The Clinical Value of Histological Femoral Artery Plaque Analysis

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The Clinical Value of Histological Femoral Artery Plaque Analysis

De klinische waarde van het analyseren van plaque histologie uit de femoraal arterie (met een samenvatting in het Nederlands)

Proefschrift

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Voor mijn ouders,
Voor Eva en Stijn

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PART III Histological femoral plaque analysis to predict clinical outcome

CHAPTER 1

General introduction

BACKGROUND

Atherosclerosis is a focal thickening of the intimal artery layer. To a cascade of inflammatory processes over the years, the luminal narrowing will eventually lead to an adverse cardiovascular clinical event.[1] Atherosclerosis is a systemic disease and can exist in all of the arteries throughout the body, with known preferred territories, including the coronary, carotid, and femoral arteries. Depending on the anatomic localization of the occlusion, atherosclerosis will lead to a myocardial infarction, cerebral infarction, or leg ischemia.

Peripheral arterial disease (PAD) has a high incidence; however, approximately two-thirds of PAD patients are asymptomatic. The prevalence of symptomatic PAD ranges between 3% and 11%.[2,3] The overall prevalence, including asymptomatic and symptomatic, increases dramatically with age and might be up to 30% in patients aged older than 70 years.[4,5] Because of the aging of the population, the incidence of patients with symptomatic PAD is expected to grow in the future.

Patients suffering from PAD have a generalized susceptibility to atherosclerosis, with more advanced disease in other arteries and vulnerability to coronary death.[6] The risk for future cardiovascular events in patients with PAD is three times higher than in individuals without PAD.[7,8] Furthermore, patients with symptomatic PAD have a 20% mortality rate after 5 years, and patients with critical limb ischemia have the same mortality rate after 1 year.[5]

Treatment of peripheral artery disease

Lifestyle modification and best medical treatment are recommended in patients with PAD. Important lifestyle modifications are regular exercise for at least 30 minutes every day, weight reduction, a diet that is low in saturated fat, and smoking cessation.[5] Strict control of blood pressure to prevent ischemic events is recommended, and all patients with PAD should be treated with a statin to lower the concentration of low-density lipoