Athero-Express
Athero-sclerotic plaque Expression in relation to vascular events and patient characteristics

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Atherosclerotische plaque expressie in relatie tot vasculaire events en patiënten kenmerken (met een samenvatting in het Nederlands)

Proefschrift
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Voor mijn ouders
Mireille en Bruno

Contents

General Introduction

Introduction

Cardiovascular disease is still the major cause of death in Western communities[1]. Every year >20 million people worldwide experience a sudden cardiac event. A large proportion of this population will not have experienced previous symptoms[2]. Patients suffering from cerebro-vascular events or ruptured abdominal aortic aneurysm will also have no prior warning. Atherosclerosis is a generalised disease and it can sometimes take decades for the first clinical symptoms to occur. After the first attack of this progressive disease, there is an increased risk of recurrence, either in the same location or another, within the arterial system. Early detection, risk stratification and proper knowledge of the pathogenesis of atherosclerosis are necessary, in order to understand and treat this disease properly. Appropriate research is therefore essential. Research can increase both understanding and the treatment of this disease and either prevent patients from becoming symptomatic in the first place or prevent a recurrence. To further understand this complex disease, it is necessary to undertake studies from varying perspectives. Whilst bench studies are undertaken to investigate the molecular basis of atherosclerosis on the one hand, an epidemiological study of the population is undertaken on the other. The translation of research from bench to patient by combining both laboratory and epidemiological science, has proven a successful approach. A good example of an epidemiological study is The Framingham Heart protection study, which has been of enormous value in identifying high risk patients over past decades[3].

Although the research to date has contributed significantly to the understanding of atherosclerotic disease, all studies, both conventional and epidemiologic, as well as those involving human atherosclerotic tissue, suffer the following drawbacks:

– Most vascular specimens of human tissue have been obtained post mortem and follow up studies are therefore precluded. So most studies involving human vascular tissue have cross-sectional designs but these fail to provide causal analysis.
– the current definition of vulnerable plaque (atherosclerotic plaque, which is held responsible for cardiovascular events) is mainly based on post mortem research[4;5]. However the definition merits careful consideration. Although plaque rupture is associated with the presence of a large lipid pool, macrophages and a thin fibrous cap, the predictive value of these histopathological determinants for the rupture of the so-called 'vulnerable plaque' is unknown. In fact, the phenotype of the plaque that gives rise to adverse cardiovascular events has yet to be investigated in longitudinal human studies.
– Tissue banks that contain large numbers of freshly obtained atherosclerotic specimens, in which both RNA and protein expression can be studied, are scarce.

Absence of variance is often explained by the lack of power, as most atherosclerotic tissue banks are too small to allow a valid comparison between patient groups. The collection of atherosclerotic tissue from a large cohort however, will help in the screening of genes/proteins, thought to play an important role in the atherogenesis of specified subgroups.
This knowledge inspired us to design a biobank study named Athero-express. The biobank would be a longitudinal study to include atherosclerotic tissue, serum samples, clinical history and endpoint evaluation. Such a study design is innovative and, in contrast to systemic markers, will study the natural history of this systemic disease at a tissue and cellular level. The study design would also allow future proteomic analyses as well as both follow-up and cross-sectional studies. Obtaining atherosclerotic tissue is essential to this study. For this reason we selected patients suffering from carotid artery disease. This disease has a relatively high incidence and the standard therapy is Carotid Endarterectomy (CEA). During the CEA procedure, atherosclerotic tissue can easily be obtained by collecting the endarterectomy specimen. Importantly, the collection of this specimen will have no adverse effect on the clinical condition of the patient.

The outline of this thesis

The main objective of this thesis is to study plaque characteristics in relation to both clinical symptoms and immediate outcomes. For this purpose a large biobank of carotid endarterectomy tissue was created, entitled Athero-Express. Chapter 2 focuses on the design and rationale of the Athero-Express study, whilst Chapters 3-7 cover the cross sectional studies. Firstly, pre-operative symptoms and medical treatment are correlated with atherosclerotic tissue obtained during CEA and serum markers. Then procedural techniques and plaque characteristics are linked with microemboli and procedural outcome. Finally, the potential role of NOGO B as a vascular marker, is studied.
In short:

– Chapter 3 evaluates and describes the difference in plaque characteristics obtainedfrom 404 patients, comparing symptomatic with asymptomatic carotid disease.
– Chapter 4 evaluates the plaque characteristics, observed in 378 patients, comparing plaque from patients, who received statin treatment with those who received no cholesterol lowering drugs at all.
– In Chapter 5 atherosclerotic plaque characteristics are related to the occurrence of per-operative microemboli and procedural related cerebro-vascular events.

– Chapter 6 describes the relation between arteriotomy closure techniques, the observation of intraoperative microemboli and the restenosis rate.
– Chapter 7 focuses on NOGO B, in relation to plaque characteristics and clinical history
– Chapter 8 provides a summary and general discussion of the overall thesis.

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Athero-Express: Differential Atherosclerotic plaque expression of mRNA and protein in relation to cardiovascular events and patient characteristics. Rationale and design

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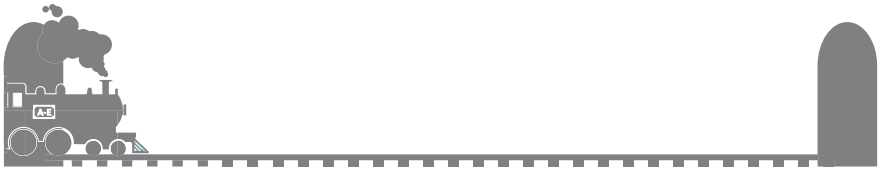

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Abstract

In clinical practice, biological markers are not available to routinely assess the progression of atherosclerotic disease or the development of restenosis following endarterectomy or catheter based interventions. Endarterectomy procedures provide an opportunity to study mechanisms of restenosis and progression of atherosclerotic disease since atherosclerotic tissue is obtained.

Athero-express is an ongoing prospective study, initiated in 2002, with the objective to investigate the etiological value of plaque characteristics for long term outcome. Patients are included who undergo an endarterectomy of the carotid artery. At baseline blood is withdrawn, patients fill in an extensive questionnaire and diagnostic examinations are performed. Atherosclerotic plaques are freshly harvested, immunohistochemically stained and examined for the presence of macrophages, smooth muscle cells, collagen and fat. Parts of the atherosclerotic plaques are freshly frozen to study protease activity and protein and RNA expressions. Patients undergo a duplex follow up to assess procedural restenosis (primary endpoint) at 3 months, 1 year and 2 years. Secondary endpoints encompass major adverse cardiovascular events.

In the future, the creation of this biobank with atherosclerotic specimen will allow the design of cross-sectional and follow up studies with the objective to investigate the expression of newly discovered genes and proteins and their interaction with patients and plaque characteristics in the progression of atherosclerotic disease.
Objective is to include 1000-1200 patients in 5 years. In January 2004, 289 patients had been included. It is expected that 250 patients will be included yearly.

Introduction

Arteriosclerosis is the leading cause of morbidity and mortality in the modern Western world. Myocardial infarction, peripheral arterial occlusive disease, cerebral infarction and aortic aneurysms are the major clinical manifestations of this inflammatory disease[1]. Atherogenesis starts early in life but may take decades before clinical manifestations become evident.

Carotid artery stenosis is a prevalent observation that is often clinically silent. In many occasions, however, embolisms may cause acute temporary occlusion of the cerebral circulation resulting in a transient ischemic attack or stroke[2,3] . Surgical intervention is indicated when diameter stenosis is 70-100% for symptomatic or progressive asymptomatic carotid atherosclerotic luminal narrowing[4-6].

Carotid endarterectomy (CEA) is a successful approach to treat carotid artery stenosis with acceptable patency rates[6]. In the longer term, restenosis is a major drawback of CEA. The incidence of restenosis is reported to vary from 2% up to 20%[7-9]. The mechanisms for carotid artery restenosis following CEA are not fully understood. In addition, no biological marker is available that is predictive for the development of restenosis following CEA.

Previous observations in coronary and carotid arteries revealed that not only atherosclerotic plaque size and subsequent luminal narrowing but also plaque phenotype is an important determinant for the development of cardiovascular events[10-13]. These observations stimulated us to design a prospective study with the objective to relate locally expressed atherosclerotic tissue derived markers with the occurrence of local restenosis and adverse cardiovascular events.

Objectives

The primary objective of Athero-Express is to investigate the relation between the presence of an vulnerable plaque phenotype and the development of restenosis following endarterectomy.

The secondary objective is to study the relation between the presence of an inflammatory lesion and the occurrence of a major adverse cardiovascular event. Not only an indicator of inflammation but also other histological features of thrombogenic unstable plaques will be assessed in the atherosclerotic plaques. Also these histological markers for plaque vulnerability (presence of a large atheromatous core, lack of collagen and lack of smooth muscle cells) will be studied in relation with the long term clinical outcome.

Another long-term objective is the creation of a biobank with atherosclerotic specimen that will allow the design of cross-sectional and follow up studies in which the role of newly discovered genes and proteins in the progression of atherosclerotic disease can be investigated.

Rationale

Restenosis still is a major drawback of surgical and catheter based interventions to treat arterial occlusive diseases. Stenting is an alternate therapeutic option for CEA but which patients will benefit from either a surgical or a catheter based intervention is unknown[10-16] . Risk stratification for restenotic lesion development or progression of atherosclerotic disease could facilitate the decision whether additional pharmaceutical therapeutic options should be considered. Sero-epidemiologic studies have been performed in search for systemically expressed markers that predict restenosis following artery catheter interventions[17-19]. Large prospective sero-epidemiological studies have gained much insight in risk factors for the progression of *de novo* atherosclerotic disease. However, for the development of restenosis following surgical or catheter based interventions no predictive biological marker is routinely assessed in clinical practice.

CEA provides a unique opportunity to study mechanisms of restenosis and progression of atherosclerotic disease since not just blood but also atherosclerotic tissue is obtained. The use of tissue derived biological markers as a predictive marker for the development of restenosis has not been considered in a prospective study previously. In addition, considering the systemic nature of atherosclerotic disease it can be hypothesized that local plaque derived biological markers could be associated with adverse cardiovascular events that do not find their origin in the carotid artery.

The idea to use the extent of carotid atherosclerosis as a surrogate marker for

systemic atherosclerotic disease is not new. Patients that suffer from carotid artery stenosis are more prone to develop clinically relevant atherosclerotic disease at other predilection sites compared with a healthy population[20-23]. For this purpose, duplex derived intima media thickness is often used as a surrogate endpoint in pharmaceutical studies[24]. It is unknown, however, whether the phenotype of the plaque is a predictor for the occurrence of adverse cardiovascular events.
The study Athero-Express is designed to investigate the expression of atherosclerotic tissue derived biological variables in relation to the long term outcome of patients undergoing CEA. Coronary post mortem studies and atherectomy procedures revealed that plaques that hide inflammatory cells, a large atheroma and lack collagen and smooth muscle cells underlie the thrombotic occlusion that has lead to an ischemic event[10-12]. In the present study we will prospectively assess the relation between these plaque characteristics that have been associated with plaque thrombosis and long term adverse outcome. Athero-Express involves a tissue bank which contains freshly frozen atherosclerotic tissues derived from surgical CEA procedures of carotid arteries, superficial femoral arteries, aorta aneurysms as well as non diseased mammary arteries. To answer the aforementioned objectives only carotid arterial segments will be used.

Study design

The Athero-Express study is a prospective cohort study that is currently being executed in two Dutch hospitals: the UMCU Utrecht and the Sint Antonius hospital Nieuwegein. Recruitment of patients started in April 2002 and will continue until at least 1000 patients have been enrolled. All cohort members will be followed for carotid restenosis for 2 years and the occurrence of adverse cardiovascular events for a minimum of 3 years.

Patients

All consecutive patients who are newly referred to the vascular surgery departments of the participating centres for treatment of carotid artery stenosis are enrolled. Patients may have been symptomatic or asymptomatic. Operation was indicated when colour Doppler assisted duplex investigation revealed a diameter reduction of > 70% on at least one side. In asymptomatic patients with stenosis >70%, the indication for surgical therapy depends on co-morbidity and vertebral-basilar (in)sufficiency. Patients with previous manifestations of carotid artery stenosis or previous surgery (restenosis) or clinical manifestations of cardiovascular disease are eligible for inclusion when CEA is indicated. Excluded are patients with a terminal malignancy and those who are

referred back to a hospital outside the Netherlands immediately following surgery.

The Medical Ethical Committees of the participating hospitals have approved the study. When the decision to operate has been taken patients receive written and oral information concerning the objectives of the study. All patients give a written informed consent and fill in a questionnaire.

Recruitment was started in one hospital April 2002. Inclusion in the second hospital started in June 2003. By January 1 2004, 289 of 296 eligible patients had been enrolled in the study. Patients were not included for the following reasons: no informed consent (3), referral to foreign hospital (2), no tissue obtained during surgery (2).

Baseline examinations

Stenosis of the carotid artery is diagnosed by carotid colour Doppler assisted duplex. In case of doubt or upon the surgeons' request the artery will be visualised with magnetic resonance angiography (MRA).

Blood samples are taken before operation. Blood samples encompass: serum, EDTA plasma, red blood cell free citrate plasma, and red blood cells that are all stored at –80 degrees. In the laboratories total cholesterol, triglycerides, high density lipoprotein cholesterol, glucose and creatinin is assessed routinely.

Patients complete a questionnaire before admission to the hospital. The questionnaire contains questions about their medical history, including history and symptoms of cardiovascular disease (coronary artery disease, peripheral artery disease, cerebral ischaemia and abdominal aneurysm), risk factors (smoking habits, alcohol consumption, hypertension, diabetes mellitus, hyperlipidemia, physical activity, vitamin intake, and family history of cardiovascular disease), and medication use. The items on coronary disease and peripheral artery disease are based on the Rose questionnaire[25].

Height, weight, body mass index and blood pressure are determined in all patients.

Atherosclerotic tissue dissection and processing

Carotid surgery consists of an endarterectomy on the bifurcation level of the carotid artery. The plaque is dissected from the bifurcation and the internal and external carotid arteries. During operation patients are monitored by EEG and, if a window is available, also by transcranial doppler (TCD). Just before the atherosclerotic segment is dissected, a technician is called who transports the atherosclerotic segment without delay to the laboratory. In the laboratory, the atherosclerotic segment is dissected in parts of 0.5 cm. The culprit lesion is numbered 0 and the adjacent segments –1 and +1 (Figure 1). The segments –1, +1 and all subsequent numbered segments (-2, +2, -3

etc) are immediately frozen in liquid nitrogen and stored in –80 degrees in numbered metal cups. Segment 0 is fixated in formaldehyde 4%. Segment 0 is then transported to the pathology department (Antonius hospital Nieuwegein) and paraffin embedded. Of each paraffin embedded segment 15 sections (5um thickness) are cut on a microtome for histological (immuno) stainings. Segment 0 is then transported to the pathology department (Antonius hospital Nieuwegein) and paraffin embedded. Of each paraffin embedded segment 15 sections (5um thickness) are cut on a microtome for histological (immuno) stainings. Segment 0 is then transported to the pathology department (Antonius hospital Nieuwegein) and paraffin embedded.

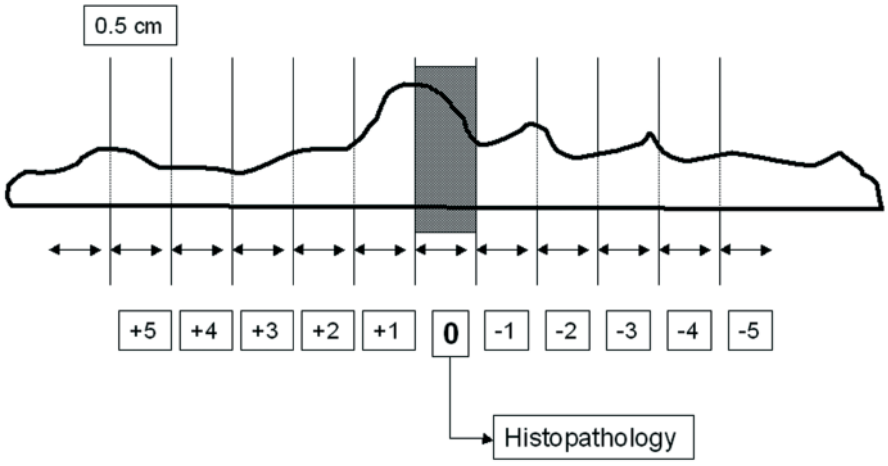

Figure 1. *Schematic presentation of segmentation of the endarterectomy specimen. The plaque is dissected in parts of 5 mm. The part with the largest plaque burden is numbered 0 and fixed in formalin for histological analyses. The other segments are numbered and freshly frozen in liquid nitrogen and frozen at –80°C.*

Of each paraffin embedded segment 15 sections (5um thickness) are cut on a microtome for histological (immuno) stainings. Segment 0 is then transported to the pathology department (Antonius hospital Nieuwegein) and paraffin embedded. Of each paraffin embedded segment 15 sections (5um thickness) are cut on a microtome for histological (immuno) stainings. Segment 0 is then transported to the pathology department (Antonius hospital Nieuwegein) and paraffin embedded. Of each paraffin embedded segment 15 sections (5um thickness) are cut on a microtome for histological (immuno) stainings. The following stainings are performed to characterise the plaque: Picro Sirius red (collagen and fat using polarised light), CD68 (macrophages), alfa actin (smooth muscle cells), haematoxilin (thrombus and calcifications) and elastin (Figure 2).

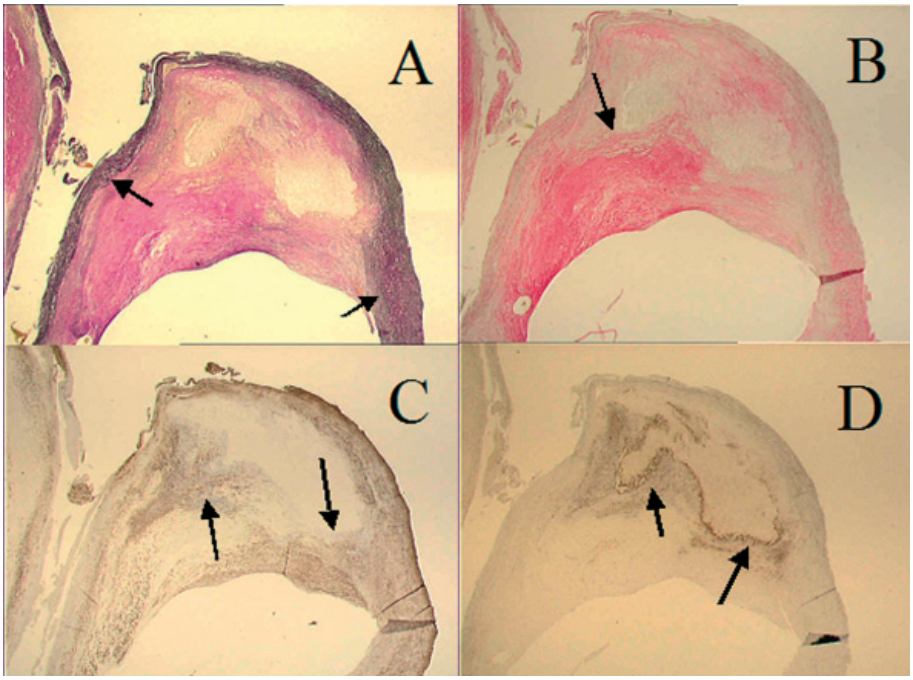

Figure 2. *Representative example of an endarterecomy specimen. A: Elastin staining. Note that also parts of the media (arrows) are often being dissected during endarterectomy. B- Collagen picro sirius red staining. The arrow indicates a thick fibrous cap overlying a small atheroma. C- Smooth muscle cell (alfa actin) staining. D- Macrophage (CD 68) staining. In this section most macrophages are observed around the lipid rich area within the plaque.*

The immunohistochemial staining for CD 68 and alfa actin stains are performed fully automated (Ventana Medical Systems, Tucson, Arizona). Ten blank 5 um thick cross-sections are cut and stored for future research purposes.
Of all +1 or –1 segments RNA and protein is isolated. For this purpose frozen arterial segments are crushed in liquid nitrogen. Total RNA and protein are isolated using 1ml Tripure™ Isolation Reagent (Boehringer Mannheim) according the manufacturers protocol. In this study we assume that expression of RNA and protein of segments –1 and +1 will be associated with the plaque phenotype as assessed by (immuno) histochemistry in segment 0. Plaques characteristics may be heterogeneous within a short disctance. Therefore, it may be that the expression of protein or RNA may not always reflect the association with the immunohistochemically assessed phenotype. This limitation merits consideration when a limited amount of samples will be used.

Plaque phenotyping. A plaque is considered more active and unstable when it reveals a strong staining for macrophages, a large atheroma and when it lacks collagen and smooth muscle cells[10-12, 26]. The more fibrous stable lesion typically lacks inflammatory cells and fat and reveals strong staining for collagen and smooth muscle cells (Figure 2). All stained sections are examined microscopically and digitally stored on CD-ROM at 2 magnifications (20x and 40x). Two observers independently score all stainings semi-quantitatively (Table I). The alfa actin (smooth muscle cell) and CD 68 (macrophage) stainings are also analyzed quantitatively by computerized analyses. For this, color thresholds are set and adjusted until the computerized detection meets the visual interpretation. The stainings are scored quantitatively as percentage of the plaque area. All sections are also analyzed by a third independent observer who is consulted when interpretations differ between observer 1 and 2.

Follow-up

Patients routinely visit the hospital at fixed points during follow-up (3-months, 1 year and 2 years). During these visits patients undergo duplex scanning of both carotid arteries. Patients fill in a questionnaire addressing the occurrence of relevant cardiovascular symptoms during follow up.

	no / minor	moderate / heavy	
Collagen (picro Sirius red)	No or only minor collagen staining along the luminal border of the plaque as visualized with and without polarized light	Moderate or strong staining for collagen along the luminal border of the plaque as with and without polarized light	
Smooth muscle cells (alfa actin staining)	Minor staining over the entire circumference with absent staining at parts of the circumference of the arterial wall	Large number of positive cells along the entire circumference of the arterial wall strongly dominating over CD68 positive cells	
Macrophages (CD 68 staining)	Absent or minor staining with negative or few scattered cells	Clusters of cells with >10 cells present.	
Cell dominance	Cell type that is dominant: CD 68 positive stained or alfa actin stained cells		
Fat (picro Sirius red and haematoxyin eosin staining)	Fibrous plaque : Less than 10% of the plaque is occupied by lipid as visualized with haematoxylin eosin stain and polarized light in picro Sirius red stained sections	Fibro -atheromatous plaque : between 10 - 40% of the plaque is occupied by fat.	Atheromatous plaque: Plaque hides an atheroma that occupies > 40% of the cross - sectional area.

Table 1. *Semi quantitative scoring of (immuno) histological stainings*

In case of doubt and when the questionnaire is not filled in, patients are called at home 12 months after surgery to insure that no relevant cardiovascular events are missed. When the patient does not respond, the general practitioner is contacted and asked for patient related information.

Vascular death	Sudden death (unexpected death occurring within 1 hour after onset of symptoms, or within 24 hours given convincing circumstantial evidence) or death from stroke, myocardial infarction, congestive failure or rupture of abdominal aortic aneurysm.
Non fatal stroke	Relevant clinical features which have caused an increase in handicap of at least one grade on the modified Ranking scale, accompanied by a fresh infarct or a hemorrhage on a repeat CT scan.
Non fatal myocardial infarction	At least two of the following: 1. Chest pain for at least 20 minutes, not disappearing after administration of nitrates; 2. ST -elevation > 1 mm in two following leads or a left bindle branch block on the ECG; 3. CK elevation of at least two times the normal value of CK and a MB fraction > 5% of the total CK.
Non fatal rupture of an abdominal aortic aneurysm.	Rupture of abdominal aortic aneurysm confirmed by laparatomy.
Vascular intervention	Any vascular intervention to treat clinically relevant ischemic disease in coronary, carotid, iliacal, femoral and crural arteries that was not yet planned at inclusion.

Table 2. *Definitions of secondary outcome events.*

End points

Primary endpoint is restenosis of the operated carotid artery. Restenosis is defined as > 70% luminal narrowing corresponding with a peak flow velocity >210 cm/second. The secondary endpoint encompasses all adverse cardiovascular events during follow up (Table 2): cardiovascular death, non fatal myocardial infarction, non fatal stroke and vascular intervention. In case an adverse event is suspected the medical files will be requested from referring centers.

	Fibrous	Fibro-Atheromatous	Atheromatous
No/minor CD 68 staining	82 (28%)	39 (14%)	35 (12%)
Moderate/heavy CD 68 staining	24 (8%)	46 (16%)	63 (22%)

Table 3. *Prevalence of different plaque phenotypes considering the amount of fat and macrophages in the plaque in the first 289 patients. For definitions see text.*

Taking the medical information into consideration, two clinicians will judge whether an endpoint has been reached.

Power of the study

When the presence of inflammatory cells will be treated as a dichotomous variable then, based on the observations in the first 289 patients, we expect an approximately 50%-50% division in non inflammatory versus inflammatory (CD 68 stained) plaques (Table 3). It is hypothesized that local inflammatory atheromatous plaques will result in a duplex defined restenosis incidence of 20% versus 10% in the non inflammatory fibrous plaques. With an estimated power (Beta) of 0.80 and alfa 0.05 we estimate that we will need to include 500 patients. The study is planned to continue until at least 1000 patients have been included.

Epilogue

This study was designed in 2001. Early 2002 the Medical Ethical Committee approved the study. The first patient was included in April 2002. January 2004, 289 patients were included. Of these patients all plaques have been characterized as described in the Methods section. It appeared that plaques characteristics varied substantially. Both, fibrous non inflammatory as well as inflammatory atheromatous lesions are observed frequently (Table 3). Table 4 summarizes the baseline characteristics of the first 289 patients.

Risk factors	
Gender	male: 197 (68.2 %)
Age	66.7 +/ - 8.5 years
Hypertension	151 (52.2 %)
Diabetes	54 (18.7 %)
Hypercholesterolemia	148 (51.2 %)
Smoker present	69 (23.7 %)
Smoker past	154 (53.3 %)
Previous manifestations of cardiovascular disease	
myocardial in farction	58 (20.1 %)
abdominal aneurysm	21 (7.3%)
Claudicatio	60(20.8 %)
Stroke	83 (28.7 %)

Table 4. *Baseline characteristics of first 289 patients.*

In the laboratory, this biobank with atherosclerotic specimen already proved its value. The expressions of proteins of interest, that were related with the progression of atherosclerotic disease *in vitro* or *in vivo* in animal studies, are currently being validated in a large number of atheroscleotic samples. Due to the relatively large sample size, Athero-Express provides the unique opportunity to study protein and RNA expression in relation to plaque phenotype after correction for potential confounders (like risk factors). Validation studies in which newly discovered proteins have been studied in relation to plaque characteristics have mostly been performed with low sample sizes due to limited availability of tissues. In those cases, retrospective correction for confounding or subgroup analyses is not feasible. Atherosclerosis is a multifactorial disease and the expression in atherosclerotic tissue of a newly discovered protein should ideally be performed in a larger population that hides patients who suffer from all recognized risk factors for atherosclerotic disease. The current study will not just allow cross-sectional studies relating plaque characteristics with local protein expression, but will also provide the opportunity to study the role of local protein and RNA expression in relation with long term outcome during follow up.

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Carotid atherosclerotic plaques in patients with TIA and Stroke have unstable characteristics compared to plaques in asymptomatic and amaurosis fugax patients

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Abstract

Introduction: Atherosclerotic carotid artery disease is responsible for a variety of clinical presentations, ranging fromasymptomatic 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.

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

Athero-Express is a prospective cohort study based on a vascular biobank that is currently being executed in two Dutch hospitals. The study design of Athero-Express has been published previously[18]. For the current study, we selected 404 patients diagnosed with symptomatic or asymptomatic carotid artery stenosis. All consecutive patients who were newly referred to the vascular surgery departments of the participating centers for treatment of carotid artery stenosis were enrolled. Patients were symptomatic or asymptomatic. The operation was indicated based upon the recommendations as published by Asymptomatic Carotid Atherosclerosis Study and the North American Symptomatic Carotid Endarterectomy Trial[1,5]. If a duplex investigation was not sufficient, an additional imaging technique was used to certify the level of carotid stenosis (magnetic resonance angiography, computed tomography angiography, or Seldinger angiography). Patients with a terminal malignancy and those who were referred back to a hospital outside The Netherlands immediately after surgery were excluded. The medical ethics committees of the participating hospitals approved the study.

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 assessed 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].

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 (5,000 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. The –1 and +1 segments, and all subsequently numbered segments (–2, +2, –3, etc) were immediately frozen in liquid nitrogen and stored in –80°C in numbered metal cups. Segment 0 was fixed in 4% formaldehyde and paraffin embedded. From each paraffin-embedded segment, 15 sections (5-_mthickness) were cut on a microtome for histologic immunostaining.

Plaque phenotyping. All stained sections were examined microscopically and digitally stored on CD-ROM at +20 and +40 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:
• Picrosirius red (collagen staining 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 intra plaque 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 Picrosirius 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 1mL 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 RPN2634, 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 χ^2 and the Fisher's exact test. All continuous variables were distributed nonparametrically. Testing was performed by the Mann-Whitney test and the Kruskal-Wallis 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 valueof $P \leq 0.05$ was considered statistically significant.

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 vasc 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
hypercholesteroleamia	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
triglycerids mmol/l (sd)	2.2	(1.3)	1.9	(0.8)	2.1	(1.1)	2.1	(1.0)	0.8
statine 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%	

Table 1: *Baseline characteristics. All values are mean+/- SD or absolute number of patients in each group with calculated percentage.*

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 influence the results (data not shown). The grades of duplex lumenal stenosis did not differ among the categories of clinical symptoms (see Table I).

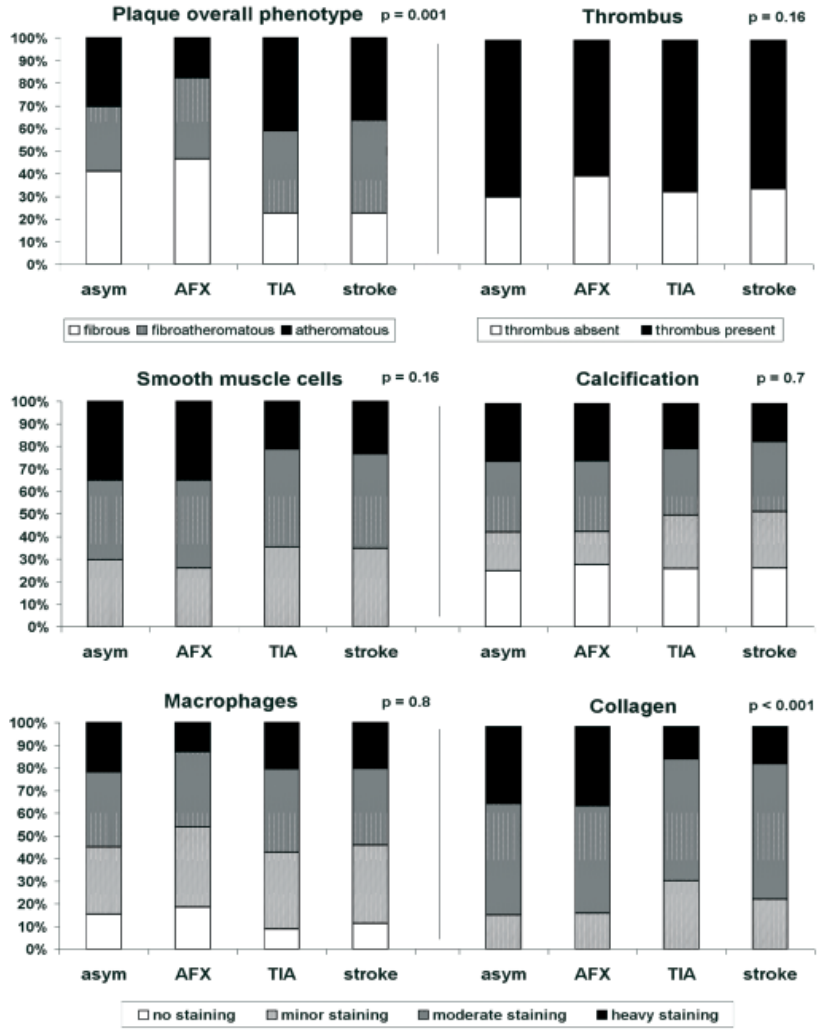


Figure 1: *Clinical symptoms and plaque characteristics. This figure shows different plaque characteristics present at the culprit lesion, for different symptomatic categories. All data are displayed in percentages.*

		Symptoms and plaque characteristics				
		No	AFX	TIA	Stroke	p - value
MP Median & QR)	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
Median & QR)	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(27.6 -171.4)	43.5(2.2 -182.4)	<0.001
ollagen	Minor	15.2%	16.1%	30.5%	22.4%	<0.001
	Moderate	50%	48.2%	54.9%	60.7%	
	Heavy	34.8%	35.7%	14.6%	16.8%	
aque verall	Fibrous	41.1%	46.4%	22.6%	22.4%	0.001
	F -atheromatous	28.4%	35.7%	36.3%	41.1%	
	Atheromatous	30.5%	17.9%	41.1%	36.4%	

Table 2*: Plaque characteristics compared to symptoms. MMP and IL data are displayed as median and interquartile range (pg/ml).*

There was no difference in symptom-free intervals between the various categories of symptoms. Fig 1 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) (Fig 1 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) (Fig 1). 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).

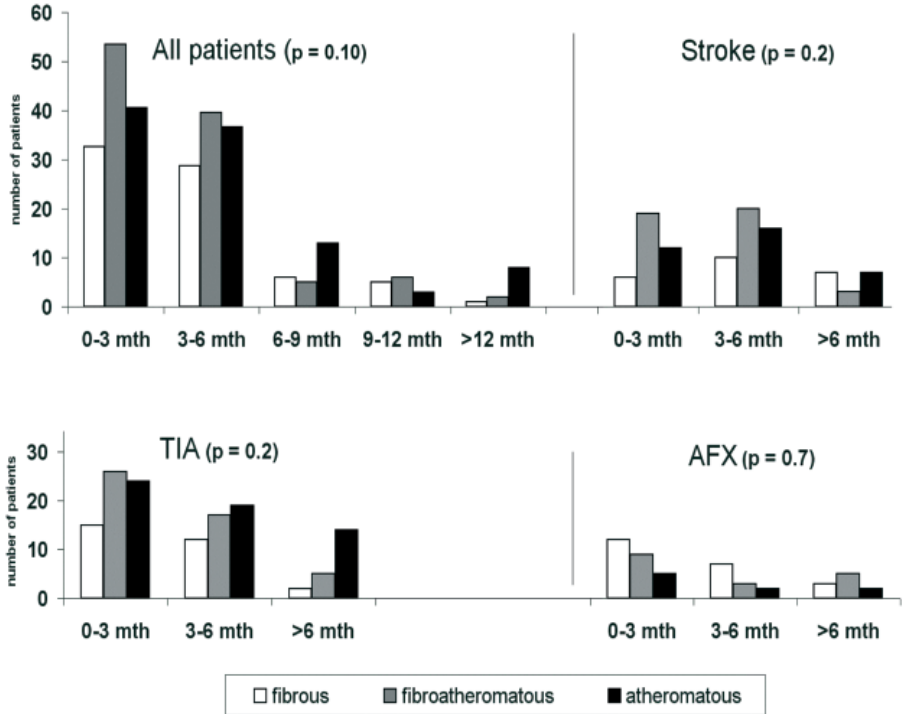
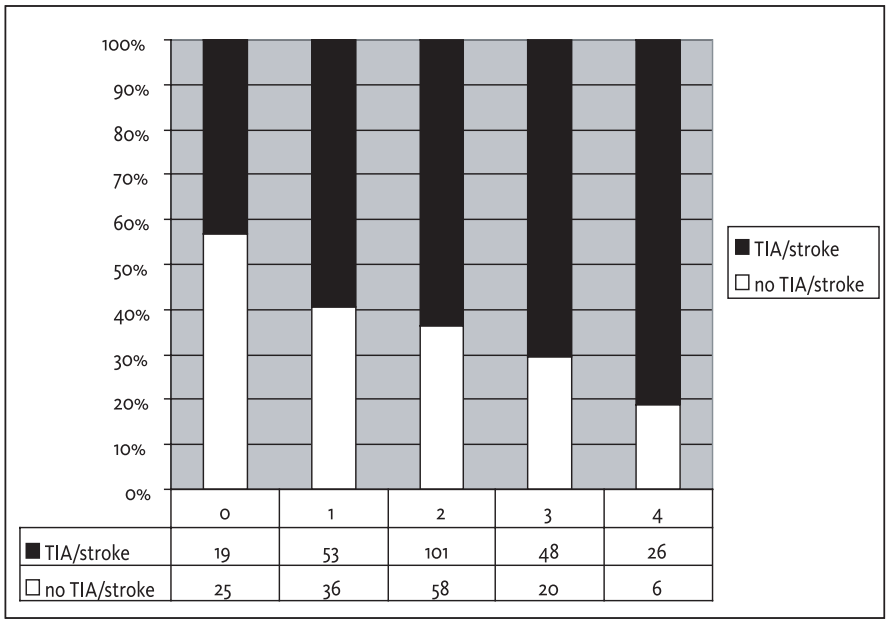


Figure 2: *Symptom free interval and plaque phenotype. Number of patients with specific plaque phenotype and symptom free interval. P-values indicate that no differences are observed between distributions of plaque phenotype and increasing symptom free interval for all patients, TIA, stroke and AFX respectively.*

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 (Fig 2). 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).



	0	1	2	3	4
TIA/stroke	19	53	101	48	26
no TIA/stroke	25	36	58	20	6

	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 3: *Plaque score model. Specific plaque characteristics based on the definition of the vulnerable plaque were given points. The percentage and number of patients with TIA and stroke related to received points are* displayed.

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 patient (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 regularly (P =.05). Fig 3 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 noninvasively. 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 post-mortem 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 considered. 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 vasospastic 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 symptomfree 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 (eg, 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 (Fig 4). The presence of one vulnerable plaque characteristic (eg, 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 (Fig 1) 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 endpoint 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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Statin treatment is not associated with consistent alterations in inflammatory status of carotid atherosclerotic plaques: a retrospective study in 378 patients undergoing carotid endarterectomy

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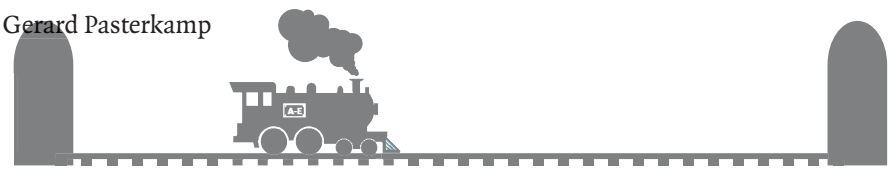

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Abstract

Purpose: Anti-Inflammatory qualities are held partially responsible for the reduction of cardiovascular events following statin treatment. We examined the phenotype of carotid atherosclerotic plaques harvested during carotid endarterectomy (CEA) in relation to the previous use of different statins prescribed in clinical practice.

Methods: Three-hundred and seventy-eight patients were included. Atherosclerotic plaques were harvested, immunohistochemically stained and semiquantitively examined for the presence of macrophages (CD68), smooth muscle cells, collagen and fat. Adjacent atherosclerotic plaques were used to study protease activity and interleukin (IL) levels. Patients' demographics were recorded and blood samples were stored.

Results: Serum cholesterol, LDL, APO-B and CRP levels were lower in patients treated with statins compared to patients without statin treatment. Atheromatous plaques were less prevalent in patients receiving statins compared to patients without statin therapy (29% vs 42%, p=0.04).

An increase of CD68 positive cells was observed in patients receiving statins compared to non-statin treatment (p=0.05). This effect was specifically related to atorvastatin treatment. In patients treated with atorvastatin, the increased amount of CD68 positive cells were not associated with increased protease activity. In contrast, a dose dependent decrease in protease activity was shown in the atorvastatin group. Interleukin 6 expression was lower in plaques obtained from patients treated with statins (p=0.04).

Conclusions: Statin use may exert pleiotropic effects on plaque phenotype. However, not the presence of macrophages but activation with subsequent protease and cytokine release may be attenuated by statin use.

Introduction

Hyperlipidemia is a risk factor for cardiovascular disease and is related to the increase of plaque atheroma[1]. Treatment with HMG-coa reductase inhibitors (statins) clearly results in reduced cardiovascular-related mortality and morbidity[2;3].

Next to the lipid lowering effects, statin treatment has potential pleiothropic effects by modulating various inflammatory responses that are involved in the initiation and progression of atherosclerotic disease[4]. Statin induced reductions of serum interleukin levels (IL), C-reactive protein (CRP) and matrix metalloproteinase (MMP) activity have been described previously[3;5-9]. However, pleiotropic effects of statin treatment on atherosclerotic plaque characteristics have mainly been described in animal studies. Pravastatin has been investigated in many experimental cardiovascular studies[10-13].

The modifying effects of atorvastatin or simvastatin on human plaque characteristics are relatively unexplored. A prospective longitudinal study including a non-treated placebo group, with the objective of studying the plaque stabilizing effects of statins, is considered unethical. In this retrospective study, we analyzed the pleiotropic effects of three regularly prescribed statins on plaque characteristics in 378 patients undergoing carotid endarterectomy (CEA).

Methods

Baseline data

Patients have been included in the longitudinal cohort bio-bank study Athero-Express. The study design of Athero-Express has been published previously[14].

Briefly, all patients receiving operative treatment for carotid artery disease in the participating centres are enrolled. Patients may have been symptomatic or asymptomatic. Operation is indicated based upon the recommendations as published by ACST and NASCET[15;16]. Patients with a terminal malignancy and those who are referred back to a hospital outside the Netherlands immediately following surgery are excluded.

The Medical Ethical Committees of the participating hospitals have approved of the study.

From patients included in Athero-express extensive baseline patient characteristics are available (history of cardiovascular disease, medication etc)[14]. In cases of doubt about medication use or dosage, the patient's general practitioner or pharmacy was consulted. Statin dosages were recorded and divided in two categories: low dose (simvastatin, pravastatin and atorvastatin 10 and 20 mg) and high dose (simvastatin and atorvastatin 40 and 80mg, pravastastin 40mg).

Blood samples of 228 patients were available. Total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL), LDL, apolipoprotein-B (Apo B) and CRP were assessed prior to surgery. Baseline patient characteristics are presented in table 1.

Tissue processing

The atherosclerotic plaque obtained during CEA, was immediately processed and dissected in parts of 0.5 cm. The culprit lesion was fixated in formaldehyde 4%, paraffin embedded and used for histological stainings. The segments adjacent to the culprit lesion were immediately frozen in liquid nitrogen and stored at –80˚C.

	No statin	All statins	p -value	Simvastatin	Pravastatin	Atorvastatin
Included in study	129	249		91	44	98
Coronary intervention in the past	13.5%	25.0%	0.01	29.1%	27.9%	20.2%
Myocardial infarct ion in the past	17.5%	22.9%	0.24	21.6%	30%	18.2%
Peripheral artery intervention ever	17.6%	25%	0.11	31.6%	18.6%	20.2%
Hypertension ever	61.2%	72.0%	0.04	71.2%	75.%	71.1%
Diabetes	13.2%	25.0%	0.01	26.3%	16.7%	26.7%
hypercholesterolemia	27.8%	78.1%	<0.001	78.5%	82.1%	74.2%
smoker	29.8%	26.0%	0.46	30.0%	16.7%	25.3%
BMI > 25	60.4%	61.7%	0.82	60.0%	60.5%	66.3%

Table 1:*Baseline patient characteristics. Numbers and percentages of patients for the displayed categories are presented*

All stained sections were independently scored by two observers as described previously[17]. Plaques stainings were categorized as followed:

• Picro Sirius red; collagen staining using polarized light microscopy: 1) no or minor staining= staining along part of the luminal border; 2) moderate or heavy staining= staining along the entire luminal border.

• CD68 positive cells: 1) absent or minor staining= negative or few scattered positive cells; 2) moderate or heavy staining= stained clusters of cells with >10 cells present.

• Alpha-actin positive cells: 1) no or minor staining= discontinuous over the entire circumference with absent staining at parts of the circumference of the arterial wall; 2) positive cells along the entire circumference of the luminal border, with at least minor staining locally with few scattered cells

• Hematoxylin and elsatin stains were used to assess overall morphology.

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 defined based on the percentage of atheroma in the plaque: fibrous plaques <10% fat, fibro-atheromatous 10-40% or atheromatous > 40% fat.

• HLA-DR staining was performed in patients with moderate or heavy CD68 stained plaques and treated with atorvastatin (n=20) or no statin (n=22). Three groups were defined based upon the percentage of cells being activated in the plaque <25%, 25-75% or >75%.

The CD68 (macrophage) staining was also analyzed quantitatively by computerized

analyses using Analysis® software. The stained sections were scored quantitatively as a percentage of the plaque area.
The adjacent segments were used for protein isolation. Protein was isolated by two protocols. One part of segment 1 was treated with 1ml Tripure™ Isolation Reagent (Boehringer Mannheim) according to the manufacturer's protocol. The other part was dissolved in 1ml 40mmol TrisHCl (ph=7.5;4˚C) and centrifuged at max rpm after which the vials were stored at -80˚C. Protein concentrations for both isolation techniques were measured according to the manufacture's protocol (Bio-Rad Laboratories, California, U.S.A). For a randomly selected subgroup of 133 plaques, MMP-2, MMP-8 and MMP-9 activities were measured in isolated protein using the Biotrak activity assays RPN 2631, RPN 2635 and RPN 2634, respectively (Amersham Biosciences, Buckinghamshire UK)[18;19]. Interleukins 6 and 8 were measured in 293 protein isolates using a multiplex suspension array system according to the manufacture's protocol (Bio-Rad Laboratories, California, U.S.A.).

Statistical analyses
Statins were associated with plaque characteristics using a Chi-square and Fisher exact test. Additionally, a binary logistic regression analysis was used in cases of confounding. In the cases of non-parametric distributed continuous variables, comparison of the variables was performed by a Mann–Whitney test and a Kruskal Wallis test. For normal distributed parameters, T-tests and Anova tests were used. P-values of <0.05 were considered statistically significant.

Results

Patients who received statin therapy showed a higher prevalence of cardiovascular morbidity and risk factors in the past (table 1). These risk factors were equally distributed among the three major statins described. We examined the relation between all baseline characteristics and the investigated markers for plaque phenotype to rule out confounding of baseline characteristics in the studied correlations between statin use and plaque phenotype. Overall, plaque phenotype (e.g. amount of fat, protease activity and Interleukin levels) was neither associated with any of the risk factors nor with clinical history (all p>0.10). CD68 positive stained cells were more prevalent in plaques obtained from patients with prior coronary intervention and patients diagnosed with high blood pressure in the past (p=0.04 and p=0.06, respectively).
Baseline characteristics of serological assessments are presented in table 2. Serum levels of cholesterol, LDL and ApoB as well as CRP were significantly lower among statin users (table 2). Atheromatous ("lipid rich") plaques were less prevalent in

patients receiving statins compared to patients without statin therapy (29% vs 42%, p=0.04, table 3). The prevalence of atheromatous plaques was not associated with statin type or dosage.

Serum characteristics (mean +/ - SD) n=228	No statin	All statins	p-value	Pravastatin	Simvastatin	Atorvastatin	p value
Cholesterol (mmol/l)	5.7 +/ - 1.3	4.7 +/ - 0.9	<0.001	4.7 +/ - 0.9	4.7 +/ - 0.9	4.7 +/ - 1.0	0.71
Triglicerydes (mmol/l)	2.0 +/ - 0.9	2.1 +/ - 1.1	0.58	2.1 +/ - 0.9	2.3 +/ - 1.0	2.0 +/ - 1.0	0.19
HDL (mmol/l)	1.2 +/ - 0.4	1.2 +/ - 0.4	0.66	1.2 +/ - 0.3	1.2 +/ - 0.3	1.2 +/ - 0.4	0.79
LDL (mmol/l)	3.7 +/ - 1. 2	2.7 +/ - 0.7	<0.001	2.9 +/ - 0.8	2.7 +/ - 0.7	2.6 +/ - 0.8	0.39
APO B (g/l)	1.1 +/ - 0.3	0.9 +/ - 0.2	<0.001	0.9 +/ - 0.2	0.9 +/ - 0.2	0.9 +/ - 0.2	0.27
CRP (median / IQR mg/l)	4.8 / 2.1 -12.9	2.8 / 1.5 -6.0	0.002	2.2 / 1.5 -5.1	2.6 / 1.5 -5.4	3.4 / 1.6 -8.9	0.31

Table 2: *Serum characteristics. Parametrically distributed variables are presented with mean and standard deviation, whereas non-parametric variables are presented with median and interquartile range (IQR).*

Plaque characteristics (%/number)	No statin	All statins	p-value	Pravastatin	Simvastatin	Atorvastatin	p value
Minor collagen	25% / 32	24% / 59	0.86	12% / 5	28% / 25	26% / 25	0.10
Heavy collagen	75% / 97	76% / 187		88% / 38	72% / 64	75% / 73	
Minor SMC	34% / 44	33% / 80	0.72	23% / 10	36% / 32	33% / 32	0.35
Heavy SMC	66% / 84	68% / 166		77% / 33	64% / 58	67% / 65	
Minor calcifications	48% / 62	47% / 117	0.97	43% / 19	49% / 45	46% / 45	0.96
Heavy calcifications	52% / 67	53% / 132		57% / 25	51% / 46	54% / 53	
Minor MØ	53% / 68	43% / 106	0.05	61% / 26	44% / 40	34% / 33	0.01
Heavy MØ	47% / 59	57% / 141		40% / 17	56% / 50	66% / 65	
Fibrous	29% / 37	33% / 83	0.04	32% / 14	31% / 28	37% / 36	0.59
F-Atheromatous	29% / 38	38% / 94		43% / 19	34% / 31	37% / 36	
Atheromatous	42% / 54	29% / 72		25% / 11	35% / 32	26% / 26	
MØ quantitative (median / IQR)	0.3 / 0.01 -0.3	0.4 / 0.1 -1.3	0.16	0.2 / 0.01 -1.0	0.4 / 0.1 -1.2	0.6 / 0.1 -1.5	0.04
MMP 8 (median / IQR)	6.3 / 2.9 -12	5.8 / 2.7 -9.8	0.32	3.6 / 0.0 -9.3	6.9 / 3.7 -9.3	6.2 / 3.1 -13.2	0.25
MMP 9 (median / IQR)	1.7 / 0.8 -5.2	2.1 / 1.0 -4.9	0.43	1.4 / 0.8 -3.8	2.6 / 0.9 -5.8	2.1 / 1.3 -4.8	0.39
IL 6 (median / IQR)	11.0 / 4.2 -23	6.9 / 1.6 -48	0.04	4.8 / 0.4 -13.9	6.9 / 2.2 -19.6	7.9 / 2.1 -19.5	0.29
IL 8 (median / IQR)	34.4 / 0.6 -97	42.9 / 7.3 -138	0.29	32.4 / 0.8 -178	43.3 / 8.8 -113	44.1 / 6.8 -152	0.95

Table 3: *Plaque characteristics. Plaque stainings are in absolute numbers and percentages. Parametrically distributed variables are presented with mean and standard deviation, whereas non-parametric variables are presented with median and interquartile range (IQR). MØ= CD 68 staining, SMC= Smooth muscle cell staining.*

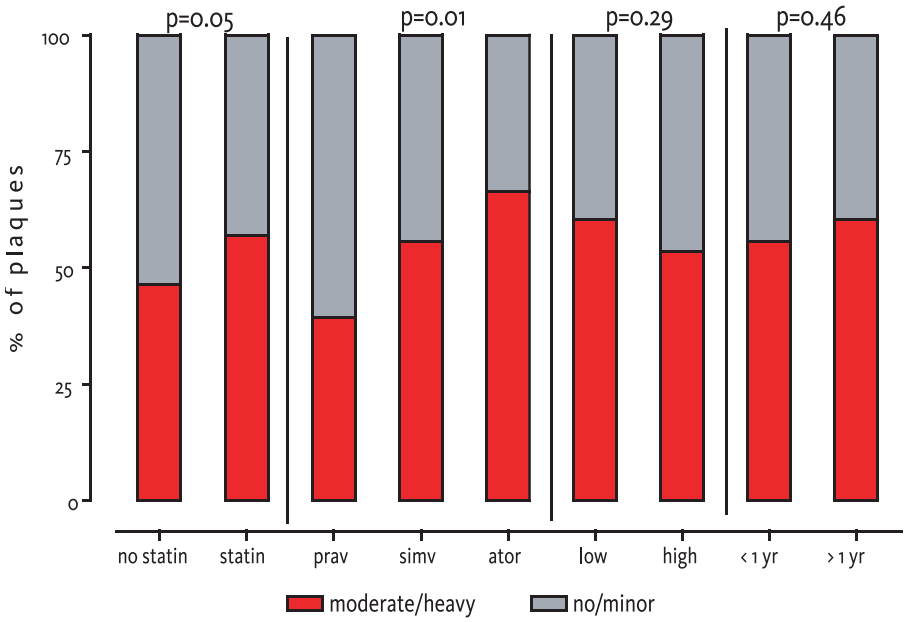

Figure 1: *Percentage of plaques with minor or heavy staining for CD 68 positive cells. Prav= pravastatin; simv= simvastatin and ator= Atorvastatin. Low= low dose and high= high dose. <1 yr= statin therapy shorter than 1 year and >1 yr= longer than 1 year*

A tendency towards a lower prevalence of atheromatous plaques was noticed for patients receiving statins for more than 1 year (no statin: 41.9% atheromatous plaques, <1 year statin 32.9% and >1 year statin 27.7%) (p=0.07).

In contrast to the decreased level of instable "lipid rich plaques", a significant increase in CD68 positive cells was observed in the statin group (p=0.05, table 3 and figure 1). To rule out confounding between statin use and the previously mentioned baseline characteristics that were related to CD68 positive cells (hypertension and prior coronary intervention), a binary logistic regression analysis was performed to identify the independent relations.

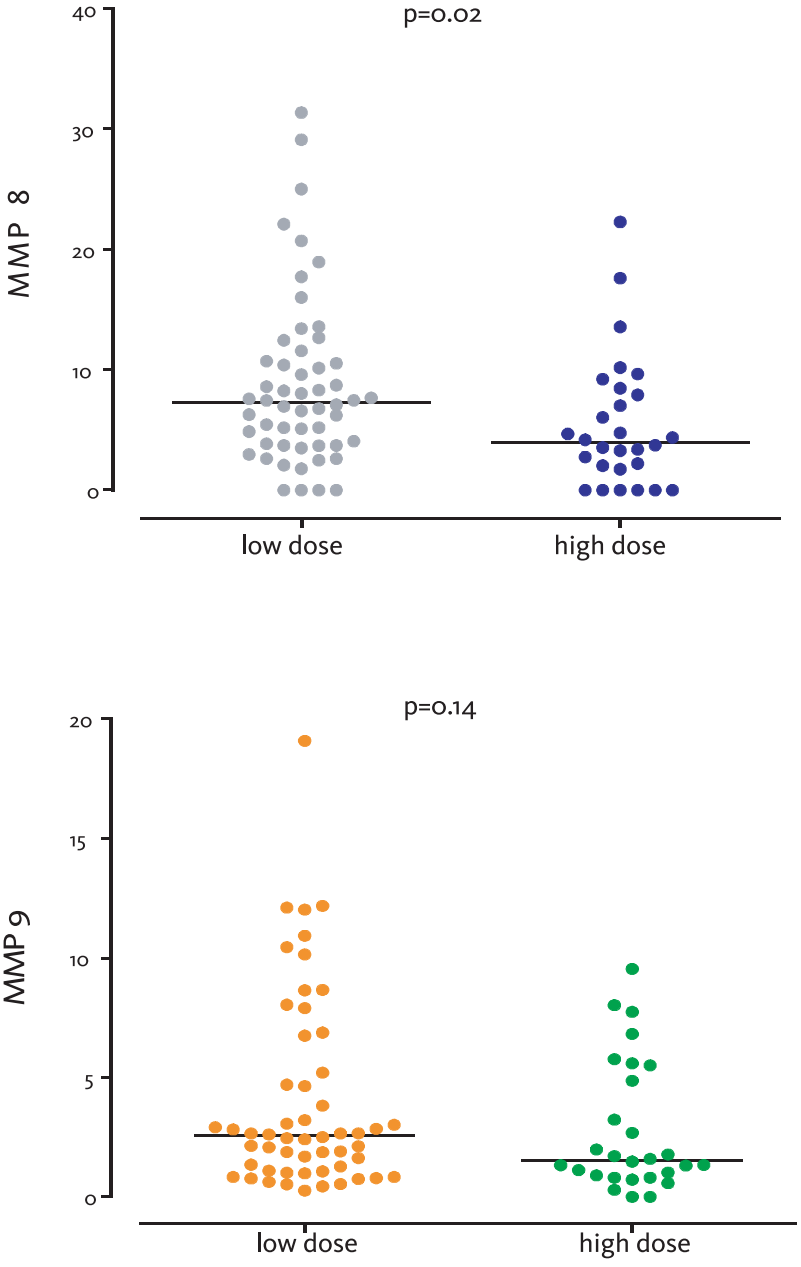

Figure 2a: *Patients treated with high statin dose showed a significantly decrease in MMP 8 activity, whereas a tendency is observed for MMP 9. Bars indicate median.*

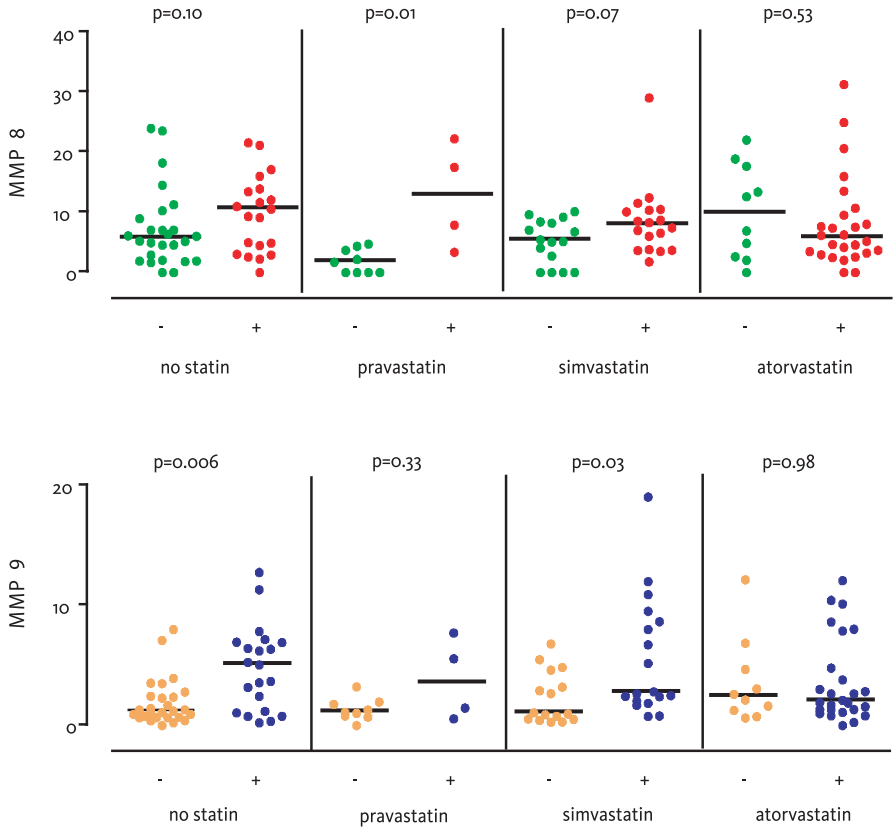

Figure 2b: *MMP 8 and 9 levels of atherosclerotic plaques with moderate/heavy (+) staining for CD 68 positive cells compared to plaques with no/minor (-) staining and different groups of statin treatment. Bars indicate median.*

Statin use was independently related to an increased number of CD 68 positive cells in the plaque (p=0.02), whereas prior coronary intervention and high blood pressure were not (p=0.25 and 0.06).
These observations would appear to conflict with previous studies demonstrating that pravastatin treatment is related to lower macrophage content in the atherosclerotic plaque.

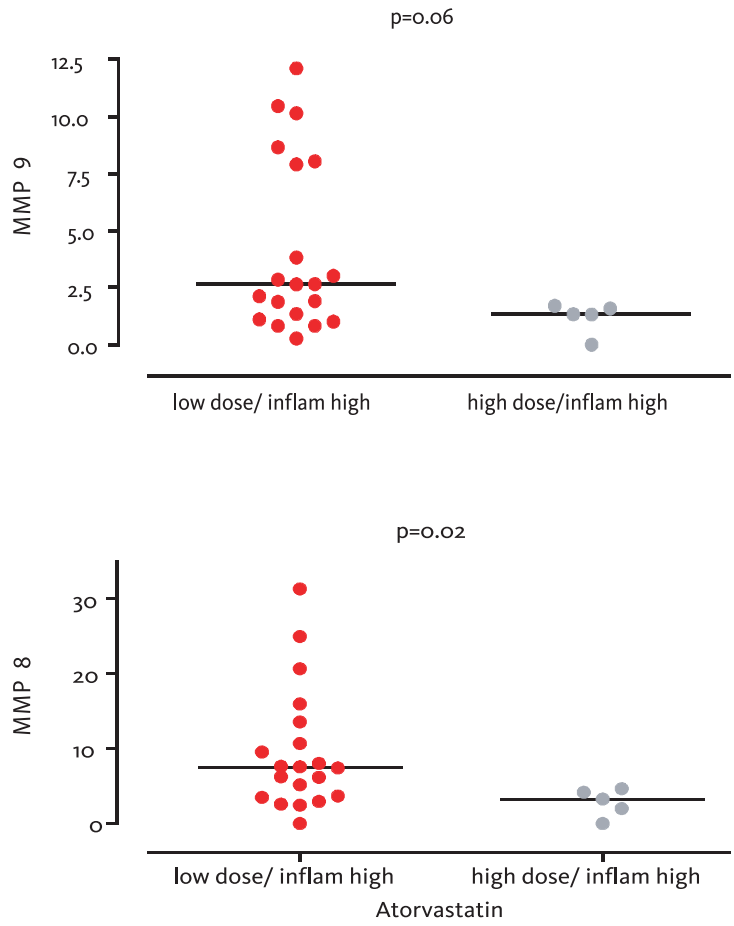

Figure 3: *MMP 8 levels for patients receiving low dosage atorvastatin and high staining for CD 68 positive cells compared to patients with high dosage. Bars indicate median. For MMP 9 a similar relation was observed. Bars indicate median.*

Therefore, we decided to perform subgroup analyses for the different, frequently prescribed, statins. Plaques derived from patients receiving atorvastatin showed significantly more CD68 positive cells than those receiving pravastatin (table 3, p=0.01). Prolonged statin therapy (>1 year) and high dosage were not related to a decreased prevalence of CD68 positive cells (figure1). The increased amount of CD68 positive cells in patients who received statin treatment was not associated with an increased MMP activity (table 3).

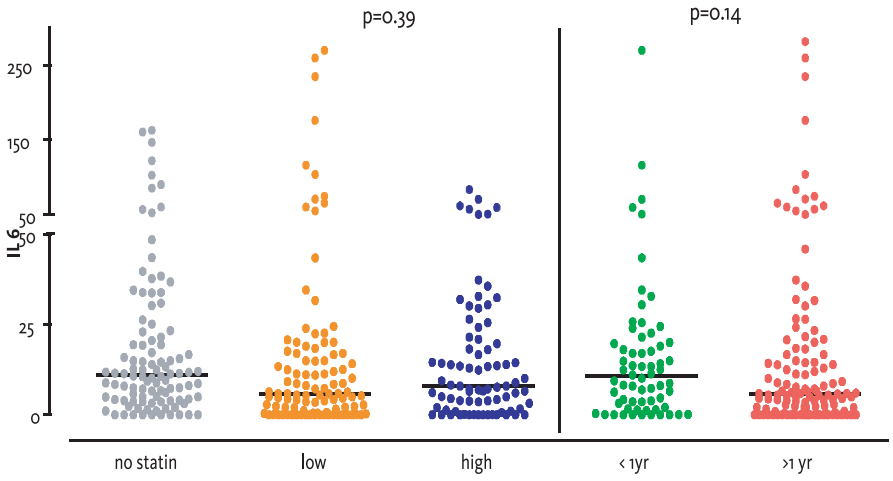

Figure 4: *IL 6 levels (pg/l) measured in plaques from patients. Low= low dose and high= high dose. <1 yr= statin therapy shorter than 1 year and >1 yr= longer than 1 year. Error bars indicate median. P-values were calculated over low and high dose patients and <1 year or >1 year treatment.. A significant difference was observed between statin and non statin users (see table 3*

In contrast, patients treated with high doses of statin showed less protease activity compared to patients treated with low doses of statin (figure 2A). In the non-statin treated group MMP8 and 9 activities were associated with the degree of CD68 positive staining. The relation between CD68 staining and MMP activities was abolished in the atorvastatin treated group but remained significant in the pravastatin treated group (figure 2B). Patients with strong CD68 staining and high dose atorvastatin revealed lower activity levels of MMP 8 and 9 compared to patients with low dose and strong CD68 staining (p=0.02 and p=0.06, figure 3). Statin use did not influence MMP2 activity (data not shown).

