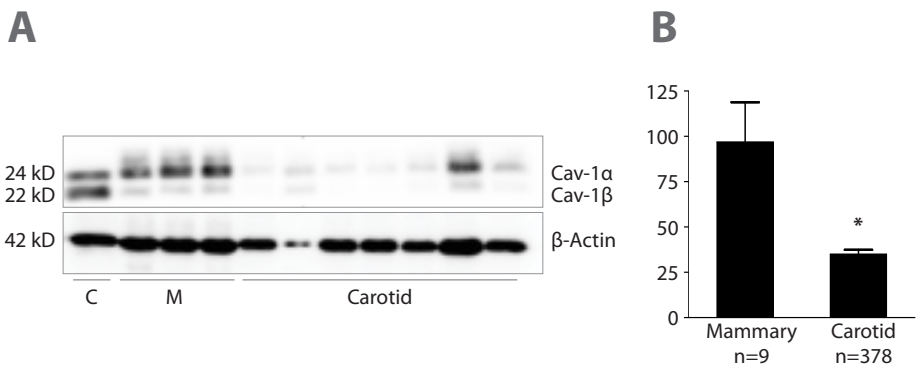

Figure I Cav-1 expression pattern in normal and atherosclerotic lesions

A: representative Western Blot. "C" denotes control (pooled sample), "M", mammary arteries, "Carotid", carotid plaques. Detection of Cav-1 α and β isoforms (22 and 24 kD; above) and β-actin (42 kD; below) are shown. **B:** Quantification of Cav-1protein levels in carotid vs. control mammary arteries (mean and SE). * p=0.001. Note that β–actin and Cav-1 expression patterns differ completely, indicating the specificity of Cav-1 down-regulation in CEA samples.

survival analysis. Cox Proportional Hazard analysis was used to compute Hazard ratios (HR) with 95% confidence intervals [CI] and to adjust for sex, gender and plaque overall phenotype. Data from animal, ex vivo and cultured cells were analyzed by Mann-Whitney U tests. P-values <0.05 were considered statistically significant.

Results

Baseline characteristics of the patient population are depicted in **Table I**. In total 378 patients were included in this report, with a mean age of 67.4+/–8.8 years.

Cav-1 expression pattern in human atherosclerotic lesions

Cav-1 antibody was able to detect two bands with a relative molecular weight of 24kD and 22 kD demonstrating the presence of the two known isoforms of Cav-1 (α and β); **(Figure I-A)** Cav-1 protein expression levels were strongly reduced in atherosclerotic plaques compared with non-atherosclerotic mammary arteries **(Figure I-B)**. The carotid plaques showed 64% lower expression levels compared to mammary arteries (95% CI: [31%–96%]) **(Figure I-B)**. By immunohistochemistry, we found that both mammary arteries and human atherosclerotic lesions expressed Cav-1 **(Figure II)**. In mammary arteries, Cav-1 staining was distributed throughout the intimal, medial and adventitial layers. In carotid plaques, Cav-1 was frequently found in

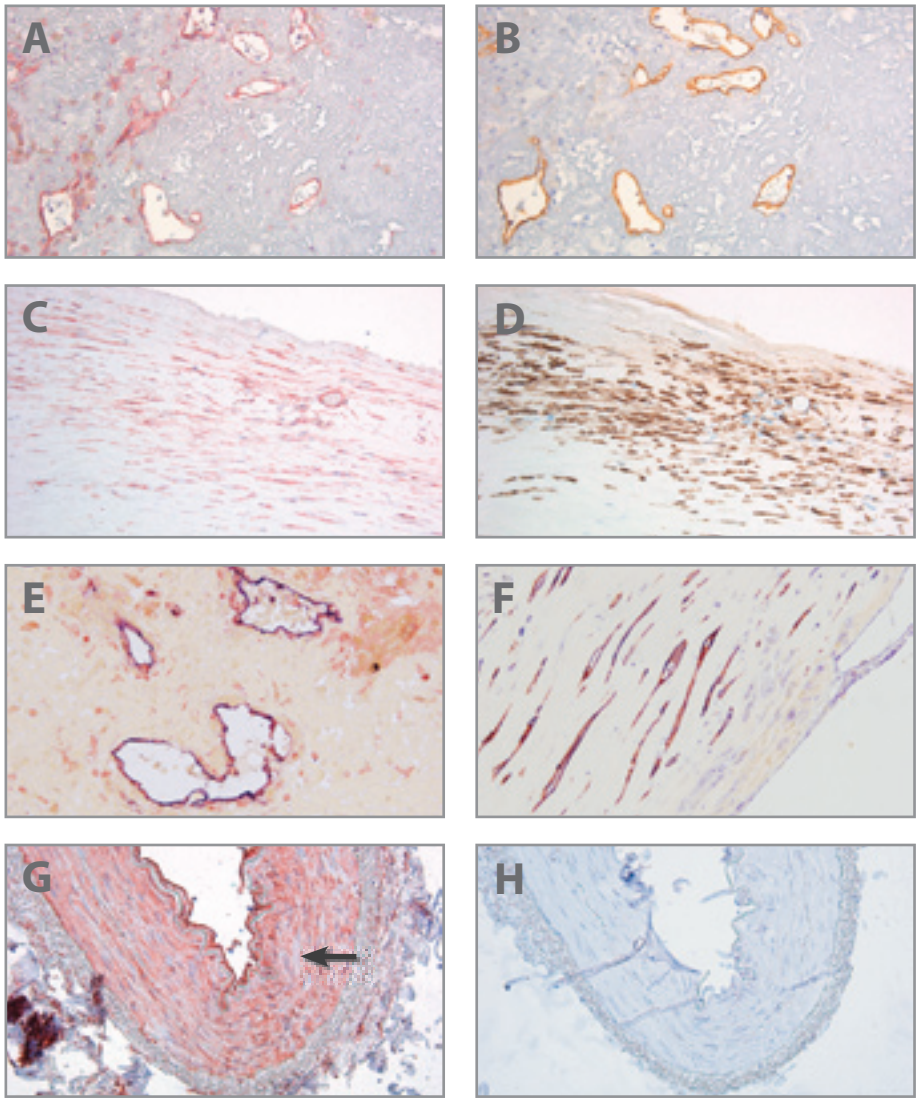

FIGURE II Cav-1 immunohistochemistry

A,C: Cav-1 staining on carotid plaques (red, 200x magnification). **B:** Endothelial staining (CD34, brown) on a consecutive section of A showing co-localization of Cav-1 and endothelium. **D:** Alpha-actin smooth muscle cell staining (brown) on a consecutive section of C, showing co-localization of Cav-1 and smooth muscle cells. **E:** Double-staining of Cav-1(red) and CD34 (blue), (200X magnification). **F:** Double staining of Cav-1 (blue) and Alpha-actin smooth muscle cell sataining (red), (200X magnification). **G:** Cav-1 staining on a mammary artery, showing staining throughout the intima, media and adventitia. (100x magnification). **H:** Negative control of Cav-1 staining in a consecutive section of G, avoiding the primary antibody. Sections were counterstained with hematoxylin except the double-stained sections (E and F) .

Table II Relationships between plaque histology and Cav-1 protein expression levels

	Cav-1 levels per staining category				P
	No staining	Minor staining	Moderate staining	Heavy staining	
Macrophage staining	24.0 (5.7 - 54.4)	23.0 (10.3 - 51.2)	20.0 (5.7 - 47.4)	14.5 (3.6 - 30.7)	0.04
Smooth muscle cell staining	5.7 (2.3 - 27.4)	11.9 (3.5 - 25.4)	19.6 (6.4 - 45.7)	34.4 (18.2 - 71.2)	<0.001
Collagen staining		15.6 (3.7 - 33.8)	20.4 (6.7 - 47.9)	26.1 (12.0 - 49.9)	0.02
Calcifications	23.5 (5.9 - 50.1)	18.4 (4.9 - 35.7)	15.9 (7.4 - 41.2)	26.2 (7.7 - 55.7)	0.57
Thrombus	30.1 (13.0 - 53.3)	17.4 (5.7 - 46.0)	14.6 (3.5 - 33.1)	10.7 (2.5 - 27)	0.005
Overall phenotype [A]		26.2 (11.7- 53.3)	25.0 (7.7 - 56.0)	13.3 (4.5 - 32.9)	<0.001

The values given are the median Cav-1 levels and interquartile range in the respective staining group. P-values were calculated comparing Cav-1 expression levels between no and minor staining vs. moderate and heavy staining, and in case of overall phenotype: fibrous vs. atheromatous.

[A] fibrous - fibro-atheromatous, atheromatous

smooth muscle cells, the endothelium of neovascularized areas and endothelium aligning the vessel lumen. Co-localization with macrophages was not observed. No staining was observed in non-immune controls **(Figure II)**.

Cav-1 abundance and plaque characteristics

We further examined the relationships between Cav-1 expression levels and different plaque characteristics. Plaques with an atheromatous phenotype showed lower Cav-1 expression levels compared with fibrous plaques ($p<0.001$; **Table II**). In addition, Cav-1 levels were significantly lowered in plaques with unstable characteristics such as high macrophage staining ($p=0.04$), low smooth muscle cell staining ($p<0.001$), low collagen staining ($p=0.02$), and high amount of thrombus ($p=0.005$). There was no association between Cav-1 expression levels and extent of calcifications.

Cav-1 abundance, proteolytic activity and inflammation, human data

Next, we investigated the relationships between Cav-1 expression levels, local MMP activity, expression of the MMP-inducer CD147/EMMPRIN and the levels of pro-inflammatory cytokines **(Figure III)**. High MMP-9 activity was observed in patients with low Cav-1 expression levels ($p<0.001$) while there was no significant correlation between Cav-1 levels and MMP-2 **(Figure III-A, B)**. Previously, we observed

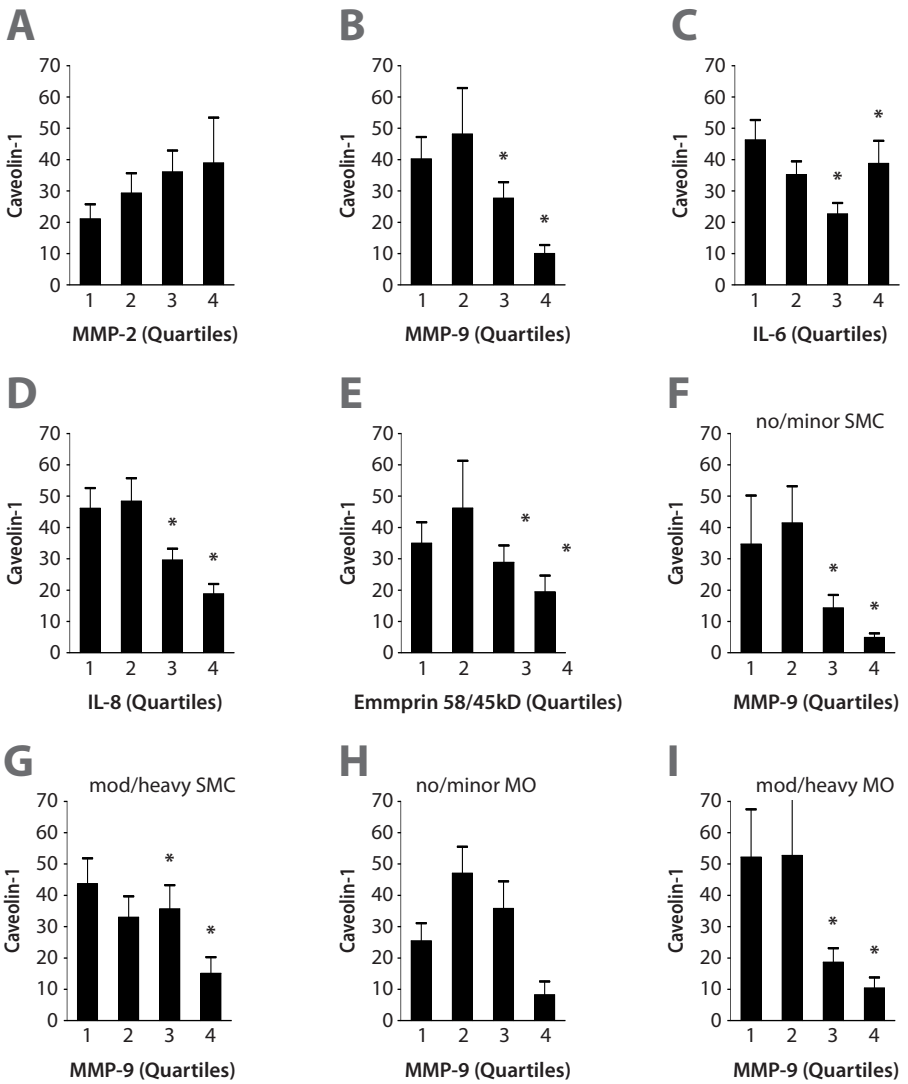

Figure III Relationships between Cav-1, Interleukin levels, MMP activity and EMMPRIN levels

A, B, C, D: MMP-2, -9 and IL-6,-8 vs. Cav-1 protein expression levels. **E:** EMMPRIN 45/58kD ratio vs. Cav-1 protein expression levels. **F, G:** Analysis of association between MMP-9 and Cav-1 levels in plaques with no or minor smooth muscle cell (SMC) staining vs. plaques with moderate or heavy smooth muscle cell staining. **H, I:** Analysis of association between MMP-9 and Cav-1 levels in plaques with no or minor macrophage (MO) staining vs. plaques with moderate or heavy macrophage staining. Cav-1 levels are given as mean and standard error. P-values were calculated comparing quartiles 1 and 2 to quartiles 3 and 4 with the Mann-Whitney U test. * denotes $p < 0.05$.

positive associations between MMP-9 expression and 58kD glycosylated EMMPRIN and MMP-2 and 45kD EMMPRIN levels, respectively.[21] Therefore, we studied the association between EMMPRIN glycosylation and Cav-1 expression. A significant association between Cav-1 expression and EMMPRIN glycosylation levels was observed (**Figure III-E**, p=0.04). Low Cav-1 levels were associated with high IL-6 levels (p=0.006) and IL-8 levels (p<0.001). These associations were not a mere re-

Table III Relations between clinical characteristics and Cav-1 expression levels

Clinical characteristics	Prevalence (%)	Cav-1 levels		P-value
		+	-	
Age>70	43%	18.0 (5.5 - 44.0)	20.5 (6.7 - 49.6)	0.29
Male	68%	15.8 (4.7 - 36.6)	36.1 (15.5 - 62.4)	<0.001
Hypertension	70%	19.5 (5.2 - 45.9)	22.3 (7.3 - 48.2)	0.42
Diabetes	20%	17.6 (7.7 - 49.6)	19.6 (5.5 - 45.6)	0.90
Smoking	26%	18.6 (6.2 - 48.3)	19.9 (5.4 - 46.0)	0.92
BMI >30 kg/m2	14%	25.3 (5.5 - 50.2)	20.0 (6.7 - 47.1)	0.98
Total cholesterol > 5.0 mmol/l	47%	22.9 (6.1 - 49.5)	19.8 (4.7 - 47.5)	0.55
HDL < 1.2 mmol/l	38%	22.1 (4.9 - 50.2)	20.1 (5.7 - 41.1)	0.58
LDL > 3.0 mmol/l	40%	20.3 (6.6 - 55.9)	22.2 (4.8 - 44.9)	0.90
Triglycerides > 2.0 mmol/l	39%	24.6 (6.2 - 25.6)	19.4 (5.2 - 44.1)	0.36
Hs-CRP > 3.4 mg/l	49%	20.0 (5.3 - 49.6)	22.3 (5.2 - 49.5)	0.94
Restenotic lesion	3%	6.1 (0.7 - 28.1)	19.7 (6.9 - 48.0)	0.04
History of myocardial infarction	20%	13.3 (3.2 -40.7)	20.5 (7.4 - 48.4)	0.04
History of angina pectoris	35%	17.2 (3.9 - 44.7)	20.5 (7.7 - 48.4)	0.21
Intermittent claudication	46%	18.1 (4.5 - 46.2)	20.5 (6.8 - 46.2)	0.46
Medication				
Statin	64%	18.0 (4.6-46.0)	22.9 (8.0 - 46.2)	0.27
Aspirin	84%	18.9 (5.4 - 48.6)	23.0 (10.2 - 45.7)	0.55
NSAID	6%	19.6 (9.8 - 53.7)	19.4 (5.7 - 46.0)	0.75
ACE inhibitor	43%	17.1 (4.8 - 45.3)	20.8 (7.2 - 49.3)	0.20
Beta blocker	45%	19.8 (5.7 - 46.0)	18.3 (5.9 - 46.7)	0.62
Clinical presentation				0.13 [A]
Asymptomatic	23%	23.6 (8.2 - 53.0)		
Ocular symptoms	14%	27.1 (10.2 - 45.9)		
TIA	35%	15.3 (4.4 - 45.2)		
Stroke	28%	17.1 (7.7 - 48.2)		

Median Cav-1 levels and interquartile range are given for patients in whom a clinical characteristic is present (+) or absent (-); e.g. age>70 (+) denotes the patient group older than 70 years.
[A]TIA and stroke compared with asymptomatic.

flection of constitutional expression of Cav-1 on certain cell types. As an example, **Figure III (panel F-I)** shows that the inverse associations between Cav-1 and MMP-9 are preserved within subgroups based on the number of smooth muscle cells and macrophages. Similar results were obtained for MMP-2, IL-6, IL-8, and EMMPRIN glycosylation (data not shown).

Cav-1 abundance, clinical presentation and follow up

Having established the inverse association between local low levels of Cav-1 and the characteristics of a local vulnerable plaque phenotype, we investigated if local Cav-1 expression levels were related to clinical presentation at baseline and the occurrence of adverse events due to progression of atherosclerotic disease during follow up. Symptomatic patients presenting with transient ischemic attack or stroke had lower Cav-1 levels than asymptomatic patients but this difference did not reach statistical significance (**Table III**; p=0.13). There was no association between hypertension, diabetes, smoking and Cav-1 **(Table III)**. Women showed clearly higher levels compared with men (36.1 vs. 15.8; p<0.001). Patients with a history of myocardial infarction had lower levels of Cav-1 than patients with no such history (13.3 vs. 20.5; p=0.04). Low levels of Cav-1 were found in patients with restenotic lesions: 6.1 vs. 19.7 (p=0.04) **(Table III)**.

Total follow-up included 625 patient-years (mean 23 months) and 13 patients were lost to follow-up (3%). In total, 92 outcome events occurred **(Table IV)**. Patients with vascular events within 30 days of surgery (8/378) had significantly lower Cav-1 levels than patients with no perioperative events (**Figure IV-B**; p=0.03) and all of these patients had Cav-1 levels lower than the median Cav-1 level (p=0.005). As shown in **Figure IV-A**, this difference tended to persist during the first year after the inter-

Table IV Occurrence of outcome events during follow-up

	Follow-up Interval	
	30 Days	**Total**
Patient-years of follow-up [A]	31	625
Vascular death	2	16
Non-fatal ischemic stroke	2	11
Non-fatal cerebral bleeding	1	1
Non-fatal myocardial infarction	1	7
Non-fatal ruptured aortic aneurysm	0	0
Coronary revascularization	2	15
Peripheral vascular intervention	0	42

[A] Years of follow-up until occurrence of primary outcome event or end of follow-up period.

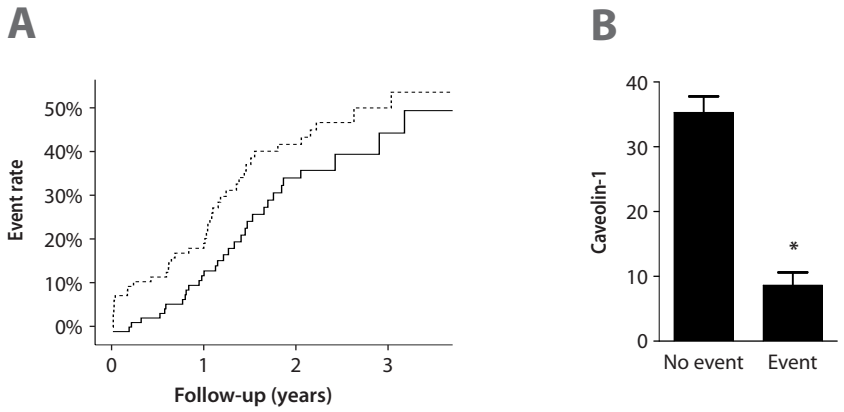

Figure IV Cav-1 and vascular outcomes during follow-up

A: Cumulative hazard of vascular events during long-term follow-up. The solid line corresponds to patients with Cav-1 levels smaller than the median, and the dashed line corresponds to patients with Cav-1 levels larger than or equal to the median. Hazard ratio = 0.77 [0.48 - 1.23] (high vs. low Caveolin-1 expression) **B:** Caveolin-1 expression levels in the plaque in relation the occurrence of an adverse vascular event within 30 days follow-up. *: p=0.03

vention. However, no significant difference persisted during longer follow-up. At longer follow up, the Hazard Ratio (HR) for vascular events (Cav-1 >=median vs. Cav-1 <median) was 0.77 [0.48–1.23] **(Figure IV-A)**. The HR for cardiac events and ischemic strokes separately were similar: 0.76 [0.36–1.59] and 0.67 [0.22–2.03] respectively. Adjusting for age, sex and overall plaque phenotype did not markedly change these associations: HR for vascular events: 0.83 [0.51–1.36], HR for cardiac events 0.68 [0.31–1.48] and HR for ischemic stroke 0.85 [0.25–2.89].

Cav-1 abundance, proteolytic activity and inflammation, in vitro and animal data

We further extended the study on the associations between Cav-1 and MMP expressions by analyzing the contribution of gelatinase activity to intimal hyperplasia in Cav-1 null mice upon femoral artery injury using peri-adventitial cuffs. Morphometric analysis revealed a significant increase in intimal area in Cav-1 null mice (WT=1325±1069 μm^2 vs Cav-1 null=3627±1121 μm^2 p=0.003, **Figure V-A**). Medial area did not differ (WT=9148±2340 μm^2 vs Cav-1 null=9449±2815 μm^2 p=0.4, **Figure V-B**) whereas intima-media ratio (WT=0.12±0.1 vs Cav-1 null=0.38±0.09 p=0.001) was significantly larger in the Cav-1 null mice (**Figure V-C**).

Treatment with the MMP inhibitor doxycycline (DOX), significantly corrected the increased intimal hyperplasia response in Cav-1 null mice (Cav-1 null+DOX=1975±620,

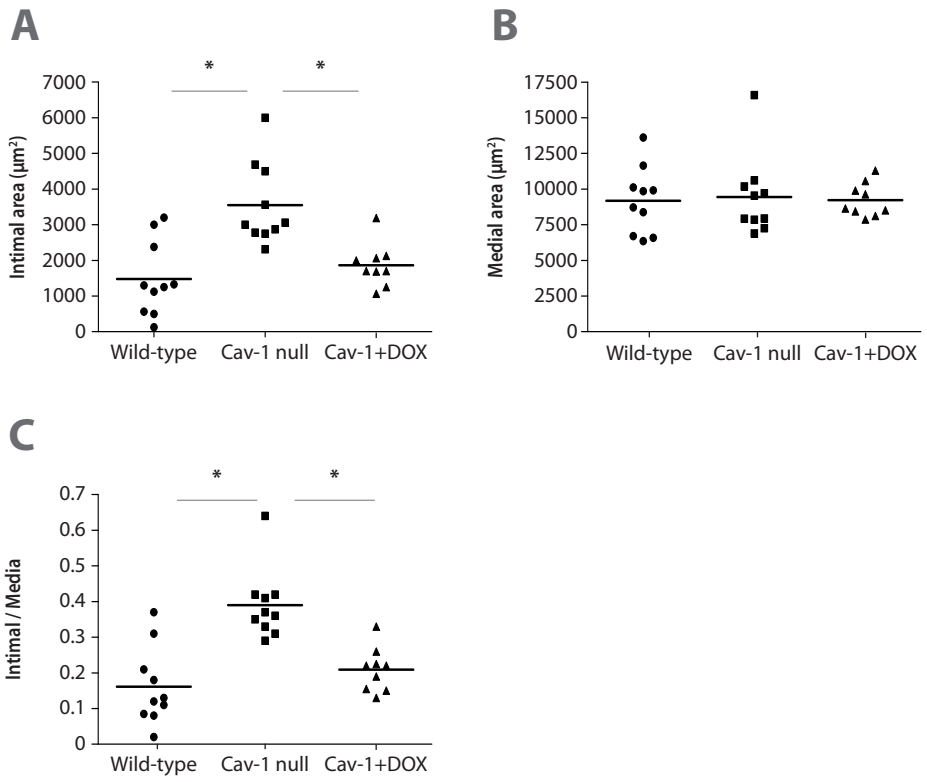

Figure V Morphometric analysis of cuffed femoral arteries

A: Total intimal area, **B:** medial area and **C:** intima-media ratio were quantified by image analysis using 6 serial sections in each cuffed artery. WT (n=11) values are represented by circles, Cav-1 null (n=10) are shown as squares and Cav-1 null+DOX (n=9) are indicate as triangles, * p<0.001.

Cav-1 null-DOX=3627±1121 µm² p<0.001, **Figure V-A**) while media area was not affected (Cav-1 null+DOX=9217±1189 Cav-1 null-DOX=9449±2815 µm² p=0.5, **Figure V-B**). Intima-media ratio also significantly reduced compared to untreated Cav-1 null mice (Cav-1 null+DOX=0.21±0.06 Cav-1-DOX=0.38±0.09 p<0.001, **Figure V-C**).

To study if Cav-1 via its scaffolding domain (CSD) is involved in MMP regulation and the expression of pro-inflammatory mediators, we next evaluated the effect of cell-permeable synthetic peptides derived from the human CSD known as Cavtratin on gelatinolytic activity and cycloxygenase-2 (COX-2) expression in cultured murine Raw-264.7 macrophages. Peptides were efficiently taken up by the cells after 6 hours incubation (data not shown). Raw-264.7 cells were treated with 10 µM of

Cavtratin or scrambled peptides for 24 hours and culture media was subjected to gelatin zymography. Cavtratin treatment significantly reduced MMP-9 levels (p=0.02) after 24 hours of incubation (**Figure VI-A,B**). The incubation of Raw-264.7 with the scrambled peptides did not affect MMP-9 levels in the culture supernatant or control β–Actin expression levels in the total lysate (**Figure VI-A,B**). Very little, almost undetectable signal from MMP-2 was detected (data not shown). Since human carotid plaques showed an inverse relationship between Cav-1 expression levels and EMMPRIN glycosylation status, we additionally investigated whether the effect of

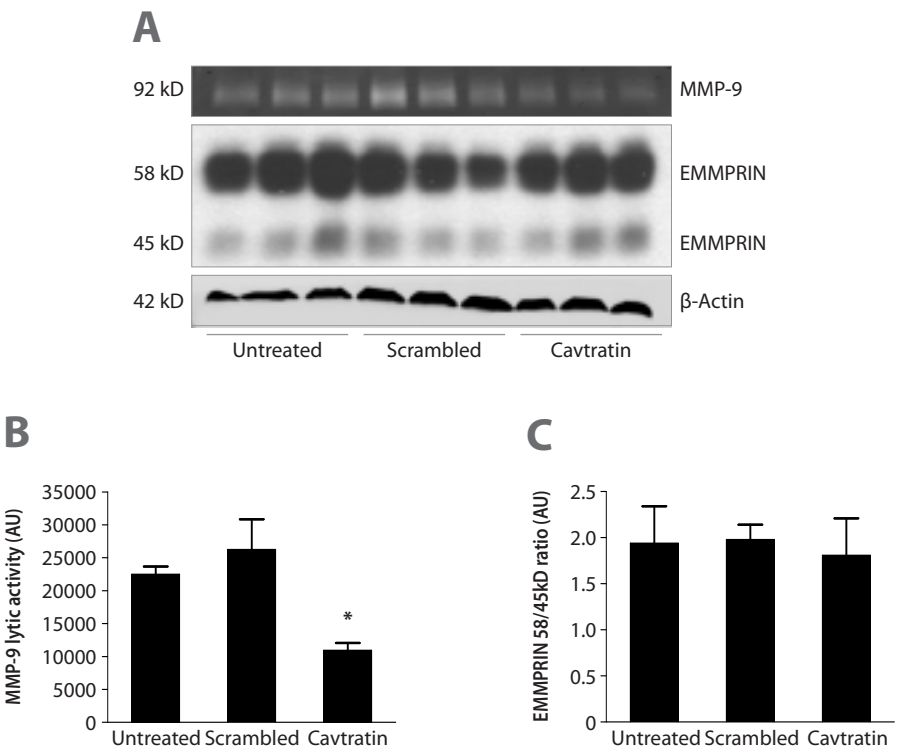

Figure VI Cavtratin, MMP-9 and EMMPRIN in Raw-264.7

A: Top, representative zymogram of culture supernatant from Raw-264.7 macrophages. 1×10^6 cells were incubated in a serum-free medium with Cavtratin or scrambled peptides (10 μM) for a period of 24 hours. Thereafter, culture media was collected and centrifuged for 5 minutes at 2000 rpm at 4°C and subjected to gelatin zymography. Little or undetectable signal was observed for MMP-2. Middle, EMMPRIN expression levels in total cell lysates obtained from Raw-264.7 cells and representative western blot for β-actin (bottom panel). **B:** Quantification of MMP-9 lytic activity in the culture supernatant. * P=0.02. **C:** Quantification of EMMPRIN expression levels. Data presented were normalized by β–actin expression.

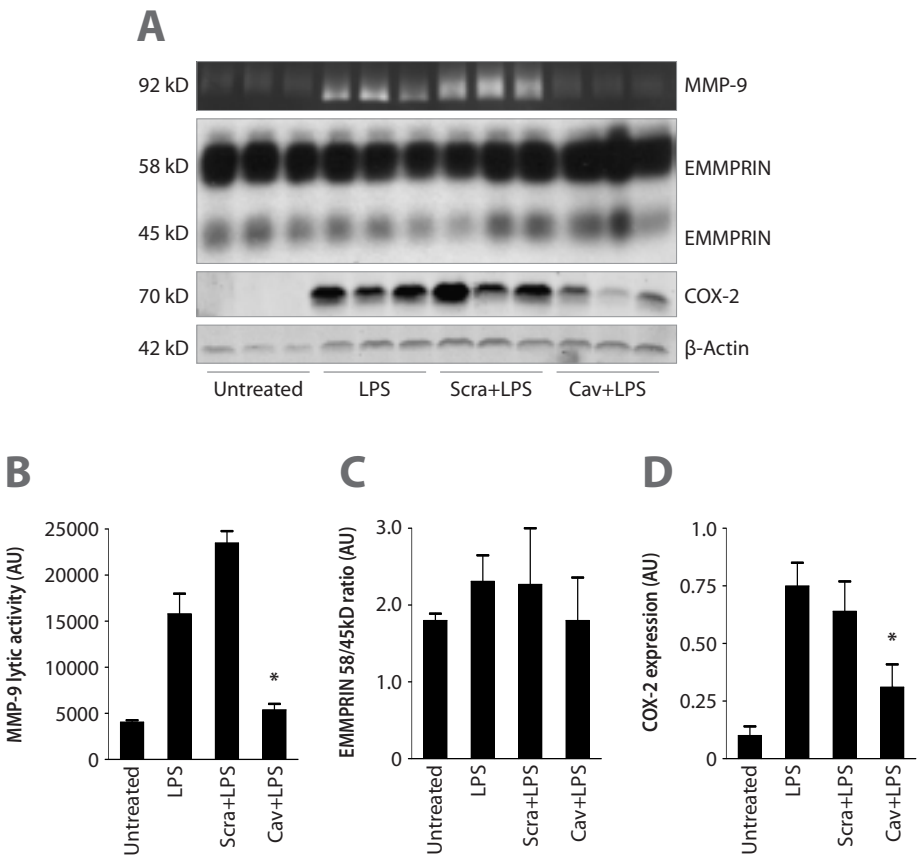

Figure VII Cavtratin, MMP-9, COX-2 and EMMPRIN in LPS-challenged Raw-264.7

A: Top, MMP-9 activity in the culture supernatant from Raw-264.7 macrophages. 1×10^6 cells were pre-incubated in a serum-free medium with Cavtratin or scrambled peptides (10 μM) for a period of 8 hours. Thereafter, LPS (10ng/mL) was added and the culture media was collected after 20 hours of LPS addition and centrifuged for 5 minutes at 2000 rpm at 4C and subjected to gelatin zymography. Middle top, representative western blot showing EMMPRIN expression in total cell lysates obtained from LPS-stimulated Raw-264.7 cells. Middle bottom, representative western blot for COX-2 expression in total cell lysates obtained from LPS-stimulated Raw-264.7 cells. Bottom panel, representative western blot showing β-actin expression. **B:** Quantification of MMP-9 lytic activity in the culture supernatant. * P<0.001. **C:** Quantification of EMMPRIN expression levels. Data presented were normalized by β–actin expression. **D:** Quantification of COX-2 expression levels. Data presented were normalized by β–actin expression. * P<0.001

Cavtratin on MMP-9 levels were mediated by changes in the glycosylation levels of EMMPRIN. As shown in **Figure VI-A,C**, the ratios between low and higly-glycosylated EMMPRIN levels were not affected by any treatment (p=0.7).

To test if Cavtratin might prevent the MMP-9 activation and the induction of COX-2, Raw-264.7 cells were challenged with E.coli Lipopolysaccharide (LPS) in order to induce gelatinase activation and COX-2 expression. As expected, addition of LPS (10 ng/mL) for 20 hours, up-regulated total MMP-9 levels in the culture medium and COX-2 expression in the cell lysates **(Figure VII)**. This up-regulation of active MMP-9 was significantly blocked by pre-incubation of cells with 10 μM Cavtratin (p=0.01) **(Figure VII-A,B)**. Induction of COX-2 expression after LPS stimulation was also markedly reduced by pre-incubation with Cavtratin (p<0.001).

In contrast, pre-incubation of Raw-264.7 with scrambled peptides did not show any inhibitory effect either on MMP-9 or COX-2 levels (p=0.4) **(Figure VII-A,B,C)**. In all cases, no differences were found in β-Actin expression **(Figure VII-A)**. We also determined EMMPRIN levels after LPS stimulation. As shown in **Figure VII-A,C** addition of LPS to Raw-264.7 did not have any impact on EMMPRIN expression levels. Pre-incubation of cells with either Cavtratin or scrambled peptides did not affect EMMPRIN levels **(Figure VII-A,C)**.

In parallel, this inhibitory effect of Cavtratin on gelatinolytic activity was also evaluated in arterial rings from porcine mammary artery that were cultured for 3 days with and without Cavtratin or scrambled peptide (10 μM). Gelatin zymography showed a significant down-regulation of the total lytic activity corresponding to MMP-2 (p=0.02) and MMP-9 levels (p=0.01) between Cavtratin and scrambled peptide treated or non-treated arteries **(Figure VIII-A,B,C)**. In situ zymography of the arteries suggests a reduction in total gelatinase activity in the presence of 10 μM of Cavtratin **(Figure VIII-D)** while treatment with scrambled peptides did not show any effect **(Figure VIII-D)**.