Interleukin 6 levels were lower in plaques obtained from patients who were on statin treatment compared to plaques derived from patients who did not receive statin treatment (p=0.04, table 3). Levels of IL 6 slightly decreased in patients with prolonged statin therapy (figure 4, p=0.14). No significant differences were observed for IL8 among patients' groups (table 3). As an indication of activated CD68 cells we measured plaque HLA-DR expression in patients with high staining for CD 68. No difference between the percentage of activated cells was observed between patients treated with atorvastatin and patients without statin treatment (p=0.99).

Discussion

Statin treatment of cardiovascular patients results in improved clinical outcome and reduction of serum LDL and CRP levels[3;20;21]. Reduction of serum interleukin levels has also been described in relation to statin treatment[8;22]. However, statin related effects on plaque characteristics, have only been described in a limited number of studies that were limited by the number of specimens analyzed [10-13;23]. In this retrospective study, we analyzed the inflammatory status of atherosclerotic plaques obtained during carotid endarterectomy (CEA) in relation to statin therapy.

Our primary findings are:

1/ Atheromatous plaques are less prevalent in patients treated with statins.

2/ Overall, the number of CD 68 positive cells does not decrease in patients receiving statins. However, this unexpected observation was mainly explained by a higher prevalence of CD68 positive stained cells that were observed in plaques obtained from patients receiving atorvastatin treatment.

3/ The relation between CD68 positive stain and MMP8 or MMP9 activities is eliminated in plaques obtained from patients who had been on atorvastatin treatment but not in plaques obtained from pravastatin treated patients. In addition, plaque IL-6 levels were lower in all statin treatment groups.

4/ The described associations between statins and plaque characteristics were related to dose and duration of treatment.

It has been acknowledged that inflammatory mechanisms couple dyslipidemia to atheroma formation[24]. Statin treatment results in reduced serum cholesterol levels, impairs inflammatory processes and might stabilize atheromatous plaques [12;23;25]. The current study confirms the earlier experimental observations that statin treatment results in lower fat content of atherosclerotic plaques. However, in contrast with previous studies, we found a slight but significant increase of CD68 positive cells in plaques obtained from statin treated patients which was mainly due to the inclusion of the atorvastatin treated patients. This was even more surprising considering the recently published clinical trial demonstrating the beneficial outcome for atorvastatin users[26]. Our observation does not imply that the inflammatory response is enhanced in atherosclerotic tissue of atorvastatin treated patients. CD68 is considered a pan macrophage marker and does not provide information about the functional status of macrophages. The question rises whether the CD68 positive cells in patients treated with statins are inactivated.

Within the atherosclerotic plaque, statins could suppress the production of cytokines and MMP by CD68 positive cells. This hypothesis is supported by our observations and is also substantiated by earlier fundamental research[12;23].

Heavy CD68 stained plaques correlated with increased protease activity. This association was eliminated in atorvastatin but not in pravastatin treated patients. Moreover, IL6 levels were lowered among patients treated with statins. It could be suggested that the pleiotropic effect of statin treatment is more reflected on a macrophage activation than on a macrophage recruitment level. For this reason, we studied HLA-DR expression, a marker for macrophage activation. HLA-DR expression did not differ significantly between statin users and non statin users. This observation makes a dramatic effect of statins on HLA-DR unlikely. However, HLA-DR expression on inflammatory cells upon immune activation is not a de novo ("on-off") feature, on activated cells the number of HLA-DR molecules increases drastically. Such a phenomenon may result in increased staining intensity, which we were unable to demonstrate, but which does not allow the tracing of discrete differences in expression (and hence activation).

Besides the above-mentioned possibilities to explain the observed differences in CD68 staining, hypercholesterolemia itself could be an explanation. Significantly more patients in the statin group had a prior history of hypercholesterolemia. However, baseline serum lipid spectra are lower in the statin group compared to the non-statin group. We also noted significantly lower serum CRP (activated plaque macrophages could produce CRP) levels in patients treated with statins compared to patients with no statin. Strictly hypothetically, the observed increased number of macrophages in the atherosclerotic plaques could be necessary for organizing plaque lipid remains.

What do these observations mean for clinical practice? First, imaging modalities are being developed to visualize the vulnerable plaque and the patient who is at risk for plaque rupture and subsequent cardiovascular symptoms. The present study suggests that in patients receiving statins, the inflammatory status of the atherosclerotic plaque should not be based on the number of inflammatory cells which may not always relate to plaque vulnerability.

Secondly, long term statin treatment may be necessary to optimally influence plaque phenotype. We report that the statin associated effects on plaque characteristics are more pronounced in patients receiving statins longer than 1 year ($p=0.07$). The reported effect of statins on LDL serum levels and incidence of myocardial infarction has also been observed after prolonged treatment[27].

Limitations

This study is based on retrospective data. No causality can be inferred from the current data and ideally a prospective study should be executed. However, it would be unethical to compare patients with no statin to statin therapy.

Besides the modifying effect of therapy duration, we also observed a dose response

effect. We report a dose response relation between plaque MMP levels and statin dose. This relation was not evident for IL-6 and IL-8. The dosages among the different statins were not equally distributed and could be of influence since the reported effects on inflammation differ among statins.
Our selected population could induce a bias. Higher rates of prior coronary intervention, hypertension, diabetes and previous cardiovascular events were resported I the statin treated group. Patients with these characteristics would be expected to have more advanced and more severe atherosclerosis and quite likely, greater inflammation and other biological derangements so typical of atherosclerosis. However, we excluded confounding and this can not explain the differences observed among simvastatin, pravastatin and atorvastatin.
Sampling error could be an issue. Adjoining tissue showed less protease and IL6 activity than would be expected from the amount of macrophages in the section examined by histology. Plaque characteristics may be heterogeneous within a short distance. Therefore, expression of protein or RNA may not always reflect the association with an immunohistochemically assessed phenotype. This limitation merits consideration when a limited number of samples is used. However, the relation we observed in control tissues in this and previous studies between MMP9 and CD68 stainings, for instance, supports the idea that inflammatory markers are present in both adjacent segments.

In summary,
Statin therapy was associated with a decreased prevalence of carotid atheromatous plaques. The number of CD68 positive cells increased slightly in patients receiving atorvasstatins. Not the presence of macrophages but activation with subsequent protease and cytokine release seem attenuated by statin use.

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Carotid **athero**sclerotic plaque characteristics are associated with microembolisation during CEA and procedural outcome

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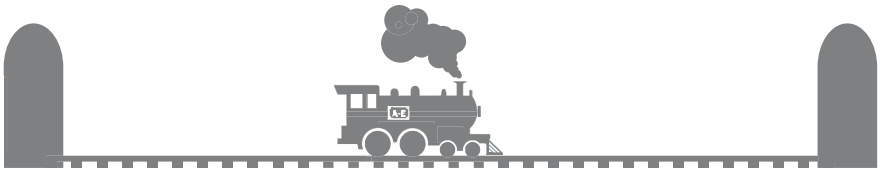

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Abstract

Background: During carotid endarterectomy (CEA) microemboli may occur resulting in peri-operative adverse cerebral events. Objective of the present study was to investigate the relation between atherosclerotic plaque characteristics and the occurrence of microemboli or adverse events during CEA.

Methods: Patients (n=200/ 205 procedures) eligible for CEA were monitored using peroperative transcranial Doppler. The following phases were discriminated during CEA: dissection, shunting, release of the clamp and wound closure. Each carotid plaque was stained for collagen, macrophages, smooth muscle cells, haematoxylin and elastin. Semi quantitative analyses were performed on all stainings. Plaques were categorized into three groups based on overall appearance (fibrous, fibro-atheromatous or atheromatous).

Results: Fibrous plaques were associated with the occurrence of more microemboli during clamp release and wound closure compared with atheromatous plaques (p=0.04 and p=0.02, respectively).

Transient ischemic attacks and minor stroke occurred in 5/205 (2.4%) and 6/205 (2.9%) patients respectively. Adverse cerebral outcome was significantly related with the number of microembolic events during dissection (p=0.003) but not during shunting, clamp release or wound closure. More cerebrovascular adverse events occurred in patients with atheromatous plaques (7/69) compared to patients with fibrous or fibro-atheromatous plaques (4/138) (p=0.04).

Conclusion: Intra-operatively, a higher number of microemboli was associated with the presence of a fibrous and not an atheromatous plaque. However, atheromatous plaques were more prevalent in patients with subsequent immediate adverse events. In addition, specifically the number of microemboli detected during the dissection phase were related with immediate adverse events.

Introduction

Carotid artery stenosis is a common disease in Western society and related with the occurrence of transient ischaemic attacks (TIA) and strokes[1]. Carotid endarterectomy (CEA) is a widely applied method to treat symptomatic and asymptomatic patients with severe carotid artery stenosis. However, 3-7% of the CEA procedures are complicated by disabling or non-disabling strokes[1-3]. Peroperative transcranial Doppler (TCD) registration of the middle cerebral artery provides online surveillance of both haemodynamic changes and passage of cerebral microemboli[4-7]. Therefore, TCD surveillance is widely applied during CEA[4,8-10]. Peroperative microembolisation detected by TCD monitoring has been related with the occurrence of adverse

neurological events[11-13]. Asymptomatic microemboli recorded by one-hour TCD registration in a group of non-operated patients with symptomatic as well as asymptomatic carotid stenosis was found to be related to TIA and stroke during follow up[14].

In cardiovascular disease, next to plaque size and luminal narrowing also plaque characteristics are considered causally related with the development of cardiovascular events[15-18]. In general, the vulnerable unstable plaque consists of inflammatory cells, accumulated lipid and a thin fibrous cap and is associated with plaque rupture, thrombosis and subsequent myocardial infarction. The relation between carotid plaque characteristics, plaque embolisation and adverse clinical outcome is unexplored.

In the current prospective study, we focussed on the association between plaque phenotype and microembolic events registered by TCD. In addition, plaque characteristics and the number of microembolic events were related with the occurrence of postoperative adverse ischemic cerebral events. In line with coronary atherothrombosis, we hypothesized that the inflammatory, atheromatous plaque is associated with an increased incidence of microemboli following CEA. The importance of such a finding is actual since the development and increasing resolution of imaging techniques like MRI, ultrasound or multi-slice CT-scan may facilitate non invasive detection of the vulnerable plaque.

Subjects and Methods

Patients

ATHERO-EXPRESS is an ongoing longitudinal study, with the objective to investigate the etiological value of plaque characteristics for long-term outcome. The design of the study has been described previously[19]. The ATHERO-EXPRESS study is currently being executed in two Dutch hospitals: the UMC Utrecht and the St. Antonius Hospital Nieuwegein. In brief, recruitment of patients started in April 2002 and will continue until at least 1000 patients have been enrolled. All consecutive patients who are newly referred to the vascular surgery departments of the participating centres for treatment of carotid artery stenosis are enrolled. Patients may have been symptomatic or asymptomatic. Operation is indicated when colour Doppler assisted duplex investigation reveals a diameter reduction of >70% on at least one side. In asymptomatic patients with stenosis >70%, the indication for surgical therapy depends on co-morbidity and vertebral-basilar insufficiency. Two hundred patients with successful TCD monitoring throughout the entire operation entered this study.

Carotid Endarterectomy

All patients were operated under general anaesthesia. Patients were monitored using TCD and EEG registration. A shunt was selectively used based on EEG and TCD criteria as described in earlier reports[20,21]. Before cross clamping, an intravenous bolus of heparin (5.000 IU) was administered. One hundred and ninety seven patients (99.5%) used anti-platelet (aspirin, plavix, persantin or a combination of these) or coumarin medication preoperative. All endarterectomies were open with carefully dissection of the bifurcation into the internal and external carotid arteries. When the vascular surgeon indicated patch closure, venous patches were preferred. A Dacron patch was only used by lack of venous material.

Atherosclerotic tissue dissection and processing

Following dissection, the atherosclerotic plaque segment was transported without delay to the laboratory. The atherosclerotic plaque was cut in 0.5 cm segments. The culprit lesion was designated as segment 0 and the adjacent segments –1 and +1. The segments –1, +1 and all subsequent numbered segments (-2, +2, -3, etc) were immediately frozen in liquid nitrogen and stored in –80 ˚C. Segment 0 was fixated in formaldehyde 4% and paraffin embedded. Of each segment 15 sections (5 µm) were cut for histological (immuno) stainings. The following stainings were performed to characterise the plaque: Picro-Sirius red (collagen and fat determined using polarised light), CD68 (macrophages), -actin (smooth muscle cells), haematoxylin (thrombus and calcifications) and elastin von Gieson (to identify the internal elastic lamina).

Plaque phenotyping

Two observers independently microscopically scored all stainings semi-quantitatively as described earlier[17].

A plaque is considered unstable when it contains high numbers of macrophages, a large atheroma and when it lacks collagen and smooth muscle cells. The more fibrous stable lesions typically lack inflammatory cells and fat and reveal strong staining for collagen and smooth muscle cells. Plaques were categorized as no/minor staining or moderate/heavy staining for the stains listed below:

- Collagen staining using polarized light microscopy: 1) no or minor staining along part of the luminal border; 2) moderate or heavy staining along the entire luminal border.
- CD68 positive cells: 1) absent or minor staining with negative or few scattered cells; 2) moderate or heavy staining, clusters of cells with >10 cells present.
- Alpha-actin positive cells: 1) no or minor 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

• Haematoxylin: 1) no signs of earlier intra plaque thrombus formation; 2) signs of earlier thrombus formation (fibrin deposition).

The percentage atheroma of the total area of the plaque was visually estimated using the picro Sirius red with polarized light and haematoxylin stains. Two groups were considered based on the percentage of atheroma in the plaque being >40% and <40%[20].

Plaques were also categorized into three groups based on their overall appearance (fibrous, fibro-atheromatous or atheromatous).

TCD monitoring

The methods of TCD monitoring have been reported previously[21,22]. In brief, blood flow velocities were measured in the middle cerebral artery. The probe is affixed to the lateral temporal region.

Doppler signals were recorded and high intensity transient signals indicating microemboli were identified. In the present study the Doppler spectra were observed in the operating room by an experienced sonographer. All microembolic events were counted during 4 different phases during operation: 1- dissection (all microembolic events during skin incision until cross clamping), 2- shunting (if a shunt was used; microemboli which occurred during introduction till removal of the shunt), 3- clamp release (the first 10 seconds after restoration of the flow through the carotid arteries) and 4- wound closure (after first 10 second of flow restoration till end of operation). Microemboli that occurred during release of the clamp and that could not be counted separately during one heartbeat were entitled shower microemboli. A shower of microemboli was given the arbitrary number of 10 microemboli, which is the maximum number of microemboli that may be discriminated during one heartbeat.

Outcome

Patients' hospital records were used to obtain information about clinical outcome. A neurologist was routinely consulted for all patients preoperatively and at the third day following operation. New neurological symptoms or worsening of existing symptoms persisting for >24 hours were regarded as stroke. Stroke was classified based on the modified Rankin Scale[23]. New neurological symptoms persisting <24 hours were regarded as a TIA.

Adverse ischaemic cerebral events were counted when they were diagnosed postoperatively till two weeks after operation.

Table 1
Baseline patient characteristics

Number of patients:	200	
Number of operations:	205	
Gender: male/female	136 (68.7%)	62 (31.3%)
Age: median/range	68 (41 - 86) years	
Smoking: yes/no	49 (29%)	121 (71%)
Diabetes: yes/no	38 (23%)	130 (87%)
Dislipidemia: yes/no	101 (60%)	60 (40%)
Symptoms: sympt/asympt	148 (72%)	57 (28%)
Side operated: left/right	109 (53%)	96 (47%)
Shunt used:	53 (26%)	

Data analysis

Data are presented as mean +/- standard error of the mean (SEM).
We used nonparametric tests for continuous variables (Mann–Whitney test, Wilcoxon signed rank test and Friedman test) and for categorical variables we used Chi-square and Fishers exact test. P values of <0.05 were considered statistically significant.

Table 2
TCD recorded microemboli during different phases of operation

Dissection	
Number of patients with embolic event:	27% (55/205)
Mean (SEM):	1.43 (0.39)
Range:	0 - 60
Sum:	294
Shunt phase	
Number of patients with embolic event:	53% (27/51)
Mean (SEM):	5.16 (1.20)
Range:	0 - 48
Sum:	263
Clamp release	
Number of patients with embolic event:	54% (111/205)
Mean (SEM):	7.27 (1.32)
Range:	0 - 180
Sum:	1482
Total number of patients with showers	24 (91 showers)
Wound closure	
Number of patients with embolic event:	32% (65/205)
Mean (SEM):	1.4 (0.26)
Range:	0 - 28
Sum:	294

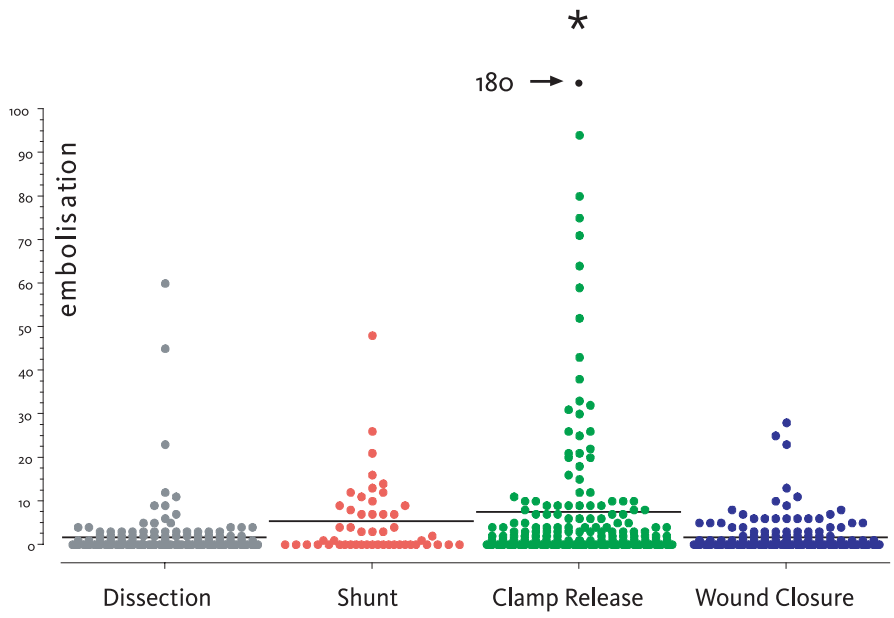

Figure 1: *Embolisation distribution for the different phases of operation; dissection, shunting, clamp release and wound closure. Black bars indicate the mean. * p<0.05*

Results

Table 1 represents the baseline patient characteristics. Table 2 shows the number of TCD registered microemboli for the different operation phases. Significantly more microemboli were recorded during clamp release compared with the other phases of operation (figure 1).

The relation between the presence of microemboli and overall plaque characteristics for the different operation phases is shown in table 3 and figure 2. During clamp release as well as wound closure the presence of microemboli was found to be associated with a fibrous plaque (p=0.04 and p=0.02, table 3). The relation between plaque characteristics and microemboli are presented in table 4. No or minor calcified plaques were related with more microemboli during dissection.

Table 3
Overall plaque phenotype (data mean(SEM)

Fibrous		
% of plaques:		30.4% (61/201)
embolic events (mean SEM):	dissection	0.90 (0.27)
	shunting	6.27 (2.10)
	clamp release	7.00 (1.85)* [1]
	wound closure	2.34 (0.65)* [2]
patients with adverse neurological outcome:		1/61 (1.6%) * [3]
Fibro - atheromatous		
% of plaques:		35.3% (71/201)
embolic events (mean SEM):	dissection	1.13 (0.40)
	shunting	5.56 (2.98)
	clamp release	10.38 (3.19) * [1]
	wound closure	1.30 (0.30) * [2]
patients with adverse neurological outcome:		3/71 (4.3%) * [3]
Atheromatous		
% of plaques:		34.3% (69/201)
embolic events:	dissection	2.30 (1.07)
	shunting	4.00 (1.23)
	clamp release	4.42 (1.39)* [1]
	wound closure	0.51 (0.140* [2]
patients with adverse neurological outcome:		7/69 (10.1%) * [3]

In accordance with overall plaque phenotype, the presence of less than 40% atheroma in the plaque was also associated with significantly more microemboli during wound closure (p=0.02). The number of microemboli was not associated with the presence of macrophages (table 3+4).

Adverse ischemic cerebral events occurred in 2.4% (TIA) and 2.9% (minor stroke) of the patients and were related with significantly more microemboli during dissection (0.92 versus 6.91, p=0.003) but not during clamp release and wound closure. More adverse events occurred in patients with atheromatous plaque (7/69 plaques) compared to patients with fibrous or fibro-atheromatous 4/138 (p=0.04). There were no significant differences in incidence of microemboli among surgeons who performed the operation (not shown).