Effect of Cav-1 scaffolding domain on arterial expansive remodeling

Since compelling evidence is pointing to arterial expansive remodeling as a major determinant of plaque vulnerability,[28,29] we next questioned whether this process might be targeted by Cavtratin. For this, we evaluated the impact of Cavtratin administration on BALB/c mice by using the carotid artery contra-lateral to the ligation as a model in which only expansive remodeling takes place. Four weeks after ligation, the contra-lateral arteries showed an increase in total EEL area (23330±5644 μm^2, p=0.001 compared to non-ligated control arteries) **(Figure IX)**. Mice treated with scrambled peptides (1.5mg/Kg/day) did not show any difference in EEL area increase compared to untreated arteries (19944±5434 μm^2) **(Figure IX)**. However, treatment of BALB/c mice with Cavtratin (1.5mg/Kg/day) resulted in a significant reduction in EEL area (735±4838 μm^2, p=0.02 compared to non-ligated control arteries) **(Figure IX)**.

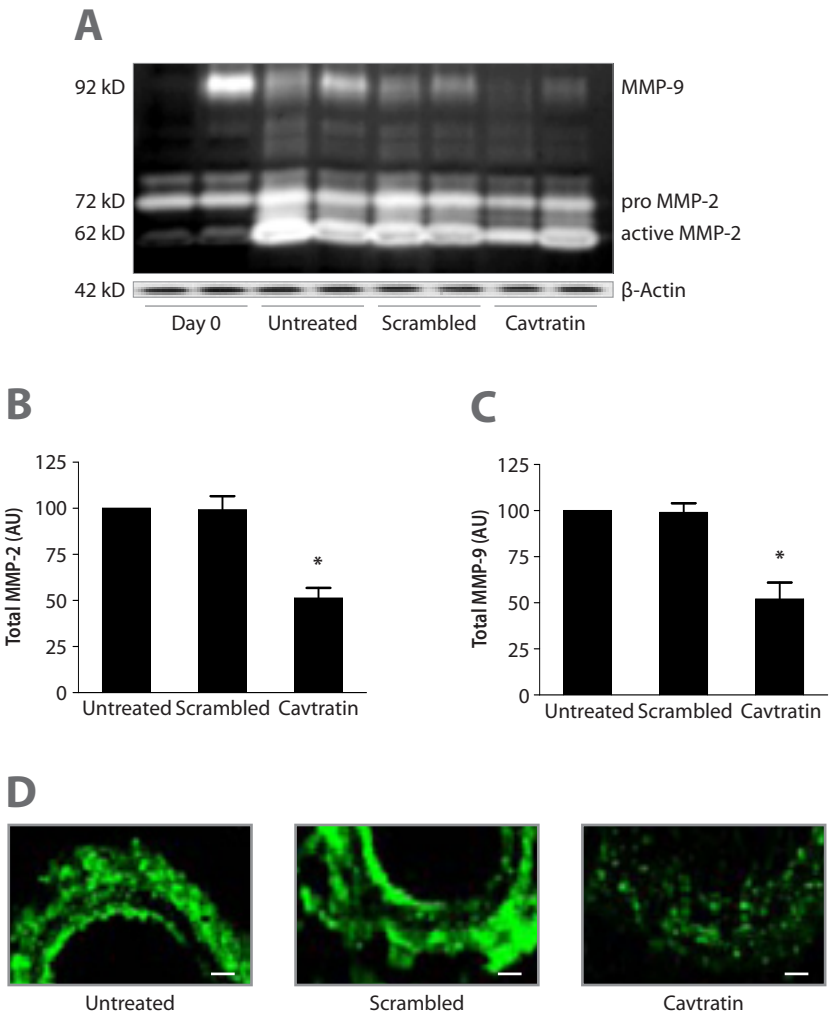

Figure VIII Cavtratin and gelatinase activity in porcine arterial rings

A: Representative zymogram of tissue homogenates from porcine arterial rings at baseline (0 days) and after 3 days in culture in the presence and/or absence of scrambled and Cavtratin peptides (10 μμ). Data represent 5 independent arterial rings. A representative western blot showing β-actin expression, demonstrating equal protein loading (bottom panel). **B:** Quantification of total MMP-2 (Pro and active MMP-2) in porcine arterial rings after 3 days in culture in the presence and/or absence of Cavtratin and scrambled (10 μμ). Data represent 5 different arterial rings. * p=0.02 compared to untreated rings. **C:** Quantification of total MMP-9 in porcine arteries after 3 days in culture in the presence or absence of Cavtratin and scrambled (10 μM). Data represent 5 different arterial rings. * p=0.01 compared to untreated rings. **D:** In situ zymography obtained from cutured rings. Gelatinolytic activity is shown in green. Scale bar=100 μm. L=Lumen.

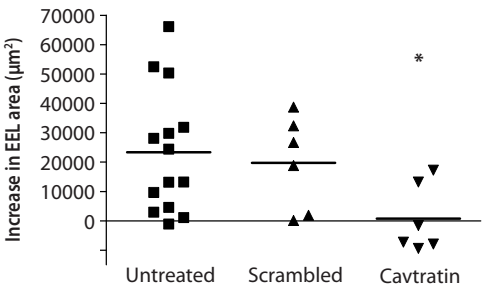

Figure IX Cavtratin and expansive remodeling

Increase in EEL area (µm2) of the left carotid arteries (contralateral arteries) after ligation of the right carotid artery in BALB/c (n=14) (circles), BALB/c+scrambled (1.5 mg/kg/day) (n=6) (squares) and BALB/c+Cavtratin (1.5 mg/kg/day) (n=6) (triangles). *p=0.02

Discussion

The present study identifies Cav-1 as a potential stabilizing factor in human atherosclerosis. Cav-1 levels were lower in atherosclerotic plaques compared to unaffected arteries and low Cav-1 levels were strongly associated with features of plaque vulnerability. Consistently, neointima formation after femoral cuff placement was increased in Cav-1 null mice, which could be reversed by addition of a MMP-inhibitor. Over-expression of the active domain of Cav-1 impaired inflammation, MMP-activity and arterial expansive remodeling. In addition to our descriptive clinical data, we show that patients with high plaque Cav-1 expression seem to be protected from cardiovascular events within 30 days after surgery, making Cav-1 the first available plaque biomarker with a prognostic value. The concept on local plaque markers that are predictive for adverse cardiovascular events that originate elsewhere in the vascular system is currently explored.

Several processes such as elevated proteolytic activity, inflammation, and expansive remodeling are directly related to plaque rupture.[4,5,28] Using different experimental approaches including cultured cells, animal models and the determination of Cav-1 levels in plaque specimens, we have evaluated the involvement of Cav-1 in the above-mentioned processes. We initially found that Cav-1 levels were inversely associated with MMP-9 activity and the glycosylation status of the MMP inducer, known as EMMPRIN, in carotid plaques. We next tested whether gelatinase activity contributes to enhanced intimal hyperplasia development in injured Cav-1 null femoral arteries. Here, we show that cuffed-arteries in Cav-1 null mice have larger intimal area and intima-media ratio than WT mice. These results are in line

with a previous study reporting that carotid artery ligation in Cav-1 null resulted in an increased intimal area response.[14] Additionally, we studied the contribution of gelatinase to intima formation in Cav-1 null mice; MMPs were pharmacologically targeted using the MMP inhibitor doxycycline. Besides its anti-microbial actions, doxycycline is able to reduce expression and activity of several MMPs, including MMP-2 and MMP-9.[30] MMP inhibition in Cav-1 null mice resulted in a significant attenuation of the increased intimal formation in Cav-1 null mice.

The existence of a positive relationship between Cav-1 and MMP-9 and the negative association with MMP-2 in the human specimens is supporting previous observations in which MMP-9 but not MMP-2 is associated with a stable plaque phenotype.[21] In a previous study, we demonstrated that MMP-2 is strongly associated with the presence of plaque stabilizing smooth muscle cells while MMP-9 is associated with the presence of inflammatory cells. Therefore, the negative association of Cav-1 with MMP-9 but not MMP-2 is supporting the hypothesis that Cav-1 is a plaque stabilizing molecule. As mentioned earlier, EMMPRIN glycosylation has previously been associated with either a stable [the 45kD glycosylated form] or an unstable plaque phenotype [the 58kD glycosylated form].[21] Therefore, we considered that EMMPRIN glycosylation could be the mechanism by which MMP-9 expression is controlled via Cav-1. For this reason the expression of MMPs and EMMPRIN glycosylation in relation to Cav-1 were studied in more depth.

Intracellular delivery of peptides that mimic Cav-1 scaffolding domain (CSD) (Cavtratin) in mouse macrophages and arterial rings impaired gelatinase activity. These results are in agreement with recently published data showing that Cav-1 inhibits MMP-2 activity in the heart and MMP-2 activity can be blocked using purified CSD.[31] Another potential mechanism by which Cav-1 might affect MMP cascade might imply changes in glycosylation status of EMMPRIN. It has been shown that up-regulation of Cav-1 prevents transition of EMMPRIN to a highly glycosylated (HG) form.[32,33] This HG-EMMPRIN forms a homodimer and augments MMP production and subsequent activation.[32,33] In our in vitro studies we could not demonstrate an effect of Cavtratin on EMMPRIN levels and therefore we could not prove that Cav-1 influences EMMPRIN glycosylation levels. Whether the reduction of Cav-1 levels leads to an increase in EMMPRIN 58kD glycosylation forms requires further investigation.

MMP-9 activity plays a major role in expansive remodeling in animal models.[5] Expansive remodeling is clinically associated with aortic aneurysms formation and a vulnerable atherosclerotic plaque phenotype.[28] Previously, it was shown that pharmacological inhibition of MMPs impaired arterial expansion upon vascular injury.[34] Therefore, we assessed the inhibitory potential of Cavtratin on this process. Here we show that treatment of mice with Cavtratin significantly reduced carotid expansive remodeling after flow increase. Although mechanically different, MMP-2 and MMP-9 are considered to play similar roles in atherosclerotic expansive remodeling

and flow induced expansion of the artery. The current results therefore point to another potential mechanism by which Cav-1 may prevent plaque growth and destabilization: expansively remodeled lesions may hide accelerated plaque growth with formation of large lipid pools with massive inflammatory response and protease activity that remains clinically silent and untreated for a long time.

We focused on protease activity as a mechanism by which Cav-1 exerts protective effects that would explain the observed outcomes in the clinical and animal studies. Although our results strongly suggest that a mechanistic link exists between Cav-1 and protease activity, we cannot rule out that Cav-1 has broader anti-inflammatory properties. The lower amount of IL-6 and IL-8 found in plaques with high Cav-1 content indeed suggests the importance of Cav-1 in the regulation of pro-inflammatory cascades associated with atherosclerosis. We also showed that incubation with Cavtratin reduced COX-2 expression in LPS-stimulated macrophages. Likewise, over-expression of Cav-1 full length in macrophages was capable of reducing inflammation via a MAPK-dependent mechanism.[35]

The factors that are driving Cav-1 down-regulation during atherogenesis need further investigation. The clinical descriptive data obtained in this study may be helpful to generate hypothesis to answer these questions. Interestingly, we have found that women showed significantly higher Cav-1 expression levels than men. It is known that Cav-1 expression levels are up-regulated upon estrogen treatment in vascular smooth muscle cells.[36] Thus, this observation could suggest that Cav-1 is a potential player in gender associated differences observed in atherosclerotic disease.[37] Low expression levels of Cav-1 were observed in restenotic plaques, although restenotic plaques in general have a fibrous phenotype with high smooth muscle cell content. However, these plaques are in a highly proliferative status. Cav-1 is now recognized as an inhibitor of smooth muscle cell proliferation and decreased Cav-1 expression has been linked to increased smooth muscle cell proliferation in human atheroma.[16] Thus, the low levels of Cav-1 found in restenotic and unstable plaques could be related to a hyperproliferative cell phenotype.

Next to the descriptive clinical observations, we also found a relation between local Cav-1 expression and new perioperative (30 days) vascular events. The ATHERO-EXPRESS is the first vascular biobank with a longitudinal design which allows studies on locally expressed biomarkers in the atherosclerotic plaque as surrogate marker to identify the so-called vulnerable patient.[9] This unique design allows investigating the process of atherosclerotic plaque progression independent of the traditionally identified vulnerable plaque characteristics as defined by post-mortem series. In the current study, all patients in the group with high Cav-1 levels (≥ median level) did not suffer any adverse event in the perioperative period. This suggests that down-regulation of Cav-1 levels in the vascular tree may induce a vulnerable status and atherothrombotic events, while patients with high Cav-1 levels seem protected. In addition, patients with a history of myocardial infarction had lower levels of Cav-1,

supporting the relation between local plaque levels of Cav-1 and vulnerability for vascular events. A systemic predisposition to vascular events and plaque irregularity beyond classical cardiovascular risk factors has been shown on basis of carotid angiograms.[38] Since the systemic vulnerability ultimately leads to formation of an unstable plaque, it is conceivable that the atherosclerotic plaque hides information regarding systemic vulnerability. Although we observed a trend towards lower risk of vascular events in patients with high Cav-1 during follow-up after 30 days, the difference was not statistically significant.

In summary, our findings demonstrate a strong reduction of Cav-1 levels in human carotid plaques in comparison to non-atherosclerotic arteries. Low Cav-1 levels were associated with signs of plaque vulnerability and conversely, plaque Cav-1 levels showed a positive correlation with plaque stabilizing elements. Clinical follow-up revealed that high Cav-1 levels were associated with absence of major adverse cardiovascular events within 30 days of surgery.

Previous reports have shown other in vivo actions of Cavtratin targeting inflammation, tumor progression and right ventricle hypertrophy.[24,25,39] We provide evidence showing that Cav-1 might act as a novel stabilizing factor in human atherosclerosis.

Although our data demonstrate that Cav-1 expression levels influence MMP activity within the plaques contributing to plaque instability, we can not rule out at this point other possibilities regarding how Cav-1 might influence plaque stability such as reduction in the total number of Caveolae and/or effects on plaque vascularization.

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CHAPTER 13

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LOCAL ATHEROSCLEROTIC PLAQUES ARE A SOURCE OF PROGNOSTIC BIOMARKERS FOR ADVERSE CARDIOVASCULAR EVENTS IN HEART, BRAIN AND PERIPHERY

Background Atherosclerotic cardiovascular disease is a major burden to health care. There is an urgent need for biomarkers that allow identification of patients who are at risk for atherothrombotic events following disease progression. Since atherosclerosis is considered a systemic disease, we hypothesized that one single atherosclerotic plaque contains molecular information that is predictive for cardiovascular events in all vascular territories.

Methods Athero-Express is a biobank collecting atherosclerotic lesions during surgery, with a 3 year follow up. The primary outcome encompassed cardiovascular death, myocardial infarction, stroke and all cardiovascular events requiring endovascular intervention. With this plaque material, a potential plaque biomarker, Osteopontin, was identified in a proteomics search. Patients undergoing carotid surgery (n=574) served as the cohort were plaque Osteopontin levels were examined in relation to the outcome during follow up and patients undergoing femoral endarterectomy (n=151) served as an external validation cohort.

Results Comparing the highest quartile of carotid plaque Osteopontin levels with quartile 1+2 showed a hazard ratio for the primary outcome of 3.9 [95% Confidence Interval (CI) 2.6-5.9]. The femoral validation cohort of 151 patients showed a hazard ratio of 3.5 [95% CI 2.1-5.9] comparing quartile 4 with quartiles 1+2. In both the carotid and femoral cohort, multivariable adjustment did not change this outcome. This demonstrated that plaque Osteopontin levels in single lesions are predictive for all different cardiovascular events during follow up.

Conclusions Local atherosclerotic plaques are a source of prognostic biomarkers with a high predictive value for secondary manifestations of atherosclerotic disease.

Introduction

Atherosclerotic cardiovascular disease continues to be the major burden to health care expenditure and its treatment requires intensive forms of medical treatment. A pressing need exists for prognostic markers to identify high-risk patients for aggressive treatment.

Proteins in the plasma are easily accessible and can serve as a surrogate measure of atherosclerotic disease progression, but existing circulating biomarkers do not provide a substantial increase in predictive value.[1,2] With respect to the identification of patients that suffer from rapidly progressing atherosclerotic disease, main focus is on the local rupture-prone (vulnerable) or recently ruptured plaque with typically a large lipid core, thin fibrous cap, a high number of inflammatory cells and thrombosis.[3-6] The pathological definition of the so-called vulnerable plaque has mainly been based on cross-sectional studies. Subsequently, molecular and cellular features that are associated with the vulnerable plaque have been considered as potential diagnostic imaging markers for plaque rupture and subsequent plaque thrombosis. However, longitudinal studies supporting the predictive power of these diagnostic markers have not been executed and information about the natural history of the atherosclerotic disease is therefore incomplete.

The systemic nature of atherosclerotic disease, however, is well established.[7-9] Histopathological observations show that within the individual, inflammation,[10] morphology,[11] and lipid content[12] correlate between different arterial segments within one individual. This gave rise to the hypothesis that local plaques contain molecular information that is predictive for atherothrombotic events in all vascular territories and that the local atherosclerotic plaque may act as a source of prognostic biomarkers that identify the patient at risk.

To provide evidence for the concept that single plaques contain molecular information that predicts future systemic events, the Athero-Express biobank was initiated.[13,14] In this longitudinal biobank study, we compared plaque proteins from patients who reached a cardiovascular endpoint and plaque proteins from patients who remained stable during follow up combined with the complementary power of proteomics. As proof of concept, expression of the identified local plaque protein Osteopontin was studied in all carotid and validated in femoral plaque samples to elucidate its predictive value for the occurrence of systemic cardiovascular events elsewhere in the body.

Methods

Study population and design

Athero-Express is a longitudinal vascular biobank study which includes biomaterials from patients undergoing carotid endarterectomy (CEA) and femoral endarterectomy in two Dutch hospitals (UMC Utrecht and St. Antonius Hospital, Nieuwegein). The primary objective of the study is to investigate the relation between single plaque characteristics at baseline and clinical outcome during follow-up. The study design has been described previously.[13] The study has been approved by the institutional review boards of both hospitals and written informed consent was obtained from all patients.

All patients undergoing CEA between April 1, 2002 and March 1, 2006 in one of the two hospitals were considered for inclusion in the Athero-Express study (n=685 carotid endarterectomy, n=200 femoral endarterectomy). Exclusion criteria for

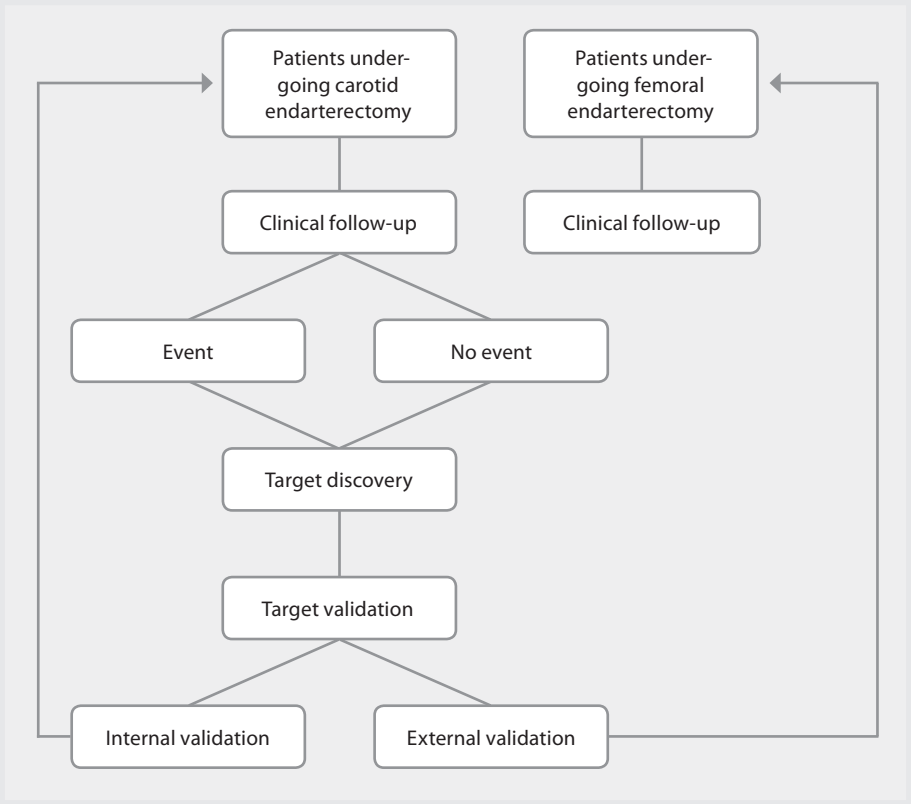


Figure I Study design

follow-up were: unwillingness or physical incapability to participate (e.g. severe dementia)
The criteria to perform carotid endarterectomy were based on the recommendations by the ACAS study for asymptomatic patients and the NASCET study for symptomatic patients.[15,16] At baseline clinical parameters including cardiovascular risk factors and medication use were recorded.

Follow-up

All patients underwent clinical follow-up 1 year after surgical intervention and filled in postal questionnaires 1, 2 and 3 years after the operation. When patients did not respond to the questionnaire, the general practitioner was contacted by phone. Adjudication of the outcome events was done by an outcome event committee, consisting of three authors FLM, JPdeV, WEH, who were blinded to laboratory results. All endpoints were independently assessed by two members of the committee; in case of disagreement, a third opinion was obtained.

Clinical outcome

The primary outcome was a composite encompassing all cardiovascular events and interventions: vascular death, non-fatal myocardial infarction, non-fatal stroke, and vascular intervention that was not planned at the time of inclusion.
Secondary endpoint was any major cardiovascular event: vascular death, non fatal myocardial infarction, non-fatal stroke and non-fatal aneurysm rupture. Definitions and assessment procedures of the outcome events were described previously.[13]

Protein extraction, purification and proteomics

Following dissection, plaques were cut in 0.5 cm segments and processed in a standardized way.[13] For the proteomics analyses, plaque proteins were compared from CEA patients who suffered from a primary outcome versus patients who were event-free. For this purpose the first 80 subsequent patients who had an event were selected in the event group. Eighty control patients were matched for age, gender and duration of follow up.

Protein extraction and purification for proteomics

Plaques were grinded in liquid nitrogen and approximately 500 µL of powder/plaque was used for extraction for proteomics, the remainder was used for RNA and protein isolation using Tripure (Roche). Proteins for proteomics were extracted with 500 µl 40 mM Tris buffer pH 7.5 plus an 1x EDTA-free proteinase inhibitor cocktail (Roche) and 20 s. sonification on ice. After centrifugation (13 krpm 4 C, 10 min.), the supernatant was stored at -80 C as the Tris fraction. The remaining pellet was extracted with 1 mL of 75% Chloroform/25% Methanol with 20 s. sonification. After centrifugation (13 krpm 4 C, 10 min.) the supernatant was stored as the Lipid

fraction and the pellet was extracted with 0.1% SDS. For this, 300 µL 0.1% SDS was added to the pellet followed by 20 s. sonification and heated for 5 min. at 95 C. After centrifugation (13 krpm 4 C, 10 min.) the supernatant was used as the SDS fraction and stored at -80°C on ice. Both the Tris and the SDS fraction were used in the analysis.

First analysis was focused on the proteomics comparing plaque material of only carotid endarterectomy from 80 patients that had a cardiovascular event and 80 sex and age matched event-free controls. For each group (event & control), 1.5 mg pooled protein of the Tris fraction was used (19 µg per patient) and 0.5 mg pooled protein of the SDS fraction was used (6.5 µg per patient). To remove the 6 most abundant serum proteins (albumin, IgG, IgA, transferrin, haptoglobin, and antitrypsin), the Multiple Affinity Removal Spin Cartridge from Agilent (Santa Clara, CA, USA #5188-5341) was used. After purification, 400 µg of the purified Tris protein and 100 µg of purified SDS protein were used for digestion. Before digestion the sample was split into two to obtain two technical replicates. Samples were reduced, alkylated, and tryptic digested as described (Washburn et al 2001). The samples were then desalted by passing the digested mixture through a conditioned Sep-Pak C-18 SPE cartridge (Waters, Milford, MA, USA), washed twice with a 3% acetonitrile (ACN) (JT Baker, Phillipsburg, NJ) and 0.1% formic acid (FA) buffer, and eluted with a 70% ACN and 0.1% FA buffer. The eluted samples were then dried to about 20 µL by removing organic solvent in a speedvac concentrator.

Liquid chromatography mass spectrometry proteomics

The samples were kept at 4°C before analysis and fractionated off-line on a BioBasic SCX column (5 µm, Thermo Electron, San Jose, USA) with an Agilent 1100 HPLC system. The Tris fractions were separated in 10 salt fractions (0, 2, 4, 6, 10, 18, 26, 45, 100, 1000 mM ammoniumchloride) loading 80 µL sample or salt with a flow of 10 µL/min. (4% Acetonitril, 0.1% Formic Acid) for 30 min. The SDS fraction was separated in 5 fractions (0, 4, 18, 100, 1000 mM). Each salt-fraction was concentrated with a speedvac concentrator and injected separately onto the second dimension for mass spectrometry (MS) analysis.

The second dimensional chromatographic separation was carried out with a home-packed nanobored C18 column (75 µm i.d x 10cm, 5 µm particles) directly into a pico-frit nanospray tip (New Objective, Wubrun, MA, USA), operating at a flow rate of 200 nL/min. with a 65 min gradient. The LTQ was operated in a data-dependent mode by performing MS/MS scans for the 5 most intense peaks from each MS scan. The MS was operated at an nanospray voltage of 1.8 kV; without shealth gas and auxiliary gas flow; ion transfer tube temperature of 180°C; collision gas pressure of 0.85 mTorr and normalized collision energy at 35% for MS/MS. Ion selection threshold was set to 500 counts for initiating MS/MS while activation q was set to 0.25 and activation time to 30 ms. The MS scan range was 400-1800 m/z. Dynamic exclusion

was activated with an exclusion duration of 1 min. For each salt-step experiment, MS/MS (dta) spectra were extracted from the raw data files with the Biowork 3.3 program (ThermoFinnigan, San Jose, CA, USA).
Protein identification was achieved by searching the data from each salt-step against the IPI human protein database (version 3.15, 58009 entries) using Sequest (Bioworks 3.3) allowing a maximum of 3 missed cleavages with trypsin. Fixed modification was carbaminomethylation and variable modification was oxidation of methionine. Results filters used: for peptides XCorr set for z=1 at 1.5, z=2 at 2.5 and z=3 at 3.5 and for proteins protein probability set at 1.00E-3.
Osteopontin was found as 1 of the proteins that was present in both technical replicas in the event group and not present in both technical replicas of the event free control group.

Bioinformatics

Using Bioworks, 1 Microsoft Excel file with identified proteins was generated per Salt fraction run. Omniviz and Excel were used to select for proteins that were only present in the events (at least in 1 salt-fraction) and not in (in none of the salt-fractions) the matched controls. To select proteins for validation in the individual patients, proteins were used that were present in the Ingenuity networks (Ingenuity Systems Inc. Redwood City CA, USA) that could be linked into one large network.
Within this network Osteopontin (OPN) was selected based on its previously described relation with atherosclerosis (Myers et al 2003, Strom et al 2004, Matsui et al 2003, Chiba et al 2002) and the availability of a commercial ELISA.

Protein extraction and Osteopontin elisa

A commercial Osteopontin ELISA (R&D systems Cat. # DOST00) assay was used according to the described procedures. From each patient, 1 µL µg Tripure (Roche) isolated protein was used per well.

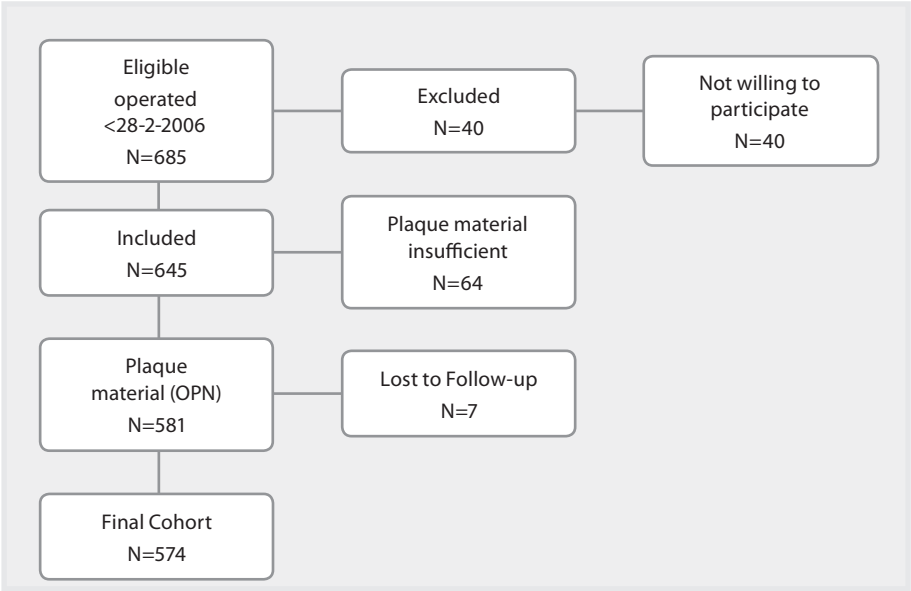
Immunohistochemistry

To make the OPN and CD68 epitopes accessible for the antibodies, sections were boiled in citrate buffer. Mouse anti-human OPN (IBL, Gunma, Japan; dilution 1 : 250) and CD68 (NovaCastra, Newcastle, UK; dilution 1 : 400) monoclonal antibodies were used. PowerVision Poly-AP-anti mouse IgG (Immunologic, Duiven, the Netherlands) and New-Fuchsin solution were used to visualize the signal. All sections were counterstained with hematoxylin. Isotype antibodies were used as negative control.

Statistical analysis

From subsequent patients who underwent carotid endarterectomy, plaques were used to assess the predictive value of plaque Osteopontin levels for reaching the primary and secondary outcome events. Plaques obtained from patients undergo-

A Carotid endarterectomy cohort



B Femoral endarterectomy cohort

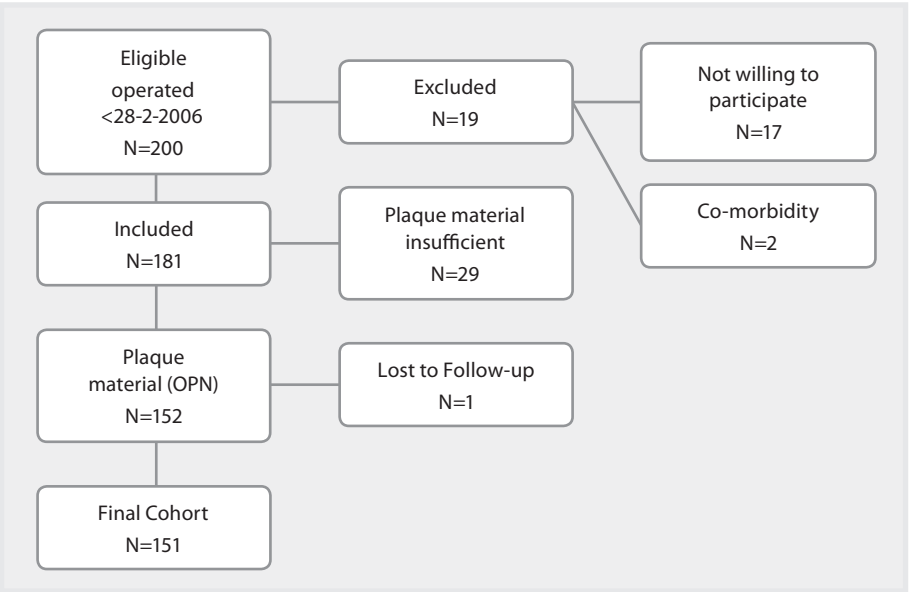


Figure II Inclusion flow diagram

A: Carotid endarterectomy cohort **B:** Femoral endarterectomy cohort

ing femoral endarterectomy were used for external validation. Since part of the carotid artery samples had been used for proteomic analyses, a subgroup analysis was performed on those patients whose plaque material had not been used for the proteomic analyses.

Plaque Osteopontin levels were divided into quartiles with cut-off values at 3.2, 8.2 and 14.9 ng/mL. In all analyses, quartiles 1 and 2 had similar risk implications; therefore data from quartiles 1 and 2 were combined and served as reference for comparison with quartiles 3 and 4. Kaplan-Meier survival analysis was used to estimate cumulative event rates after 3 years follow-up. Cox regression was used to calculate the hazard ratio (HR) with 95% confidence interval (95% CI) for the association between plaque characteristics and follow-up. In multivariable analysis, we

Table I Baseline characteristics of patients undergoing carotid endarterectomy

Patient Characteristics	n / total [A]	% [A]
Number of patients	574	
Clinical characteristics		
Age, mean/sd, y	68.0	8.8
Male gender (%)	409/574	71
Current smoker (%)	153/556	28
Diabetes (%)	468/570	18
Hypertension (%)	377/570	66
Hypercholesterolemia (%)	343/572	60
History: Vascular intervention	192/572	34
History: Myocardial infarction	120/569	21
Body mass index, mean/sd, kg/m^2	26.6	3.7
Statin use	420/570	74
Aspirin use	507/570	89
Oral anticoagulant use	83/570	15
Bilateral carotid stenosis	244/574	43
Symptomatic stenosis	456/574	79
Plaque characteristics		
Large lipid core (>40%)	198/573	35
Marked macrophage infiltration	329/569	58
Marked Calcifications	331/573	58
IL-6, mean/se, ng/g	99	12
IL-8, mean/se, ng/g	223	24
MMP-2, mean/se, a.u.	4.1	0.24
MMP-9, mean/se, a.u.	0.81	0.03

[A] Number/total and percentage unless otherwise specified

Table II Relation between plaque OPN and primary endpoint, adjusted for potential confounders