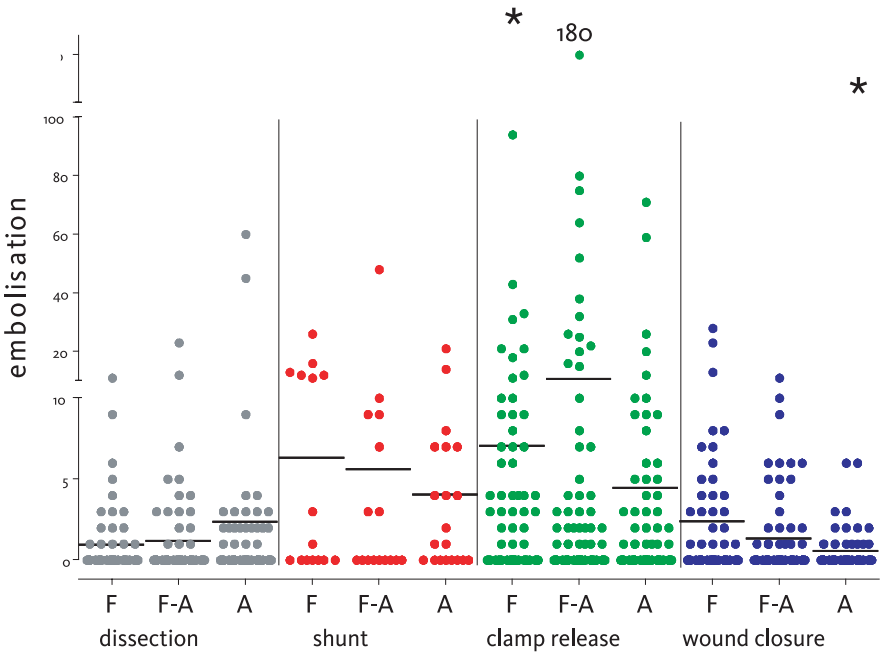

Figure 2: *Embolisation during dissection, shunting, clamp release and wound closure in relation with plaque phenotype. During clamp release and wound closure fibrous plaques have significantly more* microembolisation *then atheromatous plaques (p=0.04 and p=0.02). Black bars indicate mean values. Beware of the y-axis scaling.*

Table 4

Plaque characteristic in relation with embolic events (mean (SEM))

	No/minor Staining	Moderate/heavy Staining	p - value
Dissection			
Percentage atheroma*	1.46 (0.52)	1.44 (0.67)	0.30
Calcification staining	2.30 (0.77)	0.5 7 (0.15)	0.02
Collagen staining	1.67 (0.94)	1.39 (0.45)	0.60
Macrophage staining	1.12 (0.29)	1.73 (0.78)	0.81
SMC staining	2.43 (1.12)	0.96 (0.18)	0.59
Thrombus #	0.53 (0.14)	1.85 (0.59)	0.25
Shunt phase			
Percentage atheroma*	5.67 (1.71)	4.22 (1.36)	0.83
Calcification staining	3.81 (1.18)	6.56 (2.11)	0.42
Collagen staining	2.08 (0.790	6.10 (1.53)	0.30
Macrophage staining	3.59 (0.90)	7.23 (2.40)	0.34
SMC staining	4 .00 (1.25)	5.90 (1.81)	0.86
Thrombus #	1.78 (1.32)	5.88 (1.41)	0.13
Clamp release			
Percentage atheroma*	8.74 (1. 94)	4.74 (1.43)	0.17
Calcification staining	8.42 (2.22)	6.25 (1.52)	0.71
Collagen staining	5.69 (2.29)	7.96 (1.67)	0.50
Macrophage staining	8.10 (1.77)	6.79 (2.14)	0.30
SMC staining	7.23 (2.19)	7.58 (1.77)	0.27
Thrombus #	8.97 (3.37)	6.69 (1.34)	0.97
Wound closure			
Percentage atheroma*	1.81 (0.35)	0.50 (0.15)	0.02
Calcification staining	1.55 (0.37)	1.16 (0.29)	0.91
Collagen staining	0.73 (0.20)	1.59 (0.31)	0.56
Macrophage staining	1.63 (0.42)	1.03 (0.22)	0.32
SMC staining	0.84 (0.27)	1.67 (0.34)	0.10
Thrombus #	1.12 (0.31)	1.47 (0.32)	0.58

*percentage <40% fat or >40% fat

thrombus present no or yes

Discussion

Carotid artery stenosis is a common presentation of atherosclerotic disease[1]. Nine-12% of the patients with known atherosclerotic disease suffer from high-grade carotid artery stenosis[24]. CEA is a widely accepted method to treat patients with carotid artery stenosis. Reduction of peroperative morbidity and mortality could improve long-term outcome. It has been demonstrated that high microembolic rates during CEA are related with adverse neurological outcome[11-13]. In cardiovascular disease the vulnerable inflammatory atheromatous plaque is considered responsible for thrombotic events and subsequent myocardial infarction[15,16,18]. We hypothesized that a vulnerable plaque phenotype could also be associated with the occurrence of microemboli during CEA. Surprisingly, in this study we found a relation between the presence of fibrous plaque and the incidence of microemboli following declamping and wound closure. In

agreement with this finding, plaques containing less then 40% fat were also related with more microemboli during wound closure. There was no relation between emboli and fibrous or fibro- atheromatous plaques during dissection and shunting. In line with our hypothesis, adverse neurological outcome were related with an increase of microembolic events during dissection of the artery. Atheromatous plaque phenotype was more prevalent in patients with an adverse event.

Plaque characteristics

In coronary artery disease a strong relation is described between plaque characteristics and plaque thrombosis; deposition of free cholesterol, macrophage infiltration, enlargement of necrotic core and a thin fibrous cap are features that are related with the instable or vulnerable plaque [15-18]. Surprisingly, we observed an association between microembolic events and stable fibrous plaques instead of inflammatory lipid rich plaque during declamping and wound closure. We must take into consideration that the origin of microemboli likely alters during operation. Before arteriotomy emboli are likely due to plaque debris while during and after arteriotomy gas emboli are likely to occur. In addition, removal of a (fibrous) plaque may expose collagen to flowing blood with formation of fresh thrombi as a result. Just as atheromatous tissue, also collagen is known for its thrombotic capacity[25]. Atherosclerotic plaques with less calcification were associated with an increased number of microemboli during dissection phase. This is in line with previous observations that showed that less calcified plaques have been associated with symptomatic carotid artery stenosis[26].

Outcome

In this study we showed a significant relation between microembolic events during dissection and the occurrence of adverse neurological events. The relationship between microembolic events and adverse neurological outcome has been described by other authors[11,12]. In our study 11 adverse events (TIA and minor stroke) were recorded, a percentage that is comparable with previous studies[1-3]. Although total microemboli rate was not associated with an atheromatous plaque phenotype, the presence of atheromatous plaques was related with adverse neurological outcome. The latter would be in agreement with the idea that this plaque phenotype is related with thromboembolic events and hence with adverse outcome. In the dissection phase no association existed between fibrous plaques and microemboli. In contrast, atheromatous plaques tended to more embolic events in the dissection phase. This suggests that microembolic events during the dissection phase together with the presence of an atheromatous plaque strongly increases the risk for the development of adverse neurological events during and immediately following CEA.

Limitations

Our study is limited by the number of adverse events and by the interpretations of the TCD registered microembolic event. Since TCD is based on ultrasound it is not possible to discriminate between different kinds of embolic events (particle of the plaque, thrombo-emboli, and gaseous emboli) during the operation. We assume that microemboli during dissection are mainly plaque particles and during wound closure are mainly thrombo-embolic while during clamp release embolic events are likely gaseous[12]. For this reason, we divided embolic events during operation in the aforementioned categories.

The duration of TCD registration is another potential limitation since postoperative TCD monitoring has not been performed.

We assumed that following CEA plaque remnants at the edges reflect the same characteristics as the culprit lesion. This assumption is not proven since plaque phenotype can be quite heterogeneous and therefore merits careful consideration. However, the strongest predictive plaque marker for adverse outcomes was observed in the dissection phase when plaques were still in situ.

Conclusions

Fibrous plaques are associated with an increase of micro embolisation during clamp release and wound closure but not with immediate adverse outcome. On the other hand, the presence of an atheromatous plaque together with an embolic event during dissection was related with the occurrence of TIA or minor stroke. The presence of local inflammatory cells was not associated with embolisation or adverse outcome. Imaging modalities capable of visualizing the atheromatous lesion and peroperative embolisation may help to predict the development of adverse neurological events following CEA.

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Closure of the arteriotomy after carotid endarterectomy: patch type is related to per-operative micro-emboli and re-stenosis rate

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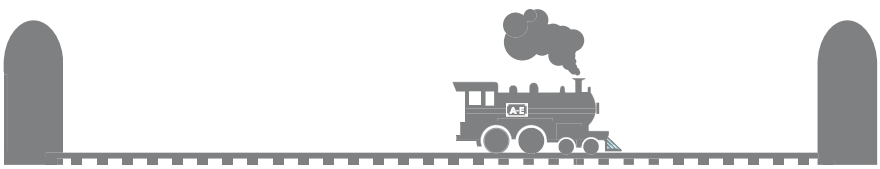

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Abstract

Objective: Patch closure after carotid endarterectomy (CEA) improves clinical outcome compared with primary closure. Whether there are differences in outcome between various patch materials is still not clear. The objective of this retrospective study was to investigate whether a relationship exists between the patch type and the number of microemboli as registered during CEA by transcranial Doppler imaging, the clinical outcome (transient ischemic attack and cerebrovascular accident), and the occurrence of restenosis.

Methods: We included 319 patients who underwent CEA. Intraoperative microembolus registration was performed in 205 procedures. Microembolization was recorded during four different periods: dissection, shunting, clamp release, and wound closure. The decision to perform primary closure or to use a patch for the closure of the arteriotomy was made by the surgeon, and Dacron patches were used when venous material was insufficient. Cerebral events were recorded within the first month after CEA, and duplex scanning was performed at 3 months (n =319) and 1 year (n=166) after CEA. A diameter reduction of more than 70% was defined as restenosis.

Results: Primary, venous, and Dacron patch closures were performed in 83 (26.0%), 171 (53.6%), and 65 (20.4%) patients, respectively. Primary closure was significantly related to sex (Dacron patch, 35 men and 30 women; venous patch, 108 men and 63 women; primary closure, 72 men and 11 women; $P < .001$). The occurrence of microemboli during wound closure was also related to sex (women, 2.5 ± 0.6; men, 1.0 ± 0.2; $P = .01$). Additionally, during clamp release, Dacron patches were associated with significantly more microemboli than venous patches (11.1 ± 3.4 vs 4.0 ± 0.9; $P < .01$), and this difference was also noted during wound closure (3.1 ± 0.9 vs 1.4 ± 0.4; $P < .05$). Transient ischemic attacks and minor strokes after CEA occurred in 5 (2.4%) of 205 and 6 (2.9%) of 205 procedures, respectively, and the degree of microembolization during dissection was related to adverse cerebral events ($P = .003$). In contrast, the type of closure was not related to immediate clinical adverse events. However, primary closure and Dacron patches were associated with an increase in the restenosis rate compared with venous patches: after 400 days, the restenosis rate for Primary closure was 11%, Dacron patch 16%, and venous patch 7% ($P = .05$; Kaplan-Meier estimates).

Conclusions: Microemboli are more prevalent during clamp releases and wound closure when Dacron patches are used. Additionally, the observed differences in embolization noted by patch type were mainly evident in women. However, the use of Dacron patches was not related to immediate ischemic cerebral events but was associated with a higher restenosis rate compared with venous patch closure. This

suggests that venous patch closure may be preferred for CEA.

Introduction

A recent systematic review concerning the type of arteriotomy closure in carotid endarterectomy (CEA) showed the benefits of patching over primary closure[1]. However, sufficient data are lacking to allow firm conclusions to be drawn regarding differences between various patch materials used for carotid closure. Synthetic patches are believed to be more thrombogenic than venous patches and may therefore theoretically produce emboli that could result in associated adverse cerebral events. During CEA, transcranial Doppler (TCD) registration of the middle cerebral artery provides online surveillance of hemodynamic changes and the passage of cerebral microemboli[2-4] and the occurrence of microemboli, recorded by TCD during CEA, has been related to the risk of immediate adverse cerebral events[3,5,6] . Therefore, different patch materials could influence the outcome of the operation as a result of differences in the number of microemboli[7]. Furthermore, several studies suggest that the type of patch used for closure of the carotid artery is also associated with late restenosis[1,7-12]. The objective of this study was to investigate whether a relationship exists between the type of patch used, the number of TCD-detected microemboli during CEA, immediate adverse cerebral events, and restenosis.

Methods

Patients

This study is part of ATHERO-EXPRESS, which is an ongoing prospective longitudinal study with characteristics in long-term outcome in patients with carotid atherosclerosis. The design of the study has been described previously[13] and ATHERO-EXPRESS is currently being executed in two Dutch hospitals. Recruitment of patients started in April 2002 and will continue until at least 1000 patients have been included. All patients receiving operative treatment for carotid stenosis in the vascular surgery departments of the participating centers are enrolled. Patients can be symptomatic or asymptomatic, and surgery is indicated when color Doppler–assisted duplex ultrasound investigation, magnetic resonance angiography, computed tomographic angiography, or angiography reveals a diameter reduction of more than 70% on at least one side. In asymptomatic patients with stenosis greater than 70%, the indication for surgery is also based on recommendations published by the Asymptomatic Carotid Surgery Trial[14]. At baseline, clinical data from patients' records, a questionnaire about medical history, blood samples, and atherosclerotic tissue harvested during CEA are collected. In this study, we analyzed 319 patients with 3 months and 1 year (n = 166) of follow-up after CEA.

Carotid endarterectomy

Preoperative antiplatelet therapy was continued during the operation. All patients underwent operation under general anesthesia, and TCD and electroencephalographic monitoring were used. Shunting was performed selectively on the basis of electroencephalogram and TCD criteria, as described in previous articles[15,16]. Before cross clamping, a bolus of heparin (5000 IU) was given intravenously. All endarterectomies were performed open, with dissection of the bifurcation into the internal and external carotid arteries. Patch closure was generally the preferred technique, especially when the lumen of the internal carotid artery was less than 3 mm or when a shunt had to be used. Venous patches were preferred and were usually obtained from the saphenous vein at the ankle or inguinal level when the vascular surgeon favored patch closure. A Dacron patch (Intervascular; DuPont, Wilmington, Del) was used only when venous material was not available or when the venous material harvested was perceived to be of insufficient strength. The medical ethics committees of the participating hospitals approved the study.

TCD monitoring

Because of logistic reasons, it was not always possible to record emboli to the highest standard during TCD recording. Therefore, we reported the TCD registrations of 205 of 319 patients. The methods of TCD monitoring have been reported previously[15,16]. Briefly, the Doppler spectra were observed online in the operating room by an experienced sonographer. Doppler signals were recorded, and high-intensity transient signals indicating microemboli were identified. All microembolic events were counted and recorded during four different phases of the surgical procedure: (1) dissection (all microembolic events from skin incision until cross clamping), (2) shunting (if a shunt was used; microemboli that occurred from the introduction to the removal of the shunt), (3) clamp release (the first 10 seconds after restoration of the flow through the carotid arteries), and (4) wound closure (after 10 seconds of flow restoration until the end of the operation). Observed microemboli that could not be counted separately during one heartbeat were entitled *shower microemboli*. A shower of microemboli was given the arbitrary number of 10 microemboli, which is the maximum number of microemboli that can be discriminated during 1 heartbeat.

Clinical events

Patients' hospital records were reviewed to obtain information concerning clinical events. A neurologist was routinely consulted for all patients before surgery and at the third day after operation. New neurologic symptoms or worsening of existing

symptoms that persisted for longer than 24 hours was regarded as a stroke, and strokes were classified according to the modified Rankin Scale[17]. New neurologic symptoms persisting less than 24 hours were regarded as transient ischemic attacks. Neurologic events were termed *immediate adverse ischemic cerebral events* when they were diagnosed during the postoperative period or when a readmission occurred for this reason within 1 month after the operation.

Restenosis and duplex criteria

Determination of the degree of recurrent stenosis during follow-up was based on duplex ultrasonographic[18] follow-up at 3 months (n = 319) and 1 or 2 years (n =166) after CEA. Restenosis was defined as greater than 70% stenosis of the endarterectomy area. Duplex criteria for restenosis are a combination of peak systolic velocity greater than 125 cm/s and a gamma (the ratio between peak systolic velocity in the stenotic area and end diastolic velocity in distal common carotid artery) greater than 12.

Table 1 Baseline patient characteristics

Number and %	Dacron Patch	Vein Patch	Primary Closure	Overall	p-value
Gender: male	54% (35/65)	63%(108/171)	87% (72/83)	215/319	<0.001
Age: median/range	67 (45 -87)	66 (40 -84)	70 (55 -85)	68 (40 -87)	0.004
Current smoker: (yes)	18% (10/57)	32% (51/157)	18% (14/80)	75/294	0.013
Diabetes: (yes)	18% (10/57)	19% (29/155)	19% (15/78)	54/290	0.9
Dislipidem ia: (yes)	65% (36/55)19	63% (97/154)	55% (42/76)	175/285227	0.4
Symptoms: (sympt)	70% (42/60)	76% 126/165	73% (59/81)	/306	0.6
Side operated: (left)	46% (30/65)	53% (91/171)	57% (47/83)	168/319	0.4
Body mass index (mean)	27	26	27	26	0.8
Myocardial infarction ever (Yes)	15% (9/59)	18% (28/153)	28% (2 2/80)	59/292	0.14
Statin use (Yes)	72% (46/64)	66% (113/170)	61% (51/83)	210/317	0.4
Angina pectoris ever? (Yes)	33% (21/63)	40% (64/162)	41% (33/81)	118/306	0.6
High blood pressure (Yes)	66% (39/59)	67% (102/152)	64% (49/77)	190/288	0.9
CABG or coronary stent in the past	17% (11/63)	20% (33/167)	27% (22/82)	66/312	0.3
Peripheral vascular intervention in the past (Pta/ stent or bypass)	29% (18/63)	19% (31/167)	18% (15/82)	64/312	0.4
Anti -platelet or anticoagulant therapy	95%(58/61)	92% (147/160)	92% (73/79)	278/300	0.7

Data analysis

Microembolic data in tables are presented as mean ± SEM. We used nonparametric tests for continuous variables (Mann-Whitney test and Kruskal- Wallis test) and χ^2 and Fisher exact tests for categorical variables. When significant differences were found with the Kruskal-Wallis test, the Dunn post hoc test was applied for their possible relationship with microemboli. Kaplan-Meier survival tables were used to assess

differences in restenosis rates among groups over time. Significance was calculated with the log-rank test. All variables as displayed in the baseline table were tested for their possible relationship with restenosis and microemboli. P values of ≤.05 were considered statistically significant.

Table 2 Patch and TCD detected microemboli

Phase of CEA	Dacron	Vein	Primary closure	p-value: Dacron vs vein	p-value: Primary vs vein	p-value: Dacron vs primary
Clamp release						
All patients	11.1 (3.4)	4.0 (0.9)	9.8 (3.5)	<0.01	n.s.	<0.05
Male	9.8 (4.2)	4.7 (1.3)	9.9 (4.1)	n.s.	n.s.	n.s.
Female	13.7 (6.0)	2.8 (0.8)	9.1 (5.7)	<0.01	n.s.	n.s.
Wound closure						
All patients	3.1 (0.9)	1.4 (0.4)	0.7 (0.2)	<0.05	n.s.	<0.01
Male	1.3 (0.5)*	1.0 (0.4)	0.8 (0.3)	n.s.	n.s.	0.08
Female	6.7 (2.1)	2.0 (0.7)	0.2 (0.1)	<0.05	n.s.	<0.01

Data are presented as mean and (SEM)

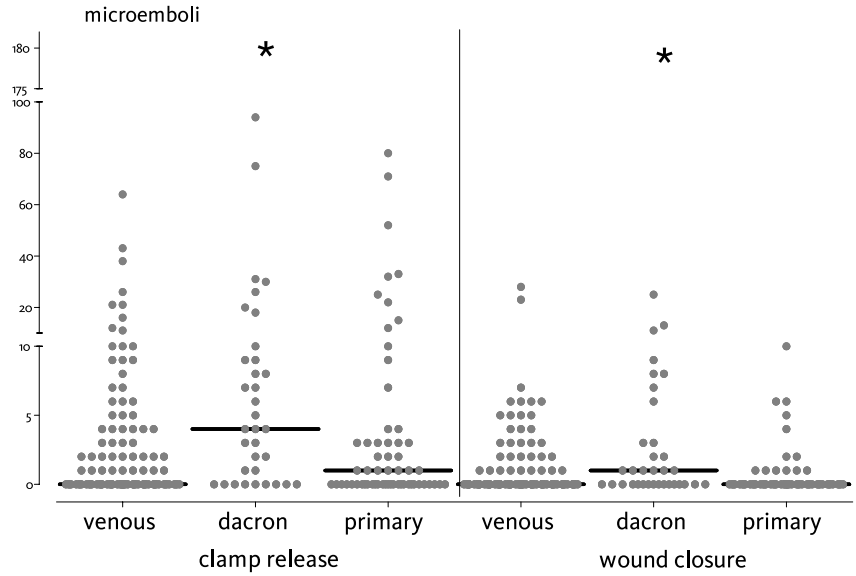


Figure 1: *Relation between microemboli recorded during clamp release and wound closure for the different kind of patches and primary closure. Dacron patch is related with an increased number of microemboli during clamp release and wound closure. Bars indicate median (for venous median=0 and also for no patch wound closure). Beware of the y-axis scaling.*

Table 3 Gender and microemboli			
Phase of CEA	Male	Female	P-value
Dissection	1.4 (0.5)	0.8 (0.2)	0.87
Shunting	3.9 (0.9)	7.1 (3.2)	0.60
Clamp release	7.7 (1.8)	6.2 (1.6)	0.76
Wound closure	1.0 (0.2)	2.5 (0.6)	0.01
Data are presented as mean and (SEM)			

Results

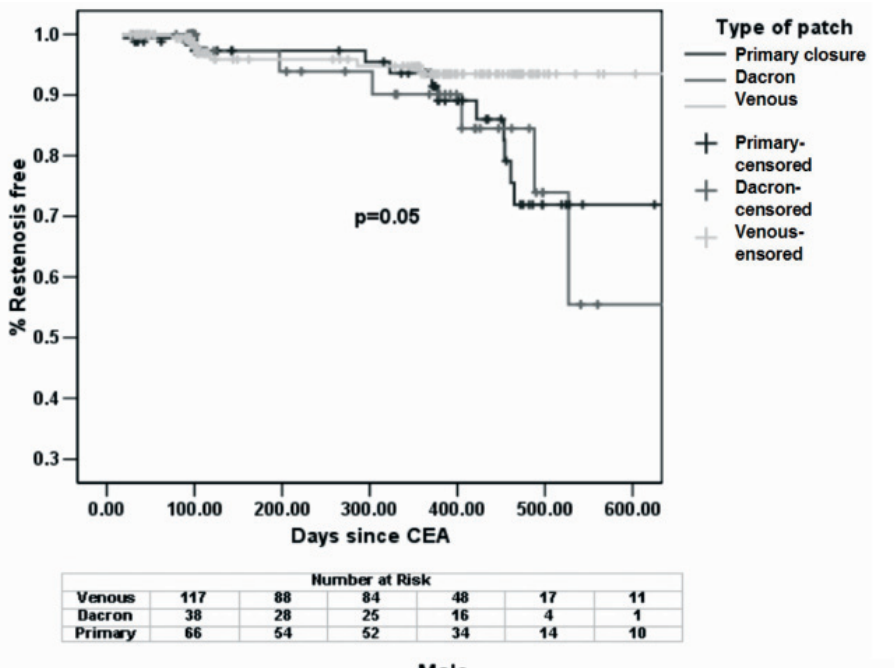
Primary, venous, and Dacron patch closures were performed in 83 (26.0%), 171 (53.6%), and 65 (20.4%) patients, respectively. Table I presents the baseline patient characteristics. Sex, smoking habits, and age differed significantly among groups. Additionally, closure type was related to sex; primary closure was significantly less common in women (Dacron patch, 35 men and 30 women; venous patch, 108 men and 63 women; and primary closure, 72 men and 11 women; $P < .001$).

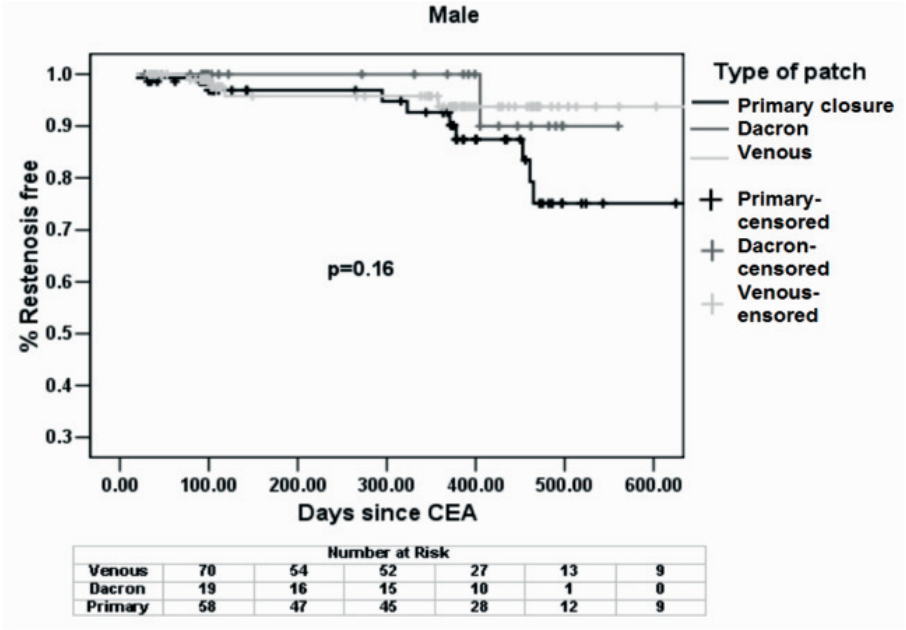
Dacron patch use was associated with significantly more microemboli compared with venous patches and primary closure during the clamp-release phase and the wound-closure phase of CEA (Table II and Fig 1). To exclude confounding of smoking, age, and sex, the analyses were repeated for these variables. Smoking and age were not related to an increased number of microemboli during clamp release and wound closure ($P = .4/P = .9$ and $P = .9/P = .8$). In contrast, women had more microemboli observed during wound closure ($P = .01$; Table III).

Additionally, the significant relationship between the type of patch used and the presence of microemboli was most evident for women (Table II), so that in women, there was clearly an increased number of recorded microemboli during clamp release and wound closure when Dacron patches were used. No such relationship was seen in men.

Table 4 Microemboli in patients with and without adverse events < 2 weeks			
Phase of CEA	Stroke and TIA	No event	p-value
Dissection	6.9 (5.3)	1.1 (0.3)	0.003
Shunting	4.4 (1.8)	5.3 (1.3)	0.8
Clamp release	15.1 (9.0)	6.6 (1.3)	0.9
Wound closure	1.3 (0.9)	1.5 (0.30	0.9
Data are presented as mean and (SEM)			

Adverse ischemic cerebral events (<1 month after CEA) occurred in 2.4% (transient ischemic attack) and 2.9% (minor strokes) of cases. These events were significantly associated with microemboli during dissection but not during shunting, clamp release, and wound closure (Table IV; $P = .003$). Furthermore, a relationship between patch use and immediate clinical adverse events was not observed (Dacron patch, two events; vein patch, four events; and primary closure, five events; $P = .5$). Dacron patches were associated with an increased restenosis rate compared with venous patches: after 400 days, Dacron patch 16%, primary closure 11%, and venous patch 7% (log-rank test; $P = .05$; Fig 2). To exclude confounding for smoking, age, and sex, analyses of these results were repeated. Restenosis was equally distributed among smokers and nonsmokers and categorized age groups. In contrast, women had an increased rate of restenosis when a Dacron patch or primary closure was used (compared with vein patching), whereas men showed an increased restenosis rate for primary closure (nonsignificant for women or men separately; log-rank test; $P = .12$ and $P = .16$; significant for men compared with women; $P=.02$). All patients but one who developed restenosis were asymptomatic. Four of the patients with restenosis were treated with carotid artery stent placement (including the symptomatic patient), and one patient underwent CEA.





Discussion

Carotid artery stenosis is a common presentation of atherosclerotic disease[19]. A total of 9% to 12% of patients with known atherosclerotic disease have high-grade carotid artery stenosis[20]. CEA is a widely accepted method of treating patients with significant carotid artery stenosis, and prevention of perioperative adverse cerebral outcome and reduction of restenosis could improve long-term results after CEA. Direct outcome of CEA is related to preoperative, intraoperative, and postoperative recorded microemboli[5,21,22]. Additionally, as demonstrated here, microemboli during CEA occur variably throughout the procedure and are related to sex and the type of closure used for carotid reconstruction. Because TCD is based on ultrasonography, it is not possible to discriminate among different kinds of emboli (particles of the plaque, thromboemboli, and gaseous emboli). We assume that microemboli during dissection are mainly plaque particles and are thus associated with plaque characteristics. In contrast, during wound closure, microemboli are likely mainly thromboembolic, whereas during clamp release, microemboli are likely to be gaseous[5]. For this reason, we divided embolic events during CEA into the aforementioned categories.

The main findings of this study are as follows: (1) Dacron patches are associated with significantly more microemboli during clamp release and wound closure compared with venous patch closure and primary closure, especially in women; (2) there is a relationship between female sex and patch use, as well as with embolization

and restenosis; (3) the different types of arterial closure were not associated with differences in adverse cerebral events before 1 month; (4) adverse cerebral events were associated with more microemboli during the dissection phase but not during shunting, clamp release, or wound closure; and (5) venous patch use was associated with a decreased long-term restenosis rate.

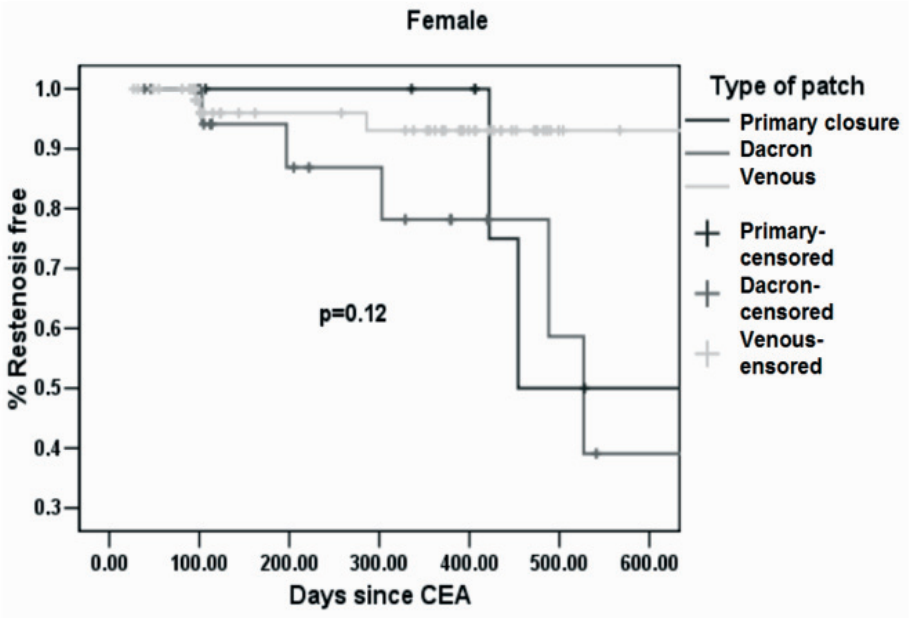


Figure 2: *Restenosis free survival in relation to closure technique used during carotid endarterectomy (CEA). Kaplan-Meier curves and estimates are presented for males and females combined (A), for males (B), and for females separately (C).*

Embolism is the principal cause of cerebrovascular complications from CEA[2]. Prevention of embolic events should contribute to a decrease in adverse cerebral events. Not only emboli occurring during dissection of the atherosclerotic plaque are associated with an increased risk of cerebral events; high numbers of emboli recorded after surgery and during closure of the vessel are also related with adverse outcome[5,6]. The occurrence of emboli may be influenced by the closure technique used, so that the closure technique with less risk of embolism should be preferable. Probably because of the dividing of microemboli in the aforementioned categories, we were able to relate microemboli during wound closure and clamp release to Dacron patches. This relationship was suggested by other authors, but until now no statistical differences have been observed between the different kinds of patch materials and the occurrence of microemboli[7]. The newly described relationship between Dacron patching and the

occurrence of microemboli could be explained by the thrombogenic characteristics of this material, as described in earlier studies[23,24]. However, neither these studies nor the current one provides a final answer to the question of whether the cause is mainly thromboembolic or gaseous or a combination of both.

Women have been shown to have an increased number of microemboli recorded after CEA[6,25]. Hayes et al[25] related this increased number of microemboli to the increased risk for stroke in women after CEA, as published by the European Carotid Surgery Trial and the North American Symptomatic Carotid Endarterectomy Trial Collaborators[26,27]. Our findings strengthen this hypothesis. The increase of microemboli in female patients might be due to an increased thromboembolic potential in women; this could be related to the influence of estrogen and progestin. The influence of these hormones on the risk for venous thrombosis has been documented[28]. Reiner et al[29] also showed an increased risk for stroke in young women with genetic variants of platelet glycoprotein receptors. We did not observe a relationship between immediate ischemic cerebral events and the type of patch being used. A recent review[1] suggested such a relationship; therefore, the small number of patients with adverse outcomes in our study could explain our findings.

In contrast, we did find a relationship between ischemic events and an increased number of microemboli during the dissection phase of the operation. This was also described in a previous article, but that study also showed a positive relationship between emboli during wound closure and outcome, and we were unable to reproduce this result in the current study[5]. This may also have been caused by the small number of patients with adverse outcomes in our study. Primary closure and Dacron patch closure were associated with an increased restenosis rate, and sex seemed to modify this outcome strongly. This difference between the sexes might be important and could influence conclusions based on three recently published studies, in which Dacron patch closure was recommended compared with primary closure, but in which sex was not taken into account[9,30,31]. The increased percentage of restenosis for Dacron patches and primary closures could probably be explained by different kinds of arterial remodeling and intimal hyperplasia. In the literature, two types of remodeling are described: inward remodeling, with a consistent outer diameter of the vessel and increasing neointima leading to restenosis, and outward remodeling, with an increase of outer diameter and an increase of neointima but a consistent luminal area[32]. The difference between restenosis after primary closure and venous patching is probably based on the difference in luminal area and, thereby, differences in remodeling mechanisms. The difference in restenosis rates between Dacron patches and venous patches could be explained by the fact that prosthetic grafts lack endothelium. These prosthetic grafts are covered with neointima consisting of

fibroblasts and fibrous matrix, which is known to induce intima hyperplasia. Finally, the luminal areas between men and women are also different, and this may contribute to the difference in restenosis rates. The consequences of restenosis after CEA are, however, still uncertain.

Limitations

Because this study was retrospective and randomization between patch materials was not performed, it is conceivable that there are differences in patient characteristics among the groups and that a selection bias led to the use of Dacron patches in patients with more severe disease. However, we studied these characteristics and could not identify such a difference. Additionally, the study was limited by the number of patients with TCD registration in which microemboli were divided into the different phases of CEA, as described previously in this article, and this study did not deal with post-CEA microembolic recordings. Finally, our long-term follow-up was limited. However, the patients will be monitored, and data will become available in the future.

Conclusions

On the basis of our results, avoiding Dacron patches used for closure of the CEA is defendable.Not only was an increased number of microemboli related to Dacron patches, but restenosis was related to Dacron patch closure as well. Thus, venous patch closure and primary closure (in men) may be the preferred techniques.

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Low Nogo B Expression Levels in Human Carotid **Athero**sclerotic Plaques are Associated with **Athero**matous Phenotype and Stenotic Lesions

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Submitted

Abstract

Background: Reticulon-4/Nogo (Nogo-B) is an endogenous inhibitor of smooth muscle cell (SMC) migration and intimal thickening in injured mouse arteries. However, its expression pattern in human normal and atherosclerotic lesions is unknown. In the present study, we determined Nogo-B expression in atherosclerotic and non atherosclerotic arterial tissues. In addition, we studied the relationship between Nogo-B expression levels and carotid atherosclerotic plaque phenotype.

Methods and Results: Nogo-B expression was measured by semiquantitative Western blotting of human atherosclerotic plaques harvested from carotid endarterectomy (CEA; n=145), femoral endarterectomy (FEA; n=19) and coronary arteries (CA; n=6). Mammary arteries were used as control (n=8). Culprit lesions of CEA samples were stained and semi-quantitatively scored for the presence of smooth muscle cells, macrophages, collagen, calcifications and fat. Overall, atherosclerotic samples showed significantly lower Nogo-B expression levels compared to control arteries (CEA= 16.4 ± 13.6%, FEA= 11.1 ± 10.4% and CA= 25 ± 14.1% compared to mammary arteries (100%); P<0.003). Immunohistochemical analysis revealed that Nogo-B staining was mainly evident in macrophage/foam cell rich lesions while SMC rich areas revealed low degree of staining. Atheromatous plaques (> 40% fat) showed a significant reduction in Nogo-B expression compared to control (p=0.002). Low Nogo-B expression levels were associated with a high degree of stenosis (>90%) (p=0.04) and restenotic lesions after prior CEA (p=0.01).

Conclusions: Here we identify for the first time Nogo-B in human atherosclerotic lesions. Our data support the concept that down-regulation of Nogo-B in atherosclerotic tissue might contribute to plaque formation and/or instability triggering luminal narrowing.

Introduction

Arterial lumen loss (stenosis) following injury is determined by geometrical arterial remodeling[1] , intimal thickening and/or atherosclerotic plaque formation[2;3]. Carotid stenosis is a typical manifestation of atherosclerotic disease that affects about 9-12 % of patients[4]. Atherosclerotic lesions are caused by smooth muscle cell proliferation and migration, enhanced infiltration of lipids and inflammatory cells and extracellular matrix accumulation[5]. Vulnerable rupture prone plaques are associated with a high inflammatory component, large lipid cores and elevated proteolytic activity[6;7]. Upon rupture of the fibrous cap, the lipid core serves as a substrate for thrombus formation triggering arterial occlusion and subsequently acute coronary syndromes or cerebrovascular events[8]. Our current knowledge of mechanisms underlying plaque growth, destabilization and rupture remains incomplete. Therefore, identification of novel players in plaque formation and progression will contribute to a better understanding of atherosclerotic disease.

Recent findings sustain the idea that a member of the reticulon familiy of proteins, reticulon 4-B, also known as Nogo-B, protects mouse arteries from lumen loss after arterial injury[9]. Acevedo et al. showed that human endothelial cells (ECs), smooth muscle cells (SMCs) and healthy murine arteries contain high levels of Nogo-B[9] and Nogo-B expression rapidly decreases following arterial injury. Consistently, Nogo A/B deficient mice exhibited an accelerated neointima formation upon femoral artery injury[9]. Functional studies revealed that the N-terminus region of Nogo-B promotes ECs adhesion and migration and blocks platelet derived growth factor -induced migration in SMCs[9] suggesting that Nogo-B serves as positive regulator of endothelial cell functions and a negative regulator of smooth muscle cell migration.

The presence or potential roles of Nogo-B have not been described in normal human blood vessels or during atherosclerotic disease. Considering the afore mentioned report on the protective role of Nogo-B in arterial occlusive disease in mice, we hypothesized that Nogo-B expression may be decreased in human atherosclerotic tissue and associated with an unstable plaque phenotype.

Here we show that Nogo-B expression levels are significantly reduced in atherosclerotic lesions compared to non-atherosclerotic tissues. This attenuation of Nogo-B expression was more evident in plaques with an atheromatous (vulnerable) phenotype. Lower levels of Nogo-B in carotid plaques were associated with a high degree of carotid stenosis. This study provides supportive evidence for a protective role of Nogo-B in human atherosclerotic lesion formation and suggests that the loss of Nogo-B during atherogenesis may be a potential biomarker for disease progression.

Methods

Subjects

Patients were enrolled in the ongoing longitudinal study ATHERO-EXPRESS[10]. The study has been approved by the ethical committees of the participating hospitals in accordance with institutional guidelines. Patients filled out questionnaires covering (among others) history of cardiovascular disease, cardiovascular risk factors, physical activity, family history of vascular disease and medication. Demographic data and risk factors of the population studied are depicted in Table1.

	N	%
Number of patients	145	
Age, y (sd)	69.1 (8.3)	
Female	47	32%
Hypertension	91	63%
Diabetes	26	18%
Prior history of:		
PTCA/CABG	35	24%
Peripheral vascular operation	32	22%
Prior carotid intervention	7	5%
Current smoker	39	27%
Smoking in the past	85	57%
Never smoked	15	10%
Hypercholesterolemia	87	60%
Cholesterol, mmol/l (sd)	4.98 (1.04)	
HDL cholesterol, mmol/l (sd)	1.17 (0.37)	
LDL cholesterol, mmol/l (sd)	2.91 (0.89)	
triglycerids, mmol/l (sd)	2.15 (1.16)	
Statin use	100	68%
Duplex stenosis		
50% - 64%	1	0.6%
65% - 89%	45	31%
90% - 99%	99	68%
Restenosis after prior CEA	10	7%

Table 1: *Baseline characteristics of subjects undergoing carotid endarterectomy*

Tissue sampling

Human mammary arteries (n=8) were surgically obtained from patients scheduled for coronary bypass surgery. Human coronary arteries (n=6) were obtained from elderly subjects at autopsy who did not die of any cardiovascular event. Femoral (n=19) and carotid atherosclerotic plaques (n=145) were obtained by endarterectomy. Carotid endarterectomy (CEA) was performed under general anesthesia by an open, non-eversion technique. Atherosclerotic plaques were dissected at the bifurcation into the internal and external carotid arteries. Immediately after dissection the atherosclerotic plaque was transported to the laboratory. Atherosclerotic segment was dissected in parts of 0.5 cm. The culprit lesion was fixated in 4% formalin solution and embedded in paraffin for plaque characterization. Adjacent segments were immediately frozen in liquid nitrogen and subsequently processed for protein isolation.

Carotid Stenosis and Duplex Criteria

The degree of luminal stenosis was based on duplex ultrasonography prior to intervention. Duplex criteria for stenosis were a combination of peak systolic velocity greater than 125 cm/s and a gamma (the ratio between peak systolic velocities in the stenotic area and end diastolic velocity in distal common carotid artery) greater than 12.

Characterization of Atherosclerotic Lesions

Plaques stainings were categorized as follows:

- Picro Sirius red; collagen staining using polarized light microscopy:
  1) no or minor staining = staining along part of the luminal border;
  2) moderate or heavy staining = staining along the entire luminal border.
- CD68 positive cells: 1) absent or minor staining = negative or few scattered positive cells; 2) moderate or heavy staining = stained clusters of cells with >10 cells present.
- Smooth muscle cells alpha-actin positive area: 1) no or minor staining = discontinuous over the entire circumference with absent staining at parts of the circumference of the arterial wall; 2) positive cells along the entire circumference of the luminal border, with at least minor staining locally with few scattered cells
- Hematoxylin and elastin staining were used to assess overall morphology.

The percentage of atheroma of the total area of the plaque was visually estimated using the picro Sirius red with polarized light and hematoxylin stainings. Three groups were defined based on the percentage of atheroma in the plaque: fibrous plaques <10% fat, fibro-atheromatous 10-40% or atheromatous > 40% fat.

Protein Isolation

Proteins were isolated from atherosclerotic segments adjacent to the paraffin embedded segment that was used for immunohistochemistry. Total proteins were extracted from samples using Tri-Pure™ Isolation Reagent (Roche) according to the manufacturer's protocol. Total protein concentration was determined using the Bio-Rad DC protein assay.

Determination of MMP activity

MMP-2, MMP-8 and MMP-9 activities were measured using Biotrack activity assays RPN 2617, RPN 2635 and RPN 2634, respectively (Amersham Biosciences, Buckinghamshire, UK).

Immunohistochemistry

Serial cross-sections (5 m) from human mammary arteries, human coronary arteries and human carotid endarterectomies samples were deparaffinized and rehydrated. After blocking with 10% (v/v) normal rabbit serum, sections were incubated overnight at 4 °C with goat anti-human Nogo-B (N-18) (Santa Cruz Biotechnology Inc, California) (2 g/ml) diluted in PBS containing 1% BSA. After washing, sections were incubated with biotinylated rabbit anti-goat (DAKO, Denmark) (1.6 g/mL). Sections were incubated with horseradish peroxidase (HRP) labeled streptavidin (Vector) (1 g/ml). Color was developed using diaminobenzidine as substrate for 10 minutes. Counterstaining was performed using Mayer's hematoxylin. Non-immune negative controls were obtained avoiding the primary antibody. Endothelial cells were identified by staining with antibody against CD31, macrophages with an antibody against CD68 and smooth muscle cells with an antibody against alpha actin.