	OPN expression			Risk of primary outcome		
	characteristic			HR	HR of Plaque OPN adjusted[A]	
	absent	present	Mean Δ [95% CI]	[95% CI]	Q3 vs Q1+Q2 [95% CI]	Q4 vs Q1+Q2 [95% CI]
Crude HR plaque OPN					2.3[1.4-3.8]	4.0[2.5-6.5]
Clinical characteristics						
Age ≥ 70	10.6 (0.56)	10.7 (0.64)	0.1[-1.6-1.8]	1.5[1.1-2.1]*	2.3[1.4-3.8]	4.0[2.5-6.5]
Male gender (%)	9.7 (0.67)	11.0 (0.53)	1.2[-0.6-3.1]	1.8[1.2-2.8]*	2.2[1.3-3.6]	3.9[2.4-6.4]
Current smoker (%)	10.1 (0.48)	12.1 (0.92)	2.0[0.1-3.9]*	1.4[0.96-2.0]	2.2[1.3-3.7]	3.9[2.4-6.3]
Diabetes (%)	10.8 (0.47)	10.1 (1.0)	-0.7[-2.9-1.5]	0.8[0.5-1.3]	2.3[1.4-3.8]	4.0[2.5-6.5]
Hypertension (%)	9.8 (0.67)	11.0 (0.54)	1.1[-0.6-2.9]	1.0[0.7-1.5]	2.3[1.4-3.8]	4.0[2.5-6.5]
Hypercholesterolemia (%)	10.4 (0.66)	10.7 (0.55)	0.4[-1.3-2.1]	1.2[0.8-1.7]	2.3[1.4-3.8]	4.0[2.5-6.4]
History: Vascular int	10.8 (0.54)	10.2 (0.65)	-0.6[-2.4-1.1]	1.4[1.0-2.0]*	2.3[1.4-3.8]	3.9[2.4-6.4]
History: MI	10.7 (0.49)	10.5 (0.89)	-0.1[-2.2-1.9]	1.6[1.1-2.3]*	2.3[1.4-3.8]	4.0[2.5-6.5]
BMI ≥ 25 kg/m^2	10.3 (0.70)	10.7 (0.59)	0.3[-1.5-2.2]	0.9[0.6-1.4]	2.3[1.4-3.8]	4.0[2.5-6.5]
Statin use	10.3 (0.87)	10.8 (0.49)	0.5[-1.4-2.4]	1.4[0.9-2.1]	2.3[1.4-3.9]	3.9[2.4-6.3]
Aspirin use	11.9 (1.2)	10.5 (0.45)	-1.4[-4.0-1.3]	0.6[0.4-1.0]*	2.3[1.4-3.8]	3.9[2.4-6.4]
Oral anticoagulant use	10.6 (0.47)	10.9 (1.1)	0.3[-2.1-2.6]	1.4[0.9-2.2]	2.3[1.4-3.8]	4.0[2.4-6.4]
Bilateral carotid stenosis	8.9 (0.50)	12.8 (0.71)	3.8[2.2-5.5]*	2.1[1.5-3.0]*	2.2[1.3-3.6]	3.7[2.3-6.0]
Symptomatic stenosis	9.7 (1.0)	10.8 (0.47)	1.1[-0.9-3.2]	1.1[0.7-1.7]	2.3[1.4-3.9]	4.1[2.5-6.6]
Other plaque characteristics						
Large lipid core (>40%)	9.7 (0.47)	12.3 (0.8)	2.5[0.8-4.3]*	1.1[0.8 - 1.5]	2.4[1.4-3.9]	4.1[2.5-6.6]
Marked MO infiltration	9.6 (0.56)	11.4 (0.61)	1.8[0.1-3.5]*	1.0[0.7-1.4]	2.3[1.4-3.8]	4.0[2.5-6.5]
Marked Calc	10.2 (0.61)	10.9 (0.59)	0.8[-0.9-2.4]	1.0[0.7-1.4]	2.3[1.4-3.8]	4.0[2.5-6.5]
IL-6 [B]	10.0 (0.58)	10.3 (0.64)	0.3[-1.4-2.0]	1.4[1.0-2.1]*	2.3[1.4-3.8]	4.1[2.5-6.6]
IL-8 [B]	9.2 (0.61)	11.1 (0.61)	1.9[0.2-3.6]*	1.2[0.9-1.7]	2.4[1.4-3.9]	4.3[2.6-6.9]
MMP-2 [B]	10.9 (0.66)	9.9 (0.55)	-1.0[-2.7-0.7]	0.6[0.4-0.9]*	2.3[1.4-3.8]	4.0[2.5-6.4]
MMP-9 [B]	10.0 (0.57)	10.8 (0.64)	0.8[-0.9-2.4]	1.1[0.8-1.5]	2.3[1.4-3.8]	4.0[2.5-6.5]
Multivariable adjustment [C]					2.0[1.2-3.4]	3.7[2.3-6.0]

Abbreviations: CI, confidence interval; Δ, difference; HR, Hazard ratio; IL, Interleukin; int, intervention; MI, myocardial infarction; MMP, Matrix Metalloproteinase; OPN, Osteopontin; Q, quartile

P<0.001 for all HR's of OPN vs. primary endpoint.

[A]Analysis restricted to patients with no missing values on any of the patient and plaque characteristics (n=431)

[B]IL-6, IL-8, MMP-2, MMP-9: ≥median vs. <median

[C]adjusted for gender, smoking, bilateral carotid stenosis, IL-8

*P < 0.05

adjusted simultaneously for those potential confounders that changed the crude HR by more than 0.1 in bivariable analysis. For the multivariable analysis, we restricted the patient selection to those with no missing values (n=431).

Results

The study design is outlined in **Figure I**. A total of 685 patients undergoing carotid endarterectomy were found to be eligible for inclusion. Forty patients were not willing to participate, and in 64 patients no plaque material was available for protein analyses. In the majority of these cases the dissected plaque sample was formalin fixed and paraffin embedded for immunohistochemistry. Seven patients (1.2%) of the remaining 581 patients were lost to follow up, resulting in a final cohort of 574 patients **(Figure II-A; Table I)**. In the cohort undergoing femoral artery surgery, 200 patients were eligible, of whom 19 were excluded (17 not willing to participate, 2 with severe dementia). In the resulting 181 patients, 29 had insufficient plaque material and 1 was lost to follow-up, yielding a final cohort of 151 patients **(Figure II-B)**. Baseline characteristics did not differ between patients of the study cohort and patients with insufficient plaque material or who were lost to follow up (data not shown).
The proteomics and bioinformatics search revealed a list of 90 candidate plaque proteins that could be upregulated in patients who suffered from an event during follow up. Osteopontin was selected for the current proof of concept study.
The mean plaque Osteopontin levels differed consistently between plaques obtained from patients who suffered from any event during follow up (mean level 15.0 +/- 1.0 ng/mL) and patients who had no event (mean level is 9.2 +/- 0.4 ng/mL). Mean OPN levels were 15.1 +/- 1.3 ng/mL for patients who had a secondary outcome, 15.5 +/- 1.6 ng/mL for those who had a stroke, 13.5 +/- 1.5 ng/mL for patients with a coronary event or intervention and 15.2 +/- 1.6 ng/mL for peripheral interventions. **Table II** shows the plaque Osteopontin levels in association with the baseline clinical characteristics and plaque characteristics.

Internal validation: Osteopontin as a predictive plaque marker in carotid plaques

Carotid plaque Osteopontin levels (n=574) were strongly related with the occurrence of the primary outcome and this relationship was stronger with higher levels of Osteopontin **(Figure III)**. The hazard ratio was 2.2 [95%CI 1.4-3.4] for the third quartile versus quartiles 1+2 and that for the highest quartile (Q4) 3.9 [95%CI 2.6-5.9]. In the patients included in the multivariable analysis, the crude hazard in the whole cohort was comparable to the crude hazard ratio for the patients in the multivariable analysis: 2.4 [1.4 – 3.8] for the third quartile versus quartiles 1+2 and that for the highest quartile (Q4) 4.0 [2.5 – 6.5] **(Table II)**. Adjustment for potential

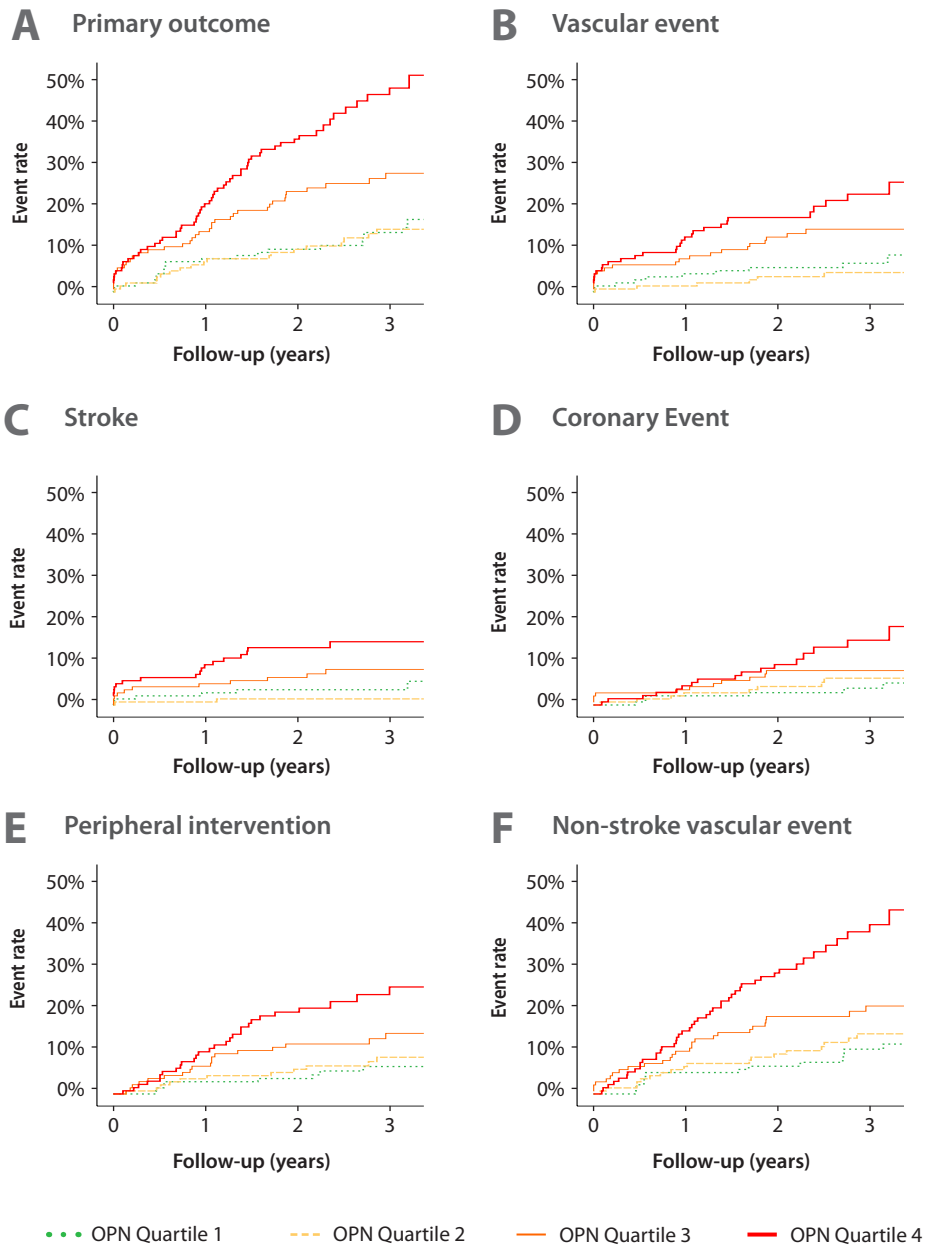


Figure III Event rate in relation to carotid plaque OPN at baseline

A: Primary outcome **B:** Secondary outcome **C:** Stroke **D:** Coronary event **E:** Peripheral vascular intervention **F:** Non-stroke vascular event

Table III Kaplan Meier survival analysis

	No. patients w. event	No. patients at risk				3 year risk KM estimate	Risk difference (vs. ref)			HR [95% CI] [A]
		baseline	1 y	2 y	3 y		1 year	2 years	3 years	
Carotid										
Primary outcome										
Q1 OPN	20	143	132	113	46	15.4%		- ref -		- ref -
Q2 OPN	20	144	130	114	46	15.1%		- ref -		- ref -
Q3 OPN	38	144	123	88	32	28.2%	+6.9%	+14.2%	+12.9%	2.2 [1.4 – 3.4]
Q4 OPN	59	143	110	64	24	47.9%	+13.5%	+27.1%	+32.6%	3.9 [2.6 – 5.9]
TOTAL	137	574	495	379	148	26.2%				
Vascular event										
Q1 OPN	10	143	137	119	49	6.8%		- ref -		- ref -
Q2 OPN	7	144	139	124	53	4.7%		- ref -		- ref -
Q3 OPN	20	144	132	100	42	14.9%	+5.5%	+9.0%	+9.2%	2.5 [1.3 – 4.8]
Q4 OPN	29	143	120	85	34	23.1%	+10.0%	+13.1%	+17.4%	4.0 [2.2 – 7.3]
TOTAL	66	574	528	428	178	15.0%				
Stroke										
Q1 OPN	6	143	138	121	50	3.6%		- ref -		- ref -
Q2 OPN	2	144	140	126	56	1.4%		- ref -		- ref -
Q3 OPN	11	144	136	104	43	8.5%	+3.1%	+4.7%	+6.0%	2.9 [1.2 – 7.1]
Q4 OPN	19	143	120	85	35	15.2%	+7.7%	+11.2%	+12.7%	5.4 [2.4 – 12.4]
TOTAL	38	574	534	436	184	8.0%				
Coronary event										
Q1 OPN	6	143	138	120	48	5.2%		- ref -		- ref -
Q2 OPN	9	144	136	122	51	6.5%		- ref -		- ref -
Q3 OPN	11	144	134	102	43	8.2%	+1.8%	+4.6%	+2.4%	1.6 [0.7 – 3.4]
Q4 OPN	18	143	124	87	34	15.5%	+1.9%	+6.1%	+9.6%	2.8 [1.4 – 5.6]
TOTAL	44	574	532	431	176	11.6%				

	No. patients w. event	No. patients at risk				3 year risk KM estimate	Risk difference (vs. ref)			HR [95% CI] [A]
		baseline	1 y	2 y	3 y		1 year	2 years	3 years	
Peripheral intervention										
Q1 OPN	8	143	136	118	49	7.0%		- ref -		- ref -
Q2 OPN	12	144	134	117	50	8.7%		- ref -		- ref -
Q3 OPN	19	144	130	97	36	14.4%	3.5%	6.8%	6.6%	2.1 [1.1 – 4.1]
Q4 OPN	28	143	113	72	27	25.1%	6.8%	15.4%	17.3%	3.8 [2.1 – 6.7]
TOTAL	67	574	513	404	162	15.5%				
Non-stroke vascular event										
Q1 OPN	14	143	134	115	46	12.1%		- ref -		- ref -
Q2 OPN	19	144	130	114	46	14.4%		- ref -		- ref -
Q3 OPN	28	144	126	92	34	20.9%	+4.5%	+10.1%	+7.7%	1.9 [1.2 – 3.2]
Q4 OPN	46	143	113	68	25	39.6%	+9.2%	+21.5%	+26.3%	3.7 [2.4 – 5.9]
TOTAL	107	574	503	389	151	24.3%				
Femoral Vascular event + intervention										
Q1 OPN	13	37	33	19	7	37.1%		- ref -		- ref -
Q2 OPN	15	38	31	20	6	46.9%		- ref -		- ref -
Q3 OPN	26	38	22	9	2	79.1%	+27.4%	+28.3%	+36.9%	2.6 [1.5 – 4.5]
Q4 OPN	28	38	17	7	1	79.1%	+40.6%	+38.1%	+37.0%	3.5 [2.1 – 5.9]
TOTAL	82	151	103	55	16	60.4%				

[A] Cox regression (Osteopontin Q3 vs. Q1+Q2 and Osteopontin Q4 vs. Q1+Q2)

confounders did not change these findings: the multiple adjusted HR for Q4 versus Q1+2 was 3.7 [95%CI 2.3-6.0].

Figure III-A, B illustrates the time-to-event curves for the primary and secondary outcome. Additional curves **(Figure III-C-F)** also demonstrate consistent outcomes for the other clinical outcomes, as well as for the primary outcome in the femoral artery cohort **(Figure IV; Table III)**. Almost half of all patients in Q4 reached the primary endpoint within 3 years. About a quarter in Q4 suffered from a vascular event during follow up compared with 6% in Q1+2. A post-hoc analysis among patients who had asymptomatic carotid artery stenosis (n=118) showed a crude hazard ratio for the primary outcome of 7.1 [95%CI 3.1 – 16.4] in Q4; the multiple adjusted HR was 7.1 [95%CI 2.9 – 17.2].

External validation: predictive value of OPN in femoral plaques

We validated these observations on basis of carotid plaque material in patients who had femoral endarterectomy. Mean follow up time in this cohort was 2.3 years+/- 0.9. Eighty two patients (54%) reached the primary outcome during follow up. The HR of Q3 versus Q1-2 was 2.6 [95%CI 1.5-4.5] and 3.5 [95%CI 2.1-5.9] for Q4. Multivariable analyses did not materially change these findings (data not shown).

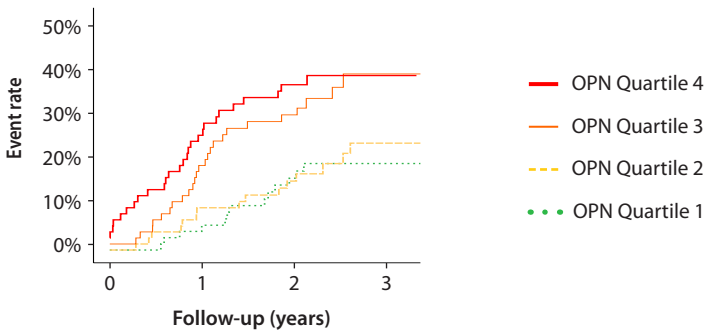


Figure IV Primary outcome in relation to femoral plaque OPN at baseline

Discussion

Our study indicates that high levels of Osteopontin from atherosclerotic plaques strongly predict the risk of new vascular complications. The findings pertain to all parts of the vascular bed and were observed for both carotid and femoral artery plaques. These observations confirm our hypothesis that local plaques contain molecular information predictive of new atherothrombotic events elsewhere in the vascular tree.