SDS-PAGE and Western Blotting

Equal amounts (10 g) of tissue samples were boiled in β-mercaptoethanol containing buffer for 5 minutes, separated on 10% polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Shuell Dasel, Germany). Equal protein loading was confirmed using Ponceau Red S staining. The membranes were blocked overnight and incubated with goat anti-human Nogo-B (N-18) (0.2 g/ml) for 1 hour at RT in PBS containing 5% non-fat dry milk /0.1% Tween-20. After several washing steps, membranes were incubated rabbit HRP-anti-goat IgG (Sigma, St Louis) (1:80000). Signal was detected by enhanced chemiluminescence. Thereafter, membranes were re-probed with mouse anti-human beta actin antibodies (Abcam) (1 g/ml) and Rabbit anti-mouse antibody (DAKO) was used as secondary antibody.

Cell lysates from neuroblastoma SH-Y5Y and extracts from pooled human mammary arteries (n=6) were used as positive controls for Nogo-B in all blots. In every gel, expression levels of Nogo-B in pooled mammary arteries (n=6) were considered as 100. Nogo-B expression levels in atherosclerotic tissues were calculated as percentages relative to control.

Data Management and Statistic

Statistical analysis was performed by the Mann–Whitney U and the Kruskal Wallis tests. Values of Nogo-B expression are presented as mean ± SEM. P values of <0.05 were considered statistically significant.

Results

Identification of Nogo-B in Human Atherosclerotic Lesions

We first studied the presence of Nogo-B in atherosclerotic tissues harvested from different vascular beds and non-atherosclerotic arteries. Nogo-B expression levels were determined by semiquantitive Western blotting in human mammary arteries (controls) (n=8), atherosclerotic human coronary arteries (n=6) and endarterectomy specimens from the femoral (FEA; n=19) and carotid arteries (CEA; n=145) (Figure 1). The Nogo-A/B antibody, N-18, detected two bands with relative molecular weights between 55KD and 50 KD indicating the existence of two variants of Nogo-B (Nogo-B1 and B2) (Figure1A) but did not detect a higher molecular weight form of the Nogo-A isoform (220 KDa), consistent with previous work in murine blood vessels (Acevedo et al). However, Nogo-A is expressed in SHY-5Y cell lysates (not shown). Western blotting cellular extracts from atherosclerotic lesions showed a significant reduction in the expression of both variants of Nogo-B compared to control mammary arteries (CEA= 16.4 ± 13.6 %, FEA= 11.1 ± 10.4 % and CA= 25 ± 14.1% of control, Figure 1B) and compared with the levels of a control for protein loading β-actin (Figure 1A).

Next, we examined the localization of Nogo-B in human arteries using immunohistochemistry, and found that Nogo-B immunoreactivity is present in both control mammary arteries and human atherosclerotic lesions (Figure 2). In mammary arteries, Nogo-B staining was mainly distributed throughout the endothelium and media with minor staining in the adventitia (Figure 2 A). In carotid plaques, Nogo-B was found in SMC-alpha-actin rich areas (Figure 2 E and F), as well as macrophages-CD68 positive areas (Figure 2 C and D). Moreover, endothelial cells and the surrounding tissue of the vasa vasorum showed positive staining for Nogo-B (Figure 2 G and H). No staining was observed in non-immune controls (Figure 2B).

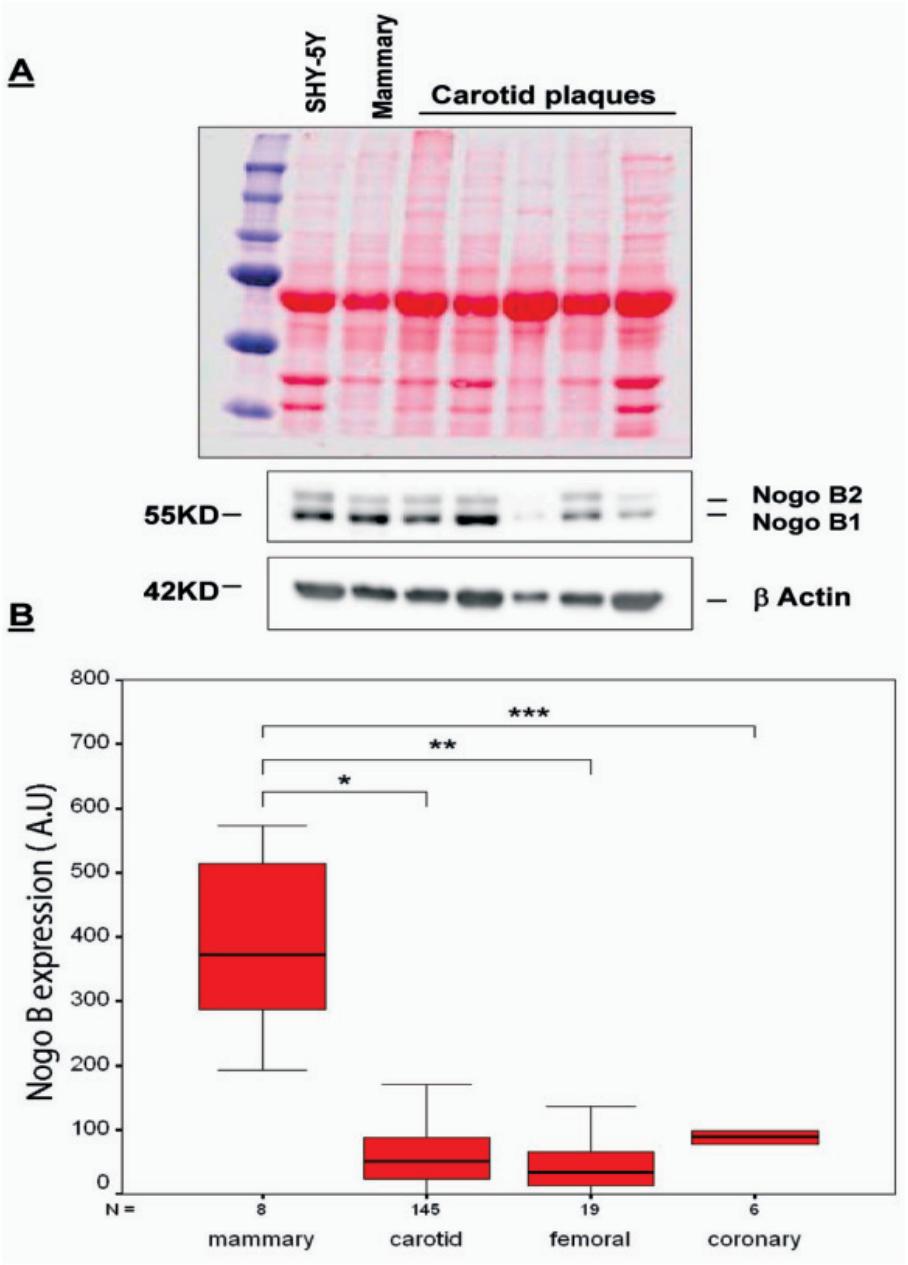

Figure 1

Figure 1

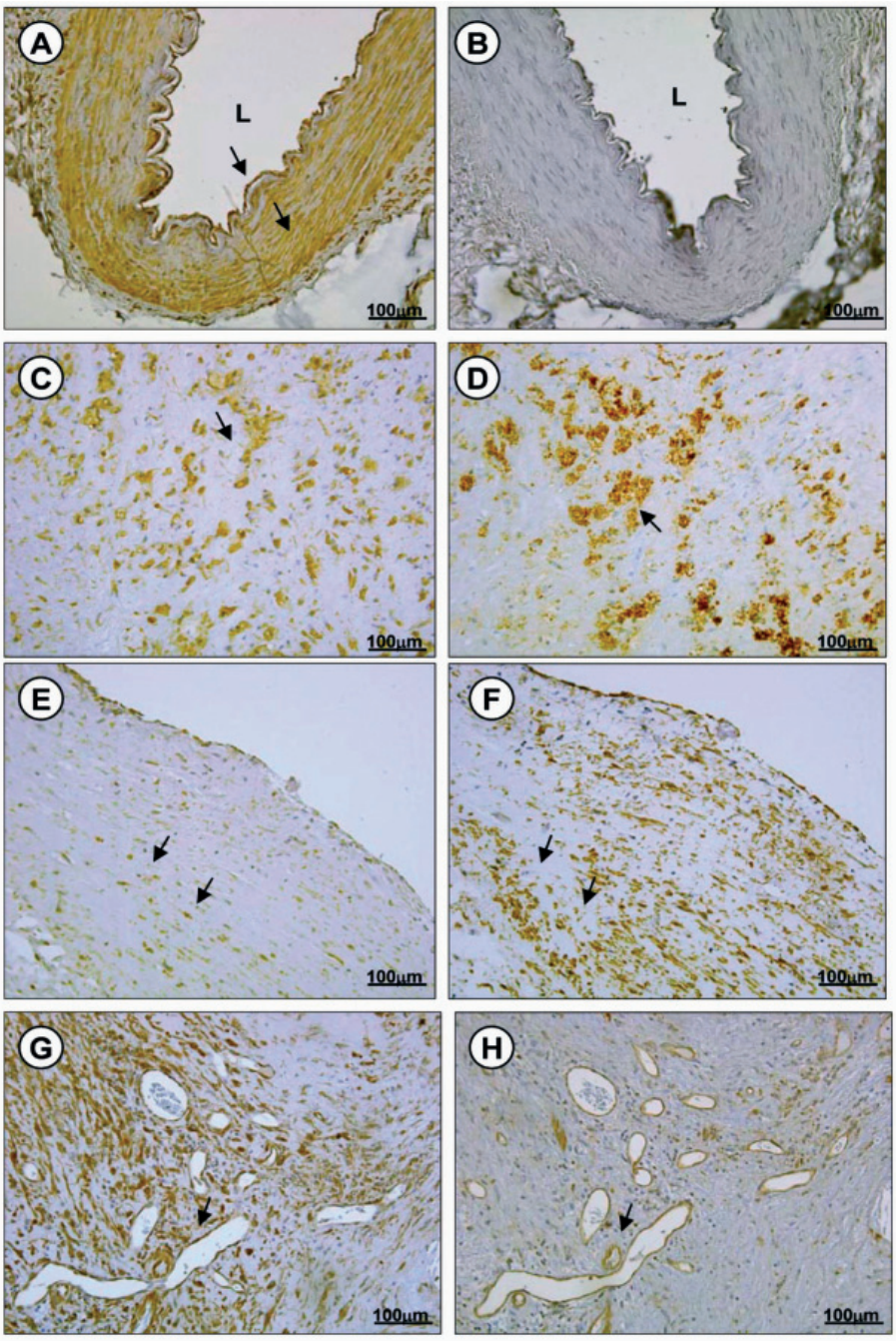
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Figure 2

Figure 1: ***Identification of Nogo-B in different human vascular beds***

A- *Representative nitrocellulose membrane stained with Ponceau Red S showing protein loading (Top). Representative Western blots for Nogo-B and β -Actin expression in human mammary and human carotid endertarectomy samples (Bottom). Lysates from human neuroblastoma cell line SHY-5Y was used as positive control for Nogo-A and B.*

B- *Densitometric analysis of Nogo-B expression levels in human mammary arteries (n=8) and atherosclerotic specimens of human carotid endertarectomies (CEA) (n=145), human femoral endertarectomies (FEA) (n=19) and human coronary arteries (CA) (n=6) * P<0.001, ** P< 0.001, *** P<0.003. A.U = Arbitrary Units.*

Figure 2: ***Immunodetection of Nogo-B in human arteries***

Paraffin-embedded sections (5□m) from human mammary arteries (A) and human carotid plaques(C, E and G). were incubated with goat anti-human Nogo-B antibody N-18. Adjacent sections were stained with anti-CD-68 (Macrophages, D) anti-alpha actin (SMC, F) and anti-CD-31 (ECs, H) antibodies. Non-immune control antisera is shown in (B). Brown staining indicates protein abundance. Magnification (x200). Scale bar = 100□m. L= lumen.

Nogo-B Expression Levels, Plaque Characteristics and MMP activity

Next, we looked into the relationship between Nogo-B levels and different plaque characteristics. Carotid plaques containing more than 40% of fat (atheromatous phenotype) showed significantly lower Nogo-B levels in comparison to plaques with little or no fat content (p=0.002), (Table 2). No relationships were found between Nogo-B expression levels and other plaque characteristics such as number of macrophages and/or smooth muscle cells, amount of collagen or presence of thrombus and/or calcifications (Table 2).

		Nogo-B		P-value
		Median	Interquartile range	P value
Fat	<40%	65	30-106	
	>40%	37	20-63	0.002
Macrophages	No/Minor	56	24-90	0.86
	Moderate/Heavy	50	26-94	
Smooth muscle cells	No/Minor	41	22-80	0.15
	Moderate/Heavy	63	25-103	
Collagen	No/Minor	63	22-101	0.99
	Moderate/Heavy	51	25-89	
Calcifications	No/Minor	47	23-88	0.67
	Moderate/Heavy	61	25-94	

Table 2: *Relation between Nogo-B expression levels and carotid plaque characteristics. Note that carotid endarterectomy samples containing large fat content (40%) are strongly associated to low Nogo-B expression levels.*

Nogo-B Expression Levels, Proteolytic activity and inflammation

We further investigated the relationship between Nogo-B expression levels and local Matrix-metalloprotease (MMP) activity and pro-inflammatory cytokines levels. A positive relationship was found between Nogo-B levels and Matrix-metalloproteinase-2 (MMP-2) activity in carotid plaques (p=0.01), (Table 3). On the contrary, no relationships were detected between Nogo-B levels and MMP-8 or MMP-9 activity (Table 3). Levels of either interleukin-6 or interleukin - 8 did not show any relation with Nogo-B levels (not shown).

		Nogo-B		P-value
		Median	Interquartile range	P value
MMP-2 activity	<median	40	20-67	0.01
	>median	71	32-115	
MMP-8 activity	<median	49	23-100	0.77
	>median	61	24-94	
MMP-9 activity	<median	58	25-106	0.36
	>median	48	22-86	

Table 3: *Relation between Nogo-B expression levels and MMP activity*

		Nogo-B		P-value
		median	interquartile range	
Carotid degree of stenosis by duplex	<90%	68	37-109	
	>90%	46	19-86	0.04
Restenosis or a denovo stenosis	Restenosis	61	26-103	0.01
	De novo	13	0-31	
History of stroke	no	49	21-100	0.38
	yes	61	25-100	
History of myocardial infarction	no	63	22-106	0.59
	yes	39	29-84	
History of angina pectoris	no	65	33-104	0.13
	yes	39	19-97	

Table 4: *Relation between Nogo-B expression levels, risk factors and clinical symptoms*

Nogo-B Expression Levels and Clinical Data

We also analyzed the relationship between Nogo-B, classical risk factors and different clinical symptoms associated with atherosclerosis (Table 3). Nogo-B expression levels were not related to any of the classical risk factors of atherosclerosis. However, Nogo-B levels were significantly lower in those patients that revealed a high percentage carotid artery stenosis (>90%) and patients with restenosis after prior CEA (Table 4).

Discussion

In the central nervous system, reticulon-4/Nogo-A has been described as a myelin-associated inhibitor that blocks axonal regeneration after damage[11,12]. In the arterial wall, it was demonstrated that the isoform Nogo-B inhibits intimal growth and subsequent luminal narrowing in mice[9]. The presence and expression patterns of Nogo-B in human atherosclerotic lesions has not been established previously.

In the present study, we observed Nogo-B in normal arteries and atherosclerotic plaques. Two variants of Nogo-B (Nogo-B1 and B2) are present in both non-atherosclerotic and atherosclerotic tissues. However, Nogo-B expression levels are significantly lower in atherosclerotic lesions in comparison to healthy mammary arteries. The mechanisms that influence Nogo-B levels in atherosclerotic plaques are presently unknown. Pressure, but not cellular stretch, have been associated with an up-regulation of Nogo-B levels[13]. It is unknown whether initiation and progression of atherosclerosis with the presence of pro-atherogenic factors trigger signaling cascades that reduce the expression or promote the degradation of Nogo-B.

As shown by immunohistochemistry, in healthy mammary arteries Nogo-B is distributed throughout the vessel wall with a strong signals in the endothelial layer and the smooth muscle cells within the media. This observation is in agreement with the previous reports which have identified expression of Nogo-B in cultured human endothelial cells and smooth muscle cells as well as mouse fibroblasts[9,14]. In carotid plaques, Nogo-B levels are lower and is present in smooth muscle cells and endothelial cells from the vasa vasorum. Interestingly, Nogo-B also seems to be concentrated in macrophage/foam cells rich areas. This expression pattern has not been reported in human tissues previously. Recently, Rosseau and coworkers did report the existence of Nogo-B in RAW264 macrophages [15], although its role remains to be established.

Another finding is that Nogo-B levels in carotid plaques are inversely related with the presence of large lipid pools. It is thought that in advanced atherosclerotic lesions, apoptosis of macrophages/foam cells contribute to lipid-rich necrotic cores formation[16]. In this context, it has been suggested that Nogo-B might promote apoptosis in cancer cells[17-19]. Despite the overall reduction in Nogo-B levels in plaques, a local accumulation of Nogo-B in macrophages might contribute to cell death facilitating lipid accumulation. However, it should be considered that other members of the Nogo family have demonstrated an anti-apoptotic effect[20]. Therefore, the involvement of these proteins in apoptotic process is still controversial and requires further investigation[21].

We have searched for associations between expression levels of Nogo-B and other established factors related to plaque vulnerability such as MMP activity and pro-inflammatory cytokines and have observed a positive relationship between Nogo-B expression levels and MMP-2 activity. Recently, we have reported that high levels of MMP-2 are associated with a smooth muscle cell-rich stable plaque phenotype[22]. Thus, this observation suggests that high Nogo-B levels may contribute to plaque stabilization. We also found that Nogo-B levels are not influenced by previous use of medication to treat cardiovascular disease like statins (not shown). The correlation between Nogo-B levels, patient's clinical history and clinical complications did reveal data supporting the idea that down-regulation of Nogo-B might accelerate luminal narrowing in atherosclerotic and restenotic disease. Indeed, we found that Nogo-B levels were negatively associated with high preoperative degree of carotid stenosis and restenosis after prior CEA. These observations are consistent with animal experimental observations of Acevedo et al, who demonstrated that Nogo impairs neointima formation following arterial ligation in mice[9].

We hypothesize that during atherosclerotic plaque formation and progression the reduction of Nogo-B levels especially in SMC promotes their migration stimulating neointima formation. The reduced levels of Nogo-B in plaques obtained from high grade stenosis supports this concept.

In summary, here we report for the first time that low Nogo-B expression levels in carotid endarterectomies are strongly associated with an atheromatous (vulnerable) phenotype, restenotic lesions and severe stenosis in subjects receiving operative treatment. Therefore, an upregulation of Nogo-B might be considered as a target to prevent human carotid stenosis associated with atherosclerotic syndrome.

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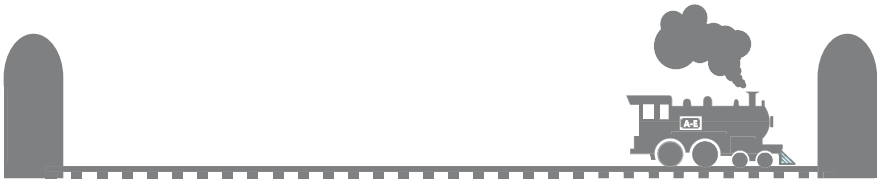
Summary and general discussion



Athero-Express is a tissue bank study, designed to investigate the expression of atherosclerotic derived biological variables in relation to the long-term outcome of patients undergoing carotid endarterectomy. Its design includes both cross-sectional and follow-up studies, the results from which should expand our current knowledge of atherosclerotic disease. Due to the relatively large sample size, it should be possible to correct potential confounders whilst exploring protein and RNA expression in relation to plaque characteristics. The objectives of Athero-Express include the identification of new plaque markers, patient risk profiles and treatment strategies as well as the early detection of "patients at risk". Follow-up studies, in which the long term outcome in relation to local protein and RNA expression are studied, will take several years to complete, due to the large sample size required to correct confounding. For this reason, cross sectional studies are likely to be undertaken in the first years of the study. Cross-sectional studies can be used to validate any proteins of interest, which, from either in vitro or in vivo studies, appear to be related to the progression of atherosclerotic disease. Future follow-up studies will focus on proteomic analyses. These, in turn, should result in the discovery of new proteins of interest.

Has Athero-Express been successful in fulfilling these objectives?

The design of Athero-Express

The study, which was first designed in 2001, was approved by the medical committee in 2002. The first patient was included in April 2002. At the present time, more then 900 patients have been included. In addition to the 650 patients, who underwent carotid endarterectomy, the study has been extended to include other types of surgical intervention. Tissue samples from patients undergoing femoral artery endarterectomy, those receiving conventional repair of either abdominal or thoracic aneurysm have also been included, as well as samples of healthy mammary arteries, obtained during coronary bypass surgery. The total number of patients included to date will approach 1000 by April 2006.

The aim is to include from every patient: a specimen of atherosclerotic tissue, a blood sample and a completed questionnaire. In addition to these baseline data, patients will continue to be followed up, for a number of years. Follow-up is by duplex scanning assessment of the affected artery and an annual questionnaire, by mail.
In practice, the tissue sampling and protein isolation elements of the study have been the most successful, with tissue inclusion approaching 100%. Protein is isolated according to protocol and has proven to be of good quality over time. Any excess tissue is frozen for preservation. Immunohistochemical stains of the offending

lesion are performed on a regular basis and comparable quality has been proven over time. The quantification method used has been validated in the past [1], but is now revalidated by comparing results of the semi-quantitative scoring of CD-68 and SMC stains with quantitative measurements (data not shown in this thesis). The second sample of material to be included is the pre-operative blood sample. At the beginning of the study, the inclusion of a blood sample from every single patient presented a logistical problem. Whilst this could potentially induce a selection bias, we believe any such bias will be overcome by the large sample size (=power) of the bio- bank. The questionnaire used in our study, is allied to that of the SMART study[2] and has proved most efficient.