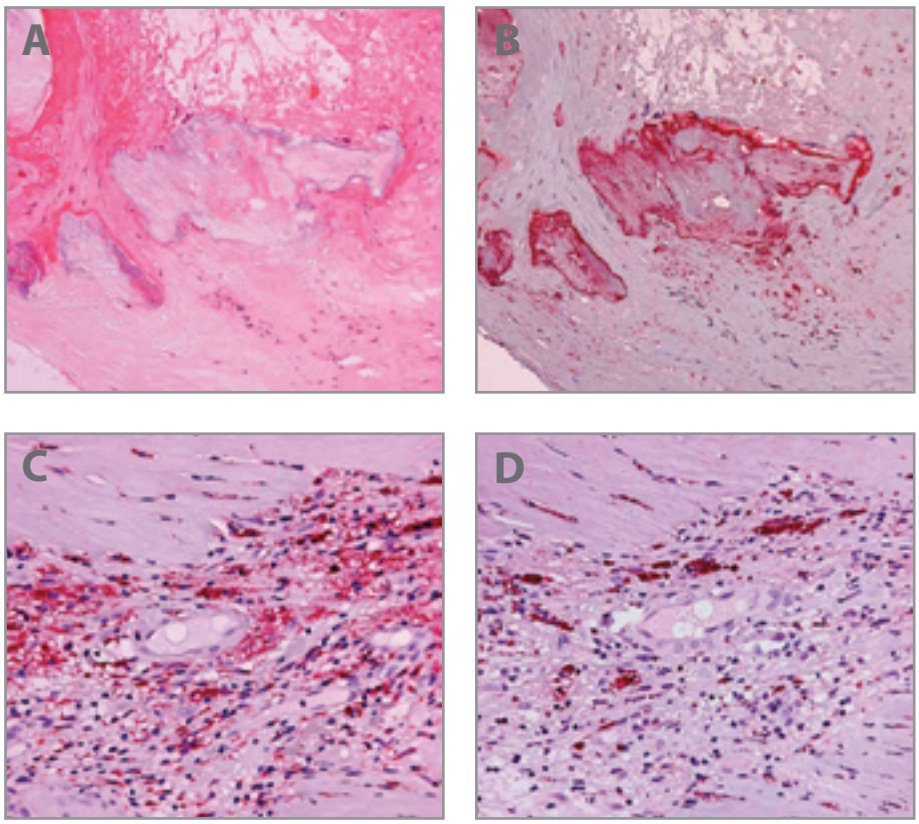


Figure V Immunohistochemistry

A: Hematoxylin and eosin staining showing calcification in an atherosclerotic plaque. **B:** Osteopontin staining at the boundary of the calcified area in the same plaque. **C:** CD68 staining showing macrophages around microvessels in the shoulder of an atherosclerotic plaque. **D:** Osteopontin staining of consecutive section in which only a subset of the macrophages and macrophage foam cells show immunoreactivity.

Our observations are in line with previous observations. The positive association between carotid-artery intima-media thickness with cardiovascular risk factors[7] and events[8] has been established. In addition, asymptomatic carotid artery stenosis is an independent predictor of vascular events,[9] and carotid plaque morphology for non-cerebral events.[11]

Our study has clinical implications with respect to the biomarker search to identify the patient at risk for secondary clinical manifestations of atherosclerotic disease. Imaging modalities like MRI, CT, SPECT, ultrasound may apply these predictive plaque proteins to stratify patients at risk for secondary events by detection of plaque levels of proteins.[17,18] Our observations also prove the concept that local plaque material may be used for prognostic screening and research since predictive plaque proteins can be detected in material of advanced plaques harvested after atherectomy, PTCA or CABG and different types of vascular surgery to stratify these patient populations for secondary events.[19]

Our results identify a new concept in the search for biomarkers of progression of atherosclerotic disease opening up a new strategy. The following observations support this view. First, following the concept that local plaques hide information on the stability of the entire atherosclerotic vascular system, we assumed that the biomarker should be detectable in every advanced atherosclerotic plaque independent of localization. We showed that besides carotid plaque Osteopontin levels also femoral plaque Osteopontin levels were predictive for cardiovascular events. This strongly suggests that there is predictive information in every advanced plaque for future cardiovascular events in other vascular territories, independent of plaque localization. Secondly, the relation of plaque Osteopontin with future adverse events was independent of plaque features that are considered the hallmark of the vulnerable plaque. The current observations do not challenge the value of the current definitions of the vulnerable plaque for the understanding of pathogenesis but reveal that the local plaque hides strong molecular biomarkers for disease progression irrespective of the pathological characterization. The weak association of Osteopontin with a large lipid core and macrophage infiltration is probably due to the presence of Osteopontin in only a subgroup of macrophages **(Figure V)**. Thirdly, the relation between Osteopontin levels and the outcome measure was not only evident in symptomatic but also in asymptomatic patients. Expression levels were studied in advanced lesions in search for markers predictive for secondary clinical manifestations. Considering the promising results in asymptomatic patients, the next phase in this biomarkers discovery approach could be a longitudinal study specifically addressing asymptomatic less advanced lesions. The high hazard ratio (7.1) for high Osteopontin plaque levels in the asymptomatic patient group was surprising. However, this is a subgroup analyses with low patient numbers and results therefore require verification to become conclusive.

Osteopontin was arbitrarily selected for this proof of concept biomarker study. The function of Osteopontin has not been explored in this study. Osteopontin, also known as early T-lymphocyte activation 1 (Eta-1), is a secreted multifunctional glycoprotein.[20] Mouse studies provided genetic evidence for a causal role of Osteopontin in the development of atherosclerotic plaques.[21-24] Osteopontin plasma levels have shown to be related to coronary artery disease[25, 26] and to be an independent predictor of future cardiovascular events in patients with chronic stable angina with an HR=1.8 underlining the high HR of plaque Osteopontin levels.[27]

Several limitations of this study need to be discussed. Plaque phenotype is associated with the clinical disease status at baseline. Patients who suffered from stroke have more plaques with unstable characteristics compared with asymptomatic patients. Although our results were independent of clinical presentation, the heterogeneity of the population merits consideration. The results presented are limited to an elderly atherosclerotic patient population which makes extrapolation to a population that suffers from traditional risk factors but without a clinical manifestation difficult. The predictive value of Osteopontin plaque levels in asymptomatic patients, however, remains high and suggests that these findings might apply to a broader population with advanced atherosclerotic plaques.

In conclusion, we show that local atherosclerotic plaques are a source of prognostic biomarkers for the occurrence of adverse cardiovascular events in other vascular territories. We identify Osteopontin as such a prognostic biomarker with a high predictive value for secondary events after endarterectomy.

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PART 6

GENERAL DISCUSSION AND SUMMARY

CHAPTER

14

GENERAL DISCUSSION

The main objective of the Athero-Express study was to investigate the predictive value of carotid plaque composition at the time of carotid endarterectomy for restenosis and clinical outcome during follow-up. Since its initiation in 2002, the Athero-Express has become a successful biobank study including over 1000 carotid plaques as well as more than 500 other vascular specimens. The study has succeeded to identify plaque characteristics that are predictive of restenosis after carotid endarterectomy and plaque characteristics with predictive value for the occurrence of cardiovascular events during follow-up. Combination of the prospective study design with a proteomics approach has enabled identification of several potential biomarkers to predict risk of future cardiovascular events. The validation process of these markers is ongoing and is expected to yield additional predictive markers in the near future. A summary of the conclusions of the thesis can be found in **Chapter 15**.

Now that the relation between atherosclerotic plaque composition and clinical outcome has been established, the next step is to incorporate these findings into clinical practice. In addition, insights obtained in the prospective studies can be translated to basic science studies. **Figure I** gives an overview of the results of this thesis as well as future research directions. Below, we will discuss the results obtained in this thesis and describe the further research that is needed to advance to clinical application of atherosclerotic plaque composition.

Influence of plaque composition on benefit and choice of vascular interventions

Influence of plaque composition on benefit and choice of vascular interventions - this study

In the present thesis we show that several important clinical characteristics are related with the composition of the atherosclerotic carotid plaque. These clinical and demographic characteristics, namely clinical presentation (symptomatic vs. asymptomatic), (male) gender, (higher) age, and (short) time interval between presenting symptoms and CEA, are the most important determinants of benefit from carotid endarterectomy.[1] The beneficial effect of carotid endarterectomy has been explained by excision of the carotid plaque as a potential source of cerebrovascular complications, caused by emboli or local thrombotic occlusion. It is plausible to assume that excision of a plaque with high risk of such complications would portray more benefit than excision of a low-risk plaque. In the coronary circulation, "high-risk" plaque characteristics have been identified that are associated with acute myocardial infarction and coronary death.[2-5] These vulnerable plaque characteristics include marked inflammation and large lipid core overlain by a thin fibrous cap,

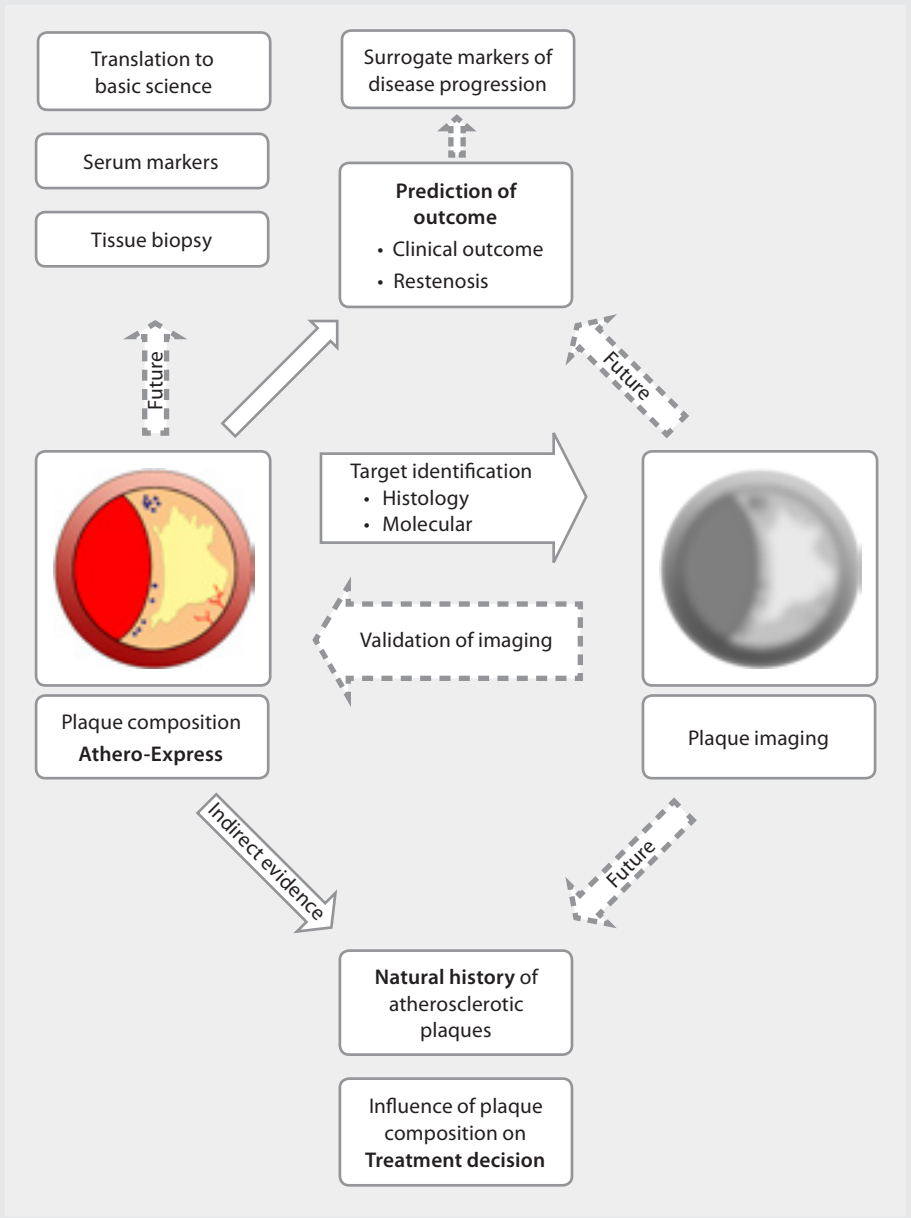

Figure I Future perspectives

Solid arrows indicate research performed in the current thesis, the dashed "FUTURE" arrows indicate future research

with decreased smooth muscle cell and collagen content. Therefore, we hypothesized that in patient groups that show more benefit from carotid endarterectomy (male, elderly, recently symptomatic), the prevalence of these plaque characteristics would be higher. **Chapters 4, 5 and 6** provide evidence supporting this hypothesis **(Table I, Figure II)**. The association with age in the Athero-Express study was described previously by Van Oostrom et al.[6] Furthermore, in **Chapter 4** we show that symptomatic presentation of carotid artery disease was associated with large lipid core and decreased collagen content, which points out that the mechanisms of symptomatic carotid disease concur with the mechanisms of symptomatic coronary artery disease. This finding was confirmed by a study in 526 patients from Redgrave et al.[7] **Table I** summarizes the evidence linking the benefit of CEA to the composition of the atherosclerotic plaque at the carotid bifurcation.

As an alternative for our explanation that plaque composition determines benefit of CEA, it could be postulated that the difference in benefit of CEA between those groups is caused by differences in disease progression after carotid endarterectomy and not by the risk of stroke that is attributable to the plaque. However, this is not in line with clinical studies published so far. For example, the gender difference is mainly caused by the higher event rate in men who undergo conservative treatment for carotid stenosis.[8] After excision of the carotid plaque, the remaining risk is equal between men and women. A part of the gender difference could be attributed to increased perioperative risk in women. However, a large meta-analysis indicated that the perioperative risk difference was only 1%, and therefore can not explain the difference between men and women in the benefit of carotid endarterectomy.[9]

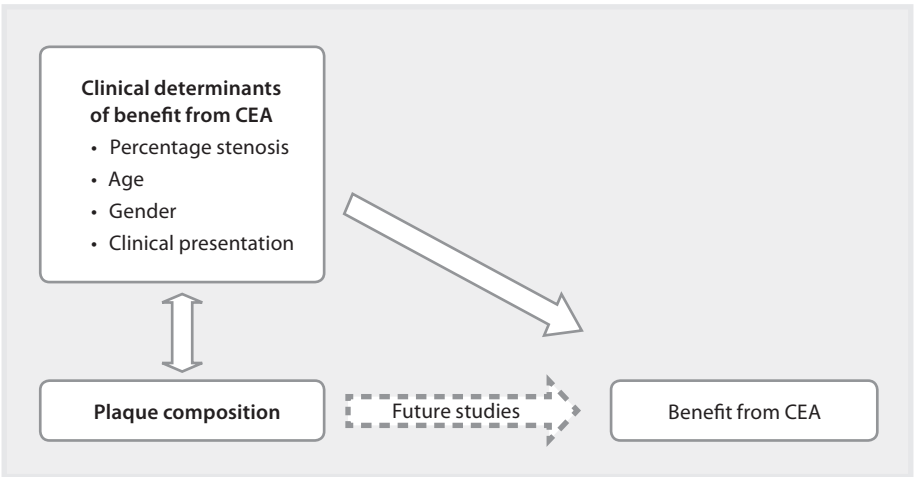

Figure II Carotid plaque composition and benefit of carotid endarterectomy

Table I Benefit of CEA versus plaque characteristics

	Benefit of CEA	Prevalence of vulnerable plaque
Symptomatic presentation	increased	increased
Older age	increased	increased
Male gender	increased	increased
Longer interval between symptoms and CEA	decreased	decreased

Influence of plaque composition on benefit and choice of vascular interventions - future research

For the next years, we can speculate that the plaque itself may become a more important determinant of benefit from CEA than the clinical risk factor "attached" to it. It is possible that the clinical risk factors influence the benefit of CEA almost completely via composition of the carotid plaque **(Figure III)**. On the other had it is likely that other factors, such as thrombogenecity of the blood, also play an important role.[10] From a pragmatic point of view, it seems plausible that the composition of the plaque is an important factor which may at least partly determine the benefit of CEA. The indirect evidence obtained by combining our results and previous data from large clinical trials warrants validation by prospective clinical trials coupled to plaque imaging, which is feasible with different imaging techniques **(Chapter 2)**. These studies should include pre-operative plaque imaging and analyze the influence of plaque composition on the number needed to treat to prevent neurologic deficit. Once pre-operative plaque imaging is linked with benefit from CEA by solid evidence, clinical application of plaque composition will gain acceptance. Those who fear that the number of surgical procedures could decline, should realize that imaging may also open up the possibility of surgical treatment for patients with less than 50%-70% stenosis who have a "vulnerable plaque" at the carotid bifurcation. At present, carotid endarterectomy for patients with less than 50% stenosis is not performed. From coronary artery disease it is known that many plaques which led to a myocardial infarction caused less than 50% stenosis before plaque thrombosis occurred.[11] Therefore, it may be well possible to identify patients with relatively low grade carotid stenosis who will benefit from carotid endarterectomy, based on noninvasively determined plaque characteristics.

Alternatively, plaque characteristics may aid in the choice of treatment modality. Vessels containing fibrous plaques are prone to develop restenosis after CEA, probably due to constrictive remodeling after CEA **(Chapter 7)**. These plaques could preferably be treated by carotid artery stenting (CAS), since stent placement inhibits constrictive remodeling.[12-14] The most important outcome measure to guide the optimal treatment strategy is overall cardiovascular event rate. Therefore, the

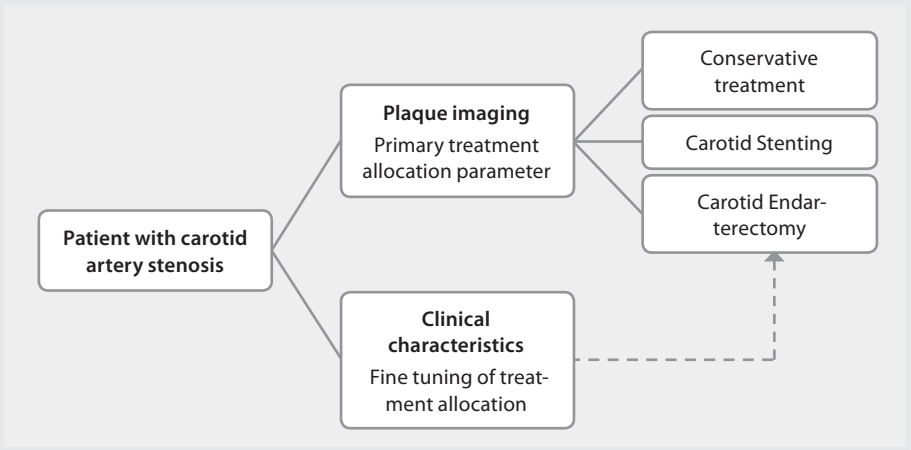

Figure III Suggested future decision scheme for treatment of carotid stenosis

concept to choose between an endovascular and open procedure based on plaque composition will have to be validated in a prospective study comparing CAS to CEA which incorporates pre-operative plaque imaging.

Prediction of local disease progression

Prediction of local disease progression - this study

In this study, we investigated the relation between local plaque composition and local restenosis after carotid endarterectomy. Previous studies investigated restenosis cross-sectionally. The histological characteristics of restenotic lesions after coronary stenting were reported in earlier studies.[15] In the current thesis **(Chapter 7)**, we demonstrate that patients whose excised carotid plaque shows marked inflammation and large lipid core are less prone to develop restenosis after CEA. This finding was somewhat unexpected, since it is generally assumed that progression of restenotic lesions is driven by inflammation.[16, 17] From the research conducted in **chapter 8** it has become clear that early restenosis is mainly caused by smooth muscle cells and collagen deposits, which is different from late restenosis which resembles primary atherosclerosis. Inflammatory arteries may be protected from early restenosis due to increased expansive geometrical remodeling of the vessel **(Chapter 7)**.

These findings may have clinical implications. Here, we present some extra analyses underscoring the predictive value of plaque composition for restenosis. From the multivariable analysis presented in **Chapter 7-Table IV**, we constructed a predic-

Table II Utrecht restenosis score

Characteristics	Points
Hypercholesterolemia (treated)	0.5
Patch	
Venous	0
Dacron	0.5
no patch	1
Plaque macrophages	
moderate or heavy	0
no or minor	1
Plaque lipid core	
>40%	0
10% - 40%	0.5
<10%	1
SUM = UTRECHT RESTENOSIS SCORE	(0 - 3.5)

tion score for the development of restenosis after carotid endarterectomy called "The Utrecht Restenosis Score" **(Table II)**. **Figure IV** shows that patients with a score <1 had 0% chance of restenosis. The intermediate group had a risk comparable to the whole population, and the group with score >2 had 3-fold increased risk of >70% restenosis (16%). Based on plaque histology, duplex follow-up could be omitted in the low-risk group and performed more frequently in the high risk group. In addition, the high risk group also showed strongly increased risk of occlusion within 3 months after CEA. Therefore, early duplex follow-up is warranted in this group. This would imply a change of clinical management in 57% of patients undergoing carotid endarterectomy (21% of patients are in the low-risk group and 36% are in the high-risk group). This regime could save useless duplex surveillance in patients who will not develop restenosis, and prevent carotid occlusion in the high-risk group. Thus, overall outcome can improve while health care costs are reduced.

Prediction of local disease progression - future research

In the current study we are not able to investigate the follow-up of the atherosclerotic plaque itself ("the natural history of the plaque"), because the plaque is excised during carotid endarterectomy. Therefore, our findings do not necessarily conflict with the definition of the local vulnerable plaque.

This investigation of the natural history of the plaque would have high clinical relevance, because we could then restrict treatment to those plaques which have a high likelihood to become unstable and cause clinical symptoms in the future. During coronary angiography, asymptomatic lesions are often identified, and immediately

treated. Since the natural history of these lesions is unknown, it is unclear whether prophylactic treatment of a lesion will be beneficial for the patient. Intra-procedural assessment of plaque composition may aid in treatment decision. With intravascular ultrasound (IVUS) it is possible to identify lipid core and calcifications.[18, 19] With optical coherence tomography (OCT), lipid core size can be measured accurately, and it is becoming feasible to perform more advanced plaque characterization such as macrophage infiltration in the fibrous cap.[20, 21] Plaque composition, determined intra-procedurally, can help in treatment decision once the relation between plaque composition and local plaque instability during follow-up has been established. In the present study we are able to show the natural history of plaque repair after an event **(Chapter 6)**. We managed this by comparing plaque composition between different patient groups, stratified by the interval between the most recent cerebrovascular event and examination of the plaque. However, this can not elucidate the natural history of asymptomatic plaques that have not yet become unstable. Therefore it is essential to conduct prospective studies with serial plaque imaging and clinical follow-up. The PROSPECT study, for example, is such a study which has enrolled 700 patients and examined the entire coronary tree with IVUS and virtual histology. The follow-up of this study is ongoing and results are expected in the near future.

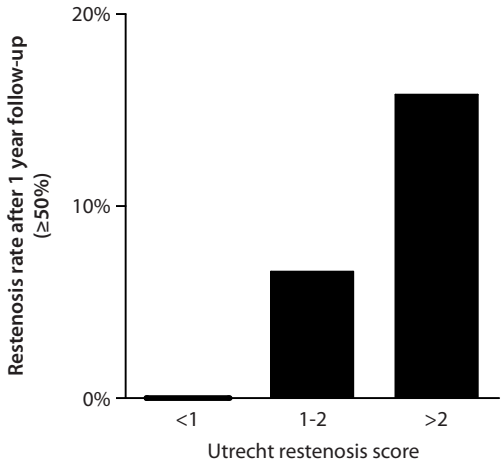

Figure IV Utrecht restenosis score

This graph shows the relation between the Utrecht restenosis score at baseline and the risk of 50% or greater restenosis determined by duplex examination after 1 year follow-up. The score was based on plaque composition and clinical risk factors. The distribution of the score among the patient cohort was: **<1:** 21% of patients; **1-2:** 42% of patients; **>2:** 36% of patients

Prediction of systemic outcome and identification of the vulnerable patient

A concept that has gained much attention is the identification of the vulnerable patient.[10, 22, 23] Each year, many patients suffer from their first cardiovascular event, such as a stroke or myocardial infarction. If it were possible to identify these patients before they become symptomatic, this might dramatically decrease the burden of cardiovascular disease. For patients with manifest atherosclerotic disease, it is important to predict the chance of a second vascular event. Since the cardiovascular events can occur anywhere in the vascular tree, the focus shifts from natural history of the local atherosclerotic plaque to markers of systemic cardiovascular vulnerability.

Prediction of systemic outcome and identification of the vulnerable patient - this study

In **Chapter 2**, we have defined the different ways to predict cardiovascular outcome **(Chapter 2, Figure I)**. Since standard epidemiological research (systemic determinants, e.g. circulating markers, for systemic outcome) has not yielded suitable biomarkers, other approaches have been attempted. As we have described, there is large interest in prospective imaging trials to predict future local plaque instability (local determinants for local outcome). In the Athero-Express study, we follow a third and novel approach. We investigate the predictive value of the local plaque composition for systemic cardiovascular outcome, regarding the plaque composition as a concentrated expression of a systemic disease. The current study shows that the presence of thrombus in the excised carotid atherosclerotic plaque predicts the occurrence of future cardiovascular events. More importantly, we identified specific protein biomarkers for systemic outcome. High expression of Osteopontin was associated with a four-fold increased risk of vascular events, which approximated 50% after 3 years in the group with high plaque Osteopontin. Confirming our hypothesis, the expression of Osteopontin in femoral endarterectomy specimens had comparable predictive value.

Prediction of systemic outcome and identification of the vulnerable patient - future research

Now that we have established that the local atherosclerotic plaque holds information that is predictive of systemic vascular outcome, further research should be focused at identifying the best markers in the plaque. Currently, we have identified a list of 90 potentially predictive protein markers in the carotid plaque. We are working on validation of these proteins in the whole carotid study cohort. Markers that are validated positively will undergo external validation in femoral or other

plaques. The next step will then be to combine multiple markers into a "plaque proteins signature" in order to identify the vulnerable patient. Whereas we are now able to discriminate between a group with approximately 10% chance of developing a cardiovascular event and a group with approximately 50% risk, it is even much more clinically relevant if this difference could be enhanced to e.g. 5% vs. 80%. This will be a great leap forwards to identify the vulnerable patient.

The predictive value of the plaque is currently limited to patients who undergo vascular surgery. To make the predictive value of the plaque available to more patients, the targets identified in our study should be translated to markers which can be measured in patients who do not undergo surgery **(Table III)**. The first possibility is to measure the circulating levels of the protein signature we identified in the plaque. As shown in **Chapter 11**, proteins from atherosclerotic carotid plaques are secreted into the systemic circulation and can be measured in a peripheral blood sample. In the case of Osteopontin, we showed that circulating levels are also predictive of future cardiovascular events, although the predictive value was lower compared to the predictive value of plaque Osteopontin. This is probably caused by production of Osteopontin by other tissues as well, which adds noise to the signal of plaque derived circulating Osteopontin. Nevertheless, when a strongly predictive signature can be obtained in the plaque, the circulating levels of these proteins may still possess good predictive value for future vascular events. The use of bioinformatics provides new possibilities to obtain an optimal predictive protein profile.

There are also other possibilities to determine markers in patients who do not undergo surgery. The first option is molecular imaging **(Chapter 2)**. In theory, plaque proteins with predictive value could be imaged with contrast agents coupled to

Table III Clinical application of plaque related biomarkers

Detection source	Advantage	Drawback
Plaque protein	High predictive value	Restricted to patients undergoing vascular surgery
Circulating protein markers	Easy to obtain in any patient	Less predictive value than plaque protein
Plaque imaging (MRI, IVUS, etc)	Easy to obtain in any patient	Unproven predictive value Some techniques (e.g. IVUS): invasive
Molecular imaging	Possibly high predictive value Non-invasive	Development difficult
Tissue biopsy	Can be obtained in any patient	Predictive value not yet proven Experimental Invasive

Abbreviations: IVUS, intravascular ultrasound; MRI, magnetic resonance imaging

specific antibodies. The development of molecular imaging techniques is still difficult and many efforts will be needed to bring this to clinical practice.
Tissue biopsy is another possibility. Possibly, the predictive markers we identified in the atherosclerotic plaque are also present in the entire vascular wall of patients at high risk for future cardiovascular events. If this is true, then taking a biopsy of an easily accessible vessel such as the radial artery could provide the same prognostic information as the atherosclerotic plaque.

Other clinical applications

Besides identification of the vulnerable patient, our predictive markers can also serve other purposes. Predictive markers are candidates to serve as surrogate endpoints in clinical trials. The intima-media thickness is one of the markers which are used as a surrogate endpoint in clinical trials investigating the effects of lipid lowering drugs. The endpoints of some of these trials are difference in intima-media thickness at the size of a single erythrocyte.[24] Plaque thrombus can be quite reliably determined with MR direct thrombus imaging and could thus serve as a more realistic surrogate endpoint for such studies.
Second, proteins associated with clinical outcome which have been identified in the carotid plaques can also be used as targets for drug development. The association of a locally expressed protein with systemic outcome indicates that the protein is involved with atherosclerotic disease throughout the vascular tree, and therefore the targeting of these proteins with specifically designed drugs could improve systemic outcome.

Conclusion

The Athero-Express study has shown for the first time that the composition of the atherosclerotic plaque contains prognostic information. Including over 1500 patients, the Athero-Express study has provided insights into the association between atherosclerotic plaque composition and clinical follow-up, both at histologic and protein level. The study has identified targets which can be clinically applied to identify patients at risk for future restenosis and cardiovascular events. Research efforts should now focus on application of plaque composition in clinical decision making and therapeutic interventions.

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CHAPTER

15

SUMMARY

SAMENVATTING

Summary

The composition of the pathologic substrate of atherosclerosis, the atherosclerotic plaque, is not taken into account in clinical practice. Atherosclerotic plaques differ greatly in their composition, and we have learned from cross-sectional pathology studies that certain plaques may be more likely to cause clinical events such as a myocardial infarction or stroke. At present, however, plaque composition has no role in clinical decision making.

The key reason for the lack of clinical application is perhaps a lack of knowledge, which this thesis aims to address. We designed a large biobank containing atherosclerotic plaques obtained during vascular surgery, called Athero-Express. The unique features of this biobank are its size and the thorough clinical follow-up. During 6 years, we have included more than 1000 patients undergoing carotid endarterectomy and another 500 patients undergoing femoral endarterectomy and aneurysm surgery. The main objectives of the study were to investigate the relation between carotid plaque composition at the time of carotid endarterectomy and occurrence of restenosis and adverse cardiovascular events during follow-up. The most important conclusions of the study are described in **Table I**. We were able to establish that the extent of benefit of carotid endarterectomy correlates with carotid plaque composition. In patients groups who benefit most from CEA, we found an increased incidence of "vulnerable" plaques. We hypothesize that the excision of a vulnerable plaque portrays more benefit than a harmless plaque which will never cause any symptoms if it remains in place. These results suggest that pre-operative assessment of plaque characteristics with non-invasive imaging is the key to obtain better overall outcome in patients with carotid artery stenosis, and probably as well in other vascular territories. Furthermore, we identified prognostic factors in the atherosclerotic plaque. A large lipid core and marked macrophage infiltration are predictive of recurrent stenosis after carotid endarterectomy. The presence of thrombus in the carotid plaque is associated with increased incidence of cardiovascular events during 3-year follow-up. Furthermore, we were able to identify more specific targets to predict outcome. Using a proteomic approach, we identified proteins that were differentially expressed in the plaques from patients who later on suffered an event during the follow-up, and controls. High expression of Osteopontin, one of these proteins, was associated with a four-fold increased risk of future cardiovascular events. This predictive value of this protein was independent of clinical risk factors and exceeds previous biomarkers such as C-reactive protein. Testing of other protein targets identified by proteomics is ongoing and we expect to present more predictive markers in the near future. Combining multiple markers could result in even better prediction of the occurrence of future cardiovascular events. These prognostic markers can be used as intermediate outcomes of clinical trials, may be used as drug targets, and allow prediction of outcome, facilitating tailor-made treatment for each patient.

In conclusion, the atherosclerotic plaque contains important prognostic information. It is essential to realize that clinical application of plaque composition can portray a major advance in treatment of advanced atherosclerotic disease. Further research on the clinical application of plaque composition, e.g. by proteomics and prospective plaque imaging studies, must be conducted and should receive high priority to improve the outcome of patients suffering from atherosclerotic disease.

Table I Conclusions of the thesis

Conclusions per section	Chapter
Introduction and study design	
Athero-Express is a large vascular biobank with the unique feature that plaque composition is linked to clinical follow-up of the patient.	1,2
There is a great need to identify patients at high risk for cardiovascular events in the near future.	2
Histologic assessment of carotid plaques is well reproducible.	3
The carotid atherosclerotic plaque and clinical presentation	
Presentation of carotid artery stenosis with stroke or TIA is associated with increased prevalence of atheromatous carotid plaque phenotype.	4
The incidence of vulnerable plaques is higher in men compared to women. This may explain the greater benefit of carotid endarterectomy in men.	5
Following a stroke, carotid plaques undergo a natural stabilization process with decrease of macrophage infiltration and inflammatory and apoptosis markers.	6
The carotid atherosclerotic plaque and restenosis	
Large plaque lipid core and marked macrophage infiltration independently predict decreased risk of restenosis after carotid endarterectomy.	7
Early restenosis after CEA is characterized by collagen and smooth muscle cells accumulation while late restenosis resembles the primary plaque.	8
Symptomatic restenotic plaques have comparable composition to symptomatic primary plaques.	8
The carotid atherosclerotic plaque and clinical outcome	
Large plaque lipid core is associated with increased incidence of peri-interventional complications in patients undergoing CAS or CEA.	9, 10
Plaque thrombus is independently associated with increased incidence of cardiovascular events during follow-up after carotid endarterectomy.	10
Biomarkers in the carotid plaque protein	
Plaque and serum NGAL expression is associated with unstable atherosclerotic disease.	11
Caveolin-1 is a potentially stabilizing factor in atherosclerosis and its expression shows a trend towards decreased risk of cardiovascular events.	12
High Osteopontin expression in atherosclerotic plaques predicts four-fold increased risk of cardiovascular events during follow-up.	13

Samenvatting

Elk jaar sterven wereldwijd vele mensen aan de gevolgen van aderverkalking (wetenschappelijke naam: atherosclerose). De belangrijkste uitingen hiervan zijn hartinfarct en beroerte. Ondanks dat er veel onderzoek gedaan wordt naar aderverkalking, kunnen we niet goed voorspellen welke patiënten nu een heel hoog risico hebben op een hartinfarct of beroerte. Het is bekend dat bijvoorbeeld hoge bloeddruk, diabetes, roken en hoog cholesterol geassocieerd zijn met een slechtere prognose, maar de voorspellende waarde van deze factoren is beperkt. Daarom zijn we naarstig op zoek naar betere manieren om deze kwetsbare patiënten op te kunnen sporen, zodat we het hartinfarct of de beroerte kunnen voorkomen. Bijvoorbeeld door preventief te dotteren of extra medicatie te geven aan die patiënten die het nodig hebben.