Cross-sectional studies, which relate plaque characteristics to clinical features and identify vulnerable plaque characteristics in Athero-Express

In addition to the studies presented in this thesis, Sluiter et al. and van Oostrom et al. have also published work based on the Athero-Express[3;4]. Plaque characteristics in relation to asymptomatic and symptomatic patients were discussed in chapter 3. We had expected to link symptomatic patients with a plaque phenotype, which is less stable, than in asymptomatic patients. From post-mortem studies we know that plaques with a lipid-rich, atheromatous core, a thin fibrous cap with macrophage and lymphocyte infiltration and production of inflammatory markers can be related to sudden cardiovascular events and are known as "vulnerable plaques"[5-8]. It is also known that symptomatic patients benefit more from CEA in comparison with asymptomatic patients[9] and that, whilst amaurosis fugax and transient ischemic attacks are both considered symptoms, the prognosis for patients with afx[10] is better. Our findings also confirmed these statements. Vulnerable plaque characteristics (IL8, MMP, high content of plaque athroma) were more frequently observed in symptomatic patients than asymptomatic patients. We also observed that amaurosis fugax and asymptomatic patients had similar plaque characteristics. Future selection of patients based on plaque characteristics determined by imaging techniques might therefore be useful.

But can these plaque characteristics be explained by the fact that asymptomatic patients are more often treated, for example, by statins? The answer is no. Symptomatic and asymptomatic patients were equally distributed among statin treated and untreated patients. But statins are associated with improved survival in cardiovascular patient groups. For this reason, in chapter 4, plaque characteristics were studied in relation to statin treatment. Statin treatment in cardiovascular disease clearly results in

reduced cardiovascular mortality and morbidity[11-13]. The beneficial effects (reduced cardiovascular events in selected patient groups) in patients treated with statins are clearer than might be expected. Apart from their direct effect on the lipid spectrum, statins are also associated with moderating various inflammatory responses involved in the initiation and progression of atherosclerotic disease. These effects are also known as pleitrophic effects[14-17]. If this is so, the plaque characteristics of patients treated with statins would differ from those of untreated individuals.
In chapter 4, we observed fewer atheromatous plaques in patients treated with statins. In addition to this, we observed higher prevalence of CD68 positive cells in the plaques obtained from patients treated with statins, especially in patients treated with atorvastatin. The increased number of CD68 positive did not subsequently lead to an increase of inflammatory reaction, in fact plaque-IL6 and CRP levels were reduced among statin users. Our results suggest a better plaque profile for patients treated with statins. This has recently been corroborated by Kennedy et al, who report an improvement in outcome for patients treated with statins prior to CEA. This difference is mainly observed in symptomatic patients treated with statins, compared to those who were untreated[18].

Are the identified 'vulnerable plaque' characteristics in Athero-Express actually vulnerable?

We identified differences in plaque characteristics among different patient categories in chapters 3 and 4. Symptomatic patients and patients not receiving statin treatment were seen to have a more lipid rich plaque core and increased inflammatory components than patients in either the asymptomatic group or the untreated patients. We would suggest that this plaque is more vulnerable and subsequently responsible for cerebro-vascular events but we needed a study to confirm this. Chapter 5 describes the relation between per operatively recorded microemboli, detected by Trans Cranial Doppler (TCD) and plaque characteristics. TCD surveillance is widely used during CEA and peroperatively detected microemboli are known to be associated with adverse neurological events[19-24]. The question therefore arises - is there a higher risk of microemboli when a "vulnerable" plaque is removed during CEA? For 205 of the procedures detailed, TCD information was available and from these, we were able to show that, during the dissection phase of CEA, an increase in the number of microemboli was related to an adverse neurological outcome. Ten of the eleven patients, who suffered such events (TIA/stroke), had a high degree of plaque atheroma. Overall, in comparison with fibrous and fibro-atheromatous plaques, a link between atheromatous plaques and an

increase of recorded microemboli during CEA, could not be established. Remarkably, however, during clamp release and wound closure, fibrous plaques were related to a higher incidence of microemboli. To conclude, athromatous plaques are more vulnerable, but are not associated with an increase in peroperative microemboli.

Procedural related outcome observed in Athero-Express

In chapter 6, Athero-express data are used to investigate both procedural related outcomes and recorded microemboli during CEA. Patch closure in CEA showed benefits over primary closure. However, there are insufficient data available to allow firm conclusions to be drawn regarding the differences between the various patch materials used for carotid closure[25]. Synthetic patches are believed to be more thrombotic and theoretically, should therefore produce TCD detectable microemboli, during CEA[26]. As already stated, these emboli are related to adverse neurological effects. In deed, Dacron patches were associated with both an increase in microemboli (during clamp release and wound-closure) and also restenosis after CEA. These differences were mainly observed in females. Females were also associated with a greater incidence of microemboli during wound-closure in general. So, in addition to the relationship between microemboli and fibrous plaques, we now observed a relationship between women and microemboli and in women, Dacron patch closure was associated with the most microemboli. To exclude the possibility of confounding, only fibrous plaques were selected and among fibrous plaques, Dacron patch closure was linked to an increase in microemboli during wound-closure (data not shown in thesis), however, both women and men had comparable numbers of microemboli. To conclude, there seems to be an independent relationship between microemboli and patch closure as well as plaque phenotype and microemboli. However, this study is retrospective and more data are needed to provide the final answer.

Search for new atherosclerotic markers

In chapter 7, the relationship is studied between NOGO-b and atherosclerosis. Recently, Acevedo et. al. showed that human endothelial cells, smooth muscle cells and healthy murine arteries all contain high levels of Nogo B[27]. Although Nogo-B is known to inhibit neuronal outgrowth and axon regeneration, until then, it had not been linked to arterial tissue. Acevedo showed that the expression of Nogo-B rapidly decreases in injured mouse arteries. Nogo-A/B deficient mice were also seen to exhibit accelerated neointima formation after femoral artery ligation. We were therefore encouraged to investigate this apparent new role of Nogo-B and its relationship to human atherosclerotic tissue. We were able to show that atherosclerotic tissue

(carotid artery, coronary artery and femoral artery) has a lower Nogo-B expression than healthy mammary arteries. At plaque level, athermatous material showed lower Nogo-B levels in comparison to plaques with no er minor fat content. Additionally MMP2 activity, measured in the plaque, was raised in those containing high levels of Nogo-B. According to clinical data, low Nogo levels are associated with both restenosis after prior CEA and also to high grade stenosis (>90%). Our findings support the role of Nogo-B in atherosclerosis.

Conclusion and future perspectives

To date, Athero-Express has proved a successful bio-bank. Several cross sectional studies have already been performed. Its power is based on the large number of patients included. At this moment, proteomic analyses are being performed in Singapore. We expect mass spectrometry proteomics to reveal new, as yet unrecognised proteins, differentially expressed in 1- traditionally defined fibrous, stable plaques versus atheromatous, inflammatory "vulnerable plaques" and 2- plaques obtained from "vulnerable patients," who reached a cardiovascular endpoint, versus controls. The identification of differentially expressed proteins between stable versus unstable and patients, who reached a cardiovascular endpoint versus controls will help identify molecular therapeutic targets and also facilitate development of new therapeutic targets and prognostic markers, to prevent destabilisation of both plaque and patient. The data continue to increase and the first follow-up studies will soon be available. Other cross-sectional studies have still to be performed, to evaluate other plaque characteristics with clinical data.

From the first cross sectional studies we can conclude that vulnerable plaque characteristics, atheromatous plaque and plaque with increased MMP or cytokine levels are each associated with symptomatic carotid disease (TIA and stroke, not amaurosis fugax). but are less frequently observed in patients treated with statins. Based on our results, athromatous plaque is associated with adverse neurological outcomes, but does not increase the number of microemboli during plaque dissection. Nogo-B was indentified as having a new role in atherosclerosis. As well as overall lower levels of Nogo-B, low expression levels of Nogo-B in particular are associated with atheromatous plaques and high grade stenotic lesions. The avoidance of Dacron patch closure after CEA, especially in women, would seem to be supported.

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Samenvatting in het Nederlands
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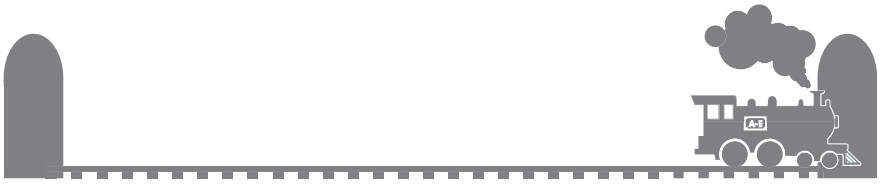

Met aderverkalking wordt eigenlijk slagaderverkalking bedoeld, de ziekte die medisch atherosclerose heet. Dit is een ziekte die het hele lichaam aantast en vele tientallen jaren nodig heeft om manifest te worden. Bekende symptomen zijn een beroerte, etalagebenen en het hartinfarct. Indien klachten van deze ziekte zich presenteren is de slagaderverkalking in een ver gevorderd stadium en richt de behandeling zich op symptoom bestrijding en voorkomen van verergering.
Athero-Express is een weefselbank (bio-bank) waarin slagaderverkalkingsweefs el (atherosclerotische plaques), verkregen tijdens de operatie van de halsslagader (carotid endarterectomy=CEA; ter voorkoming van beroertes), wordt opgeslagen en verder wordt onderzocht. Met dit materiaal wordt onderzoek gedaan om biologische variabelen en markers te vervolgen in relatie tot de uitkomst van ziekten bij patiënten op lange termijn. Het onderzoek richt zich naast patiënt variabelen (denk aan risico factoren zoals te hoog cholesterol-gehalte, overgewicht, roken etc.) op plaque specifieke aspecten. Het onderzoek van de plaque richt zich naast de microscopische kenmerken (gehalte kalk, vet en ontstekingscellen) op aanwezigheid van verschillende eiwitten en RNA (genetisch materiaal) expressie in relatie tot de atherosclerose. De studie is zo ontwikkeld dat naast de lange termijn uitkomst (follow-up) er ook tussentijds studies verricht kunnen worden, die specifieke markers/factoren bestuderen (cross-sectionele studies). Beide manieren van onderzoek moeten bijdragen tot een betere begripsvorming over de ontstaanswijze van atherosclerose. De kracht van de studie bestaat voornamelijk uit het grote aantal deelnemende patiënten. Hierdoor is het mogelijk om te corrigeren voor confounding (vertekening) tijdens het initiële onderzoek naar eiwit en RNA expressie in relatie tot plaque karakteristieken. De doelstellingen van de studie zijn: identificeren van nieuwe plaque markers, van patiënten met een instabiele plaque, het optimaliseren van de behandelings strategieën en vroege detectie van patiënten die later zeer waarschijnlijk ziekte ontwikkelen.

In hoofdstuk 1 wordt de ontwikkeling van de biobank en zijn kenmerken beschreven. De studie is gestart in 2002 nadat deze was goedgekeurd door de medisch ethische commissie. Op dit moment zijn meer dan 900 patiënten geïncludeerd in de bank. Van die 900 patiënten zijn er 650 geopereerd in verband met slagaderverkalking van de halsslagader. Naast de verzameling van dit weefsel werd gestart met de verzameling van weefsel verkregen tijdens operatie van de vergrote lichaamsslagader (aneurysma aorta abdominalis en thoracalis), dijbeenslagader (arteria femoralis superficialis/ communis), en van een slagader die vaak onaangetast is en om die reden wordt gebruikt bij omleidingen van het hart (arteria mammaria interna/ coronary bypass graft).

Per patiënt dient naast een stukje atherosclerotische plaque ook bloed afgenomen te worden. Tevens vullen patiënten een uitgebreide vragenlijst in bij initiële deelname aan de studie en ontvangen zij jaarlijks een korte vragenlijst per post aangaande de medische conditie van de patiënt. Poliklinisch worden patiënten na de operatie terug gezien en wordt met behulp van ultra geluidsgolven onderzoek (duplex/ echo) de geopereerde slagader bekeken. Weefsel en bloed verkregen tijdens de operatie worden bewerkt volgens een vast protocol. Een gedeelte van het weefsel wordt direct bewerkt voor microscopische beoordeling en classificatie, en voor het isoleren van eiwit. Restanten van het weefsel en de bloedproducten worden opgeslagen in vloeibare stikstof zodat deze goed gepreserveerd zijn en bruikbaar voor onderzoek in de toekomst.
In de praktijk is het verzamelen van weefsel en de bewerking ervan een groot succes. Van bijna alle geïncludeerde patiënten is er daadwerkelijk weefsel voor handen. De kwaliteit van het ingevroren weefsel blijkt ook op lange termijn goed te zijn. Met de verzameling van het bloed loopt de studie achter. Vooral omdat de logistiek in het begin van de studie nog niet was afgestemd op het verkrijgen van bloed, is niet van alle patiënten ingevrorenbloed voorhanden.

Plaque eigenschappen in relatie tot de symptomen voorafgaande aan de operatie van de halsslagader, worden besproken in hoofdstuk 3. Patiënten kunnen symptomatisch geweest zijn wat wil zeggen dat ze voorafgaand aan de operatie een beroerte (CVA) en/of een tijdelijke beroerte (TIA) gehad hebben. Tevens hebben al deze patiënten een ernstige vernauwing van de halsslagader. Een tijdelijke beroerte is nog onder te verdelen in twee groepen, als eerste een beroerte van het brein, als tweede een beroerte van het oog, beter bekend als tijdelijke blindheid of amaurosis fugax (AFX). Asymptomatische patiënten hebben geen CVA of TIA gehad, maar hebben wel een ernstige vernauwing van de halsslagader. Symptomatische en asymptomatische patiënten hebben een verhoogd risico op het krijgen van een herhaling van symptomen dan wel verergering. De operatie waarbij de plaque wordt verwijderd uit de halsslagader, verlaagt het risico op deze gebeurtenissen.
Uit onderzoek bij overleden patiënten was reeds bekend dat bij patiënten met een plotse hartdood vaker plaques worden aangetroffen met een hoog vetgehalte en veel ontstekingsreactie. Tevens is bekend uit de literatuur dat symptomatische patiënten meer profijt van een halsslagader operatie hebben dan asymptomatische en dat van de symptomatische patiënten, patiënten met AFX een betere prognose hebben dan patiënten met een TIA. We verwachtten in deze studie een link te vinden tussen de plaque karakteristieken en het wel of niet symptomatisch zijn van de patiënt.

Onze bevindingen bevestigen deze stellingen. Plaques met ontstekings kenmerken en een hoog percentage vet werden vaker geobserveerd in symptomatische patiënten vergeleken met asymptomatische patiënten. Patiënten die tevoren AFX hadden, kwamen met plaque karakteristieken overeen met asymptomatische patiënten.
Zou er een relatie kunnen bestaan met medicijn gebruik in een van beide groepen. Van cholesterol verlagende middelen (statines) is het bekend dat het een gunstig effect heeft bij patiënten met hart en vaatziekten. Statines werden echter door zowel asymptomatische als symptomatische patiënten evenveel gebruikt. Zijn statines wel geassocieerd met plaque karakteristieken? In hoofdstuk 4 wordt de relatie tussen statine gebruik en plaque karakteristieken nader onderzocht. Naast hun bekende cholesterol verlagende werking zouden deze medicijnen de ontsteking in de plaque verminderen. Wij beschrijven dat patiënten behandeld met een statine lager percentage vet in de plaques hebben, maar een toegenomen aantal ontstekingscellen. De ontstekingsprodukten geproduceerd door deze cellen is echter over de hele linie gedempt in vergelijking met niet behandelde patiënten. Onze resultaten suggereren dat patiënten die behandeld worden met een statine een beter plaque profiel hebben dan niet behandelde patiënten.

In hoofdstuk 3 en 4 worden verschillen in plaque karakteristieken beschreven voor symptomatische versus asymptomatische patiënten en patiënten behandeld met statines. We zijn van mening dat een plaque met een hoog gehalte aan ontsteking en vet beschouwd kan worden als een hoogrisico plaque. Patiënten in het bezit van deze plaque zouden dan vaker symptomen van de atherosclerose moeten hebben. In hoofdstuk 5 hebben we onderzocht of het door ons geïdentificeerde plaque type gerelateerd is met een toegenomen frequentie van atherosclerotische ziekte. Tijdens de CEA worden patiënten bewaakt door het meten van de hersengolf activiteit en de bloeddoorstroming. De meetapparatuur voor de bloeddoorstroming biedt de mogelijkheid om bloedpropjes (embolieën) te detecteren. Uit voorgaand onderzoek is bekend dat het voorkomen van deze embolieën gerelateerd is aan het (als complicatie van een CEA) voorkomen van een CVA of TIA. In onze studie zien we ook meer embolieëin bij patiënten die een CVA's en/of TIA's kregen rondom de operatie. Daarnaast bleken 10 van de 11 patiënten met deze complicatie een plaque met een hoog gehalte aan vet te hebben. De enige relatie die we konden aantonen was dat na het verwijderen van de plaque, dud tijdens het sluiten van de slagader meer embolieën werden gezien indien de verwijderde plaque een laag vetgehalte had.

Hoofdstuk 6 onderzoekt de verschillende technieken die worden gebruikt bij het sluiten van de halsslagader in relatie tot het opnieuw verstoppen van de halsslagader en voorkomen van emboliën. In onze studie blijkt kunststof materiaal (Dacron patch), gebruikt voor het sluiten van de slagader, minder geschikt te zijn dan eigen materiaal (ader uit het bovenbeen of enkel) of primair sluiten. Bij Dacron komt het vaker voor dat de slagader weer dicht gaat zitten, dit wordt voornamelijk gezien bij vrouwen. Daarnaast blijkt het gebruik van Dacron vaker gepaard te gaan met het voorkomen van emboliën tijdens het sluiten van de wond.

Hoofdstuk 7 bestudeert Nogo-B expressie in atherosclerose. Nogo-B was onlangs beschreven wegens zijn belangrijke functie bij beschadigde slagaders. Bij deze studie was gebruikt gemaakt van diermodellen. Deze studie inspireerde ons om de verdere functie van Nogo-B in atherosclerose te bestuderen. In dit hoofdstuk beschrijven we dat Nogo-B minder tot expressie komt in slagaders die atherosclerotisch veranderd zijn. Tevens wordt een relatie tussen Nogo-B en stenose graad van de slagader beschreven. Daarnaast bestaat er ook een relatie met een specifiek ontstekingseiwit (MMP).

Concluderend
Athero-Express heeft zich inmiddels bewezen als een succesvolle biobank. Diverse studies zijn reeds gepubliceerd en vele zullen nog volgen. De kracht van de bank schuilt voornamelijk in het grote aantal patiënten dat reeds geïncludeerd is en alleen nog maar toe gaat nemen. Op dit moment worden proteomics (grootschalige eiwit analyse) studies verricht in Singapore. We verwachten dat deze analyse nieuwe, voor atherosclerose belangrijke, eiwitten zal identificeren. Naast de studies die zijn gepresenteerd in deze dissertatie, worden op niet al te lange termijn de eerste resultaten van de follow-up studies verwacht.

Uit dit proefschrift kan geconcludeerd worden dat plaques met een hoog percentage vet en specifieke ontstekingsverschijnselen geassocieerd zijn met symptomatische halsslagader verkalking. Tevens wordt dit type plaque minder gezien bij patiënten die behandeld worden met een statine. Gebaseerd op onze studie resultaten zijn vetrijke plaques geassocieerd met complicaties na CEA, maar zijn tijdens operaties niet direct verantwoordelijk voor een toegenomen aantal embolieën.
Dat Nogo-B een belangrijk eiwit is in atherosclerose wordt in dit proefschrift bekrachtigd. Bij het sluiten van de CEA wordt bij voorkeur lichaams eigen materiaal gebruikt, in het bijzonder bij vrouwen.

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Curriculum vitae auctoris

Bart Arnoldus Nicolaas Verhoeven werd op 1 juni 1973 geboren te Gouda. Aan het Christelijk Lyceum in Gouda behaalde hij in 1991 zijn VWO diploma. Aansluitend begon hij met zijn studie geneeskunde aan de Erasmus universiteit in Rotterdam. In 1996 werd het doctoraal examen met succes afgelegd. Tijdens de wachttijd tot aan zijn co-schappen heeft hij wetenschappelijk onderzoek verricht aan de Erasmus universiteit, met als resultaat een publicatie in The Lancet (tweede auteur). In 1999 werd het arts examen met succes afgelegd (cum laude). Na aanvankelijk als AGNIO Chirurgie in Rotterdam en Breda gewerkt te hebben, begon hij 1 januari 2002 als AGNIO Chirurgie in het St. Antonius ziekenhuis te Nieuwegein. Aldaar verrichte hij aanvankelijk onderzoek naar de ontwikkeling en invoering van het elektronisch patiënten dossier samen met dr. P.M.N.Y.H. Go. Later werd onder leiding van prof.dr. F.L. Moll en prof.dr. G. Pasterkamp geparticipeerd in de Athero-express studie. In juli 2003 is de auteur gestart met de opleiding Heelkunde in het UMC Utrecht onder leiding van prof.dr. I.H.M. Borel Rinkes. Van 1 juli 2004 tot en met 1 juli 2005 werd de auteur de gelegenheid gegeven zich volledig te concentreren op deze dissertatie. Gedurende deze periode was hij werkzaam bij de afdeling Experimentele Cardiologie van het UMC Utrecht, onder leiding van prof.dr. G. Pasterkamp. Vanaf 1 juli 2006 zal hij zijn opleiding Heelkunde vervolgen in het St. Antonius ziekenhuis te Nieuwegein onder leiding van dr. P.M.N.Y.H. Go.