Atherosclerose is een ziekte die leidt tot vorming van afzettingen in de slagaders ("atherosclerotische plaques") en daarmee het dichtslibben van slagaders. In tegenstelling tot de benaming "aderverkalking", is het dus geen ziekte van de aders, maar van de slagaders. Aders, in het medisch jargon ook wel "venen" genoemd, zijn de bloedvaten die het bloed afvoeren vanuit het lichaam terug naar het hart. Het zijn vaten met een dunne wand waardoor het bloed onder lage druk terugstroomt naar het hart. Slagaders, in het medisch jargon "arteriën" genoemd, zijn de grote bloedvaten die het bloed aanvoeren van het hart naar de rest van het lichaam. Daarbij valt te denken aan de kransslagaders, die het hart zelf van bloed voorzien, de lichaamsslagader (aorta), en de halsslagaders, die het bloed aanvoeren naar de hersenen toe. Dit zijn vaten met een gespierde wand waar het bloed onder hoge druk doorheen stroomt. Het dichtslibben van de vaten leidt uiteindelijk tot een tekort aan bloed in de organen die door het beteffende vat van bloed worden voorzien, hetgeen weer kan leiden tot het afsterven van een deel van het orgaan. Het is een systeemziekte; als het op een bepaalde plaats zit (bijvoorbeeld dichtgeslibde slagaders in het been) zit het heel vaak ook op een andere plaats (bijvoorbeeld dichtgeslibde halsslagaders).

In dit proefschrift onderzoeken we of de samenstelling van de atherosclerotische plaque kan helpen bij het voorspellen welke patiënten een hoog risico hebben om op korte termijn een hoog risico lopen op symptomen van atherosclerose, zoals een hartinfarct of beroerte. Om een parallel te trekken: bij patiënten met een tumor weten we allemaal dat er een onderscheid is tussen "goedaardig" en "kwaadaardig". Het één betekent dat er niks aan de hand is (goede prognose) en het ander betekent dat er een ernstige ziekte aanwezig (slechte prognose). Dit onderscheid wordt gemaakt op basis van de samenstelling van de tumor, en heeft verregaande consequenties voor de behandeling, en de prognose (toekomstverwachting) van de patiënt.

Bij atherosclerose doen we dit op het moment niet zo. We houden er geen rekening mee, of de samenstelling van de atherosclerotische plaque "goed"- danwel "kwaadaardig" is. De reden daarvan is, dat de relatie tussen plaque-samenstelling en prognose nooit onderzocht is. Het is dus eigenlijk niet bekend welke kenmerken een kwaadaardige plaque onderscheiden van een goedaardige plaque.
Vanuit deze gedachte is de Athero-Express weefselbank geïnitieerd. Het is een weefselbank met stukjes weefsel van vaatvernauwingen die bij vaatoperaties werden verwijderd. De kracht van deze studie is dat de weefselbank is gekoppeld aan het vervolgen van de patiënt. Op die manier kunnen we bepalen welke weefselkenmerken van de vaatwandvernauwing gerelateerd zijn aan een slechte prognose van de patiënt. En kunnen we dus de behandeling van de individuele patiënt beter gaan afstemmen, aan de hand van de samenstelling van de vaatwand.
In dit proefschrift bestudeerden we de uitkomsten van patiënten die werden geopereerd aan de halsslagader. In 6 jaar tijd zijn er inmiddels meer dan 1000 patiënten in de studie opgenomen, waarvan de vaatwand werd geanalyseerd en het ziekteverloop gedurende 3 jaar vervolgd.
De conclusie van het proefschrift staan in **Tabel II**. In het eerste deel wordt de studieopzet verder uitgewerkt **(Hoofdstuk 1-3)**. In **Hoofdstuk 3** wordt vervolgens de methode van het beoordelen van atherosclerotische plaques gevalideerd. Het blijkt dat onze beoordelingsmethode goed reproduceerbaar is en dat de beoordeling door een geoefende onderzoeker goed overeenkomt met een computergestuurde meting. Deze bevindingen vormen de basis voor het onderzoek.
In het volgende gedeelte richt het onderzoek zich op de relatie tussen kenmerken van de patiënt en de samenstelling van de atherosclerotische plaque in de halsslagader. Het nut van het operatief verwijderen van een plaque in de halsslagader is namelijk afhankelijk van een aantal risicofactoren, namelijk leeftijd, geslacht, en het recent symptomatisch zijn van de plaque. Met het laatste wordt bedoeld dat de patiënt kort geleden (< 6 maanden) klachten heeft gehad, nl. een kleine beroerte, die toegeschreven kunnen worden aan de atherosclerotische plaque in de halsslagader. Als het klopt dat de samenstelling van de plaque relevant is, dan zouden deze factoren ook gerelateerd moeten zijn aan de samenstelling van de plaque. Inderdaad laten we in **Hoofstuk 4 tot en met 6** zien dat al deze factoren gerelateerd zijn met de plaque samenstelling, op de manier die verwacht zou kunnen worden. Deze bevindingen onderstrepen dat de samenstelling van de plaque zeer belangrijk is, en dat we ook bij de beslissing om een plaque in de halsslagader te opereren de samenstelling van de plaque zouden moeten betrekken. Omdat het steeds beter mogelijk wordt om de samenstelling voor de operatie al te bepalen via beeldvormende technieken zoals MRI, moeten we er ons nu op gaan richten het gebruik van deze MRI technieken voor het optimaliseren van de behandelingskeuze in de kliniek te gaan onderzoeken.

Het volgende deel van het onderzoek richt zich op restenose **(Hoofdstuk 7,8)**. Restenose is het terugkeren van een vaatvernauwing na de operatieve verwijdering ervan. Tot nu toe was de voorspelling van het terugkeren van een vaatvernauwing niet mogelijk. Door het bestuderen van de samenstelling van de plaque kan het ontstaan van terugkeer van de vaatvernauwing vaatoperatie aan de halsslagader voorspeld worden. In **Hoofdstuk 7** en in **Hoofdstuk 14** wordt dit besproken. Momenteel worden patiënten vervolgd na de operatie om te kijken of het vat openblijft. Uit onze studie blijkt dat een groot deel van de patiënten vervolgd wordt terwijl op basis van de plaque-samenstelling al voorspeld had kunnen worden dat bij deze patiënten de vaatvernauwing vrijwel nooit terugkomt. Aan de andere kant is er ook een groep patiënten bij wie het vat na de eerste controle al weer deels of zelfs geheel dichtzit. Door de samenstelling van de plaque is het goed mogelijk deze risicopatiënten al op voorhand op te sporen en deze intensiever in de gaten te houden met het doel om opnieuw dichtslibben van het vat te voorkomen. Door de plaquesamenstelling te gaan betrekken kunnen kosten bepaard worden door het weglaten van zinloze vervolgonderzoeken en resultaten verbeterd door die patiënten die het wel nodig hebben extra goed in de gaten te houden.

Daarna ontdekten we dat de samenstelling van plaque ook voorspelt welke patiënten in de toekomst een hoge kans hebben op een hartinfarct of een beroerte **(Hoofstuk 9-13)**. Als de plaque (resten van) een stolsel bevatte had de patiënt een tweevoudig verhoogde kans op het krijgen van een uiting van atherosclerose, dus ofwel een beroerte, hartinfarct, of klachten van dichtslibbende vaten in de benen.

Om deze voorspelling verder te optimaliseren werd ook een ander soort analyse van de plaque gedaan, namelijk een analyse van de eiwitsamenstelling in de plaque, een zogenaamde proteomics-analyse **(Hoofdstuk 13)**. Door deze analyse, uitgevoerd in samenwerking met een onderzoeksgroep in Singapore, slaagden we erin eiwitten in de vaatwand te ontdekken die de prognose van de patiënt voorspellen. De eerste voorspellende "marker" die we in dit proefschrift laten zien, Osteopontine, is geassocieerd met een viervoudig verhoogd risico op een hartinfarct of beroerte in de eerstvolgende 3 jaar. We verwachten dat een combinatie van meerdere van verschillende van deze markers een nog veel betere voorspelling van de prognose gaan leveren.

De volgende stappen zullen nu zijn om de bevindingen van dit onderzoek te gaan vertalen naar direct bij patiënten toepasbare methoden van onderzoek en behandeling. Belangrijk daarvoor is de beeldvorming van de plaques, waarmee het mogelijk moet worden om de samenstelling van de plaque te betrekken bij de keuze van behandeling voor een vaatvernauwing. Bij patiënten die niet geopereerd worden aan een plaque kunnen onze bevingen helpen om het risico op een uiting van aderverkalking zoals een beroerte of hartinfarct te voorspellen. Om dit toe te passen valt naast beeldvorming van de plaque te denken aan het bepalen van de voorspellende eiwitten in het bloed, omdat bekend is dat deze van de plaque kun-

nen overgaan in het bloed. Verder zou het nut van een vaatbiopt kunnen worden onderzocht. De toekomst zal uitwijzen in hoeverre we hiermee in staat zullen zijn om de prognose van patiënten met atherosclerose te voorspellen en de behandeling hier optimaal op af te stemmen.

Tabel II Conclusies van het proefschrift

Conclusies per sectie	Hoofdstuk
Introduction and study design [introductie en studieopzet]	
Athero-Express is een grote vaatbiobank met het unieke kenmerk dat de samenstelling van afzettingen in de vaatwand (atherosclerotische plaque) wordt gekoppeld aan vervolgen van de patiënt.	1,2
Er is een grote behoeft om patiënten met een sterk verhoogd risico op een beroerte of hartinfarct op te kunnen sporen; de samenstelling van de atherosclerotische plaque kan daarin een belangrijke rol spelen.	2
Beoordeling van plaque-samenstelling door histologie (onder de microscoop) is goed reproduceerbaar.	3
The carotid atherosclerotic plaque and clinical presentation [plaquesamenstelling en klinische presentatie]	
Patiënten bij wie een plaque in de halsslagader zich presenteert door een TIA of beroerte hebben vaak een vetrijk plaque-type in de halsslagader.	4
Mannen hebben vaker een fragiele, vetrijke plaque dan vrouwen. Dit kan verklaren waarom mannen meer baat hebben bij operatieve verwijdering van de plaque.	5
Na veroorzaken van een beroerte ondergaat de plaque in de halsslagader een stabilisatieproces waarbij ontstekingsactiviteit afneemt.	6
The carotid atherosclerotic plaque and restenosis [plaquesamenstelling en restenose]	
Bij een vetrijke plaque met veel ontsteking komt vaatvernauwing niet snel terug na operatieve verwijdering.	7
Vroege terugkeer van een vaatvernauwing kenmerkt zich door bindweefselrijke plaques, terwijl late terugkeer lijkt op een primaire plaque met veel vet en ontsteking.	8
The carotid atherosclerotic plaque and clinical outcome [plaquesamenstelling en klinische uitkomst]	
Vetrijke plaques veroorzaken meer complicaties tijdens operatie of dotteren dan bindweefselrijke, vetarme plaques.	9, 10
Patiënten met een plaque die een stolsel bevat hebben een twee keer zo hoge kans op nieuwe symptomen van atherosclerose.	10
Biomarkers in the carotid plaque protein [biomarkers in het eiwit van de plaque]	
Verhoogde aanwezigheid van NGAL in de plaque en in het bloed is gerelateerd aan onstabiele atherosclerose.	11
Caveoline-1 is een stabiliserende factor bij atherosclerose en verhoogde aanwezigheid in de plaque lijkt gerelateerd aan lagere kans op toekomstige uitingen van atherosclerose.	12
Een hoog gehalte van Osteopontine atherosclerotische plaques voorspelt een viervoudig verhoogd risico op toekomstige uitingen van atherosclerose zoals hartinfarct en beroerte.	13

CHAPTER

16

APPENDIX

LIST OF ABBREVIATIONS

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List of abbreviations

Abbreviation	Explanation
ACAS	Asymptomatic Carotid Atherosclerosis Study
ACE	Angiotensin Converting Enzyme
ACST	Asymptomatic Carotid Surgery Trial
AEC	3-Amino-9-ethylcarbazole
AFX	Amaurosis fugax
AP	Angina Pectoris
APO	Apolipoprotein
AR	Absolute Risk
ASA	Acetylsalycylic Acid
ATHERO-EXPRESS	Differential ATHEROsclerotic plaque EXPRESSion of mRNA and protein in relation to cardiovascular events and patient characteristics
AU	Arbitrary Unit
BMI	Body mass index
CABG	Coronary Artery Bypass Grafting
CAD	Coronary Artery Disease
CALC	Calcifications
CAS	Carotid Angioplasty and Stenting
CAV-1	Caveolin-1
CCA	Common Carotid Artery
CEA	Carotid endarterectomy
CI	confidence interval
CK-MB	Creatin Kinase, MB isoform
COLL	Collagen
COX	Cyclooxygenase
CRP	C-reactive protein

Abbreviation	Explanation
CSD	Caveolin-1 Scaffolding Domain
CT	Computed tomography
Δ	Delta (difference)
DAB	Diaminobenzidine
DM	Diabetes Mellitus
DMSO	Dimethylsulfoxide
DOX	Doxycycline
DTSSP	Dithiobis sulfosuccinimidyl propionate
DWI	Diffusion-weighted imaging
D-MEM	Dulbecco's Modified Eagle's Medium
EC	Endothelial Cell
ECA	external carotid artery
EDTA	Ethylenediaminetetraacetic Acid
ECST	European Carotid Surgery Trial
EEL	External Elastic Lamina
ELISA	Enzyme-Linked Immunosorbent Essay
EMMPRIN	Extracellular Matrix Metalloproteinase Inducer
EVG	Elastin von Gieson
FBS	Fetal Bovine Serum
fMLP	Formyl-methionyl-leucyl-phenylalanine
F-ATHEROMATOUS	Fibro-Atheromatous
GSM	Grayscale Median
HDL	High-density Lipoprotein
HE, H&E	Hematoxylin and Eosin

Abbreviation	Explanation
HG	Highly Glycosylated
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
HR	Hazard Ratio
HRP	Horseradish Peroxidase
HRT	Hormone Replacement Therapy
hs-CRP	High Sensitivity (measurement of) C-reactive protein
IBIS	Integrated Biomarker and Imaging Study
ICA	Internal Carotid Artery
ICAROS	Imaging in Carotid Angioplasty and Risk of Stroke
IFN	Interferon
IG	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IMT	Intima-media thickness
IQR	Interquartile range
IVUS	Intravascular Ultrasound
KM	Kaplan Meier
LDL	Low-density Lipoprotein
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MeSH	Medical Subject Heading
MCA	Middle Cerebral Artery
MCP-1	Monocyte Chemoattractant Protein 1
MI	Myocardial Infarction
MMP	Matrix Metalloproteinase

Abbreviation	Explanation
MO	Macrophage
MRDTI	Magnetic Resonance Direct Thrombus Imaging
MRI	Magnetic Resonance imaging
MS	Mass Spect
N	Number (of subjects)
NASCET	North American Symptomatic Carotid Endarterectomy Trial
NFκB	Nuclear Factor kappa B
NPV	Negative Predictive Value
NR	Not reported
NS	Not Significant
NSAID	Nonsteroid Anti-Inflammatory Drug
OCT	Optical Coherence Tomography
OPN	Osteopontin
OR	Odds Ratio
P	Probability
PBS	Phosphate-buffered Saline
PBSA	Bovine Serum Albumin dissolved in PBS
PPV	Positive Predictive Value
PROT	Protection device
PROSPECT	Providing Regional Observations to Study Predictors of Events in the Coronary Tree
PS	Picro-Sirius
PSV	Peak Systolic Velocity
PTCA	Percutaneous Transluminal Coronary Angioplasty

Abbreviation	Explanation
PTFE	Polytetrafluoroethylene
Q	Quartile
RD	Risk Difference
REF	Reference
ROC	Receive Operator Characteristic
RPM	Rounds per minute
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SE	Sensitivity
SEM	Standard Error of the mean
SMC	Smooth Muscle Cell
SP	Specificity
SPECT	Single photon emission computed tomography
TCD	Transcranial Doppler
TIA	Transient Ischemic Attack
TIMP	Tissue inhibitor of metalloproteinases
TLR	Target Lesion restenosis / Toll-like receptor
TNF	Tumor Necrosis Factor
THRO	Thrombus
WB	Western Blot
WT	Wild Type
Y	Year
ZY	Zymography

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Curriculum Vitae

Willem Hellings was born on June 13, 1979 in Utrecht, The Netherlands. After graduating from secondary school in 1997 (Jacob Roelandslyceum, Boxtel) he studied computer science at Technical University Eindhoven and Utrecht University from 1997 to 1999, during which he finished the first two years of the programme and was awarded a grant at the TU Eindhoven. From 1999 to 2005 he studied medicine at Utrecht University. During this period he was an active member of the medical students society. He took part in organization of activities for medical students and was co-initiator and designer of the medical students' website. As a student he started his research in the field of carotid artery surgery under the supervision of dr. Ackerstaff, dr. De Borst and prof. Moll. In 2005 he graduated for his medical training and joined the Department of Surgery and the Experimental Cardiology laboratory at the University Medical Center Utrecht, The Netherlands. He worked three years in a PhD programme which led to this thesis. His work has been awarded with a fellowship from the Dutch Atherosclerosis society and the second price for best Dutch publication in the field of Vascular Biology in 2007. After finishing his PhD programme he will start his residence in general surgery (University Medical Center Utrecht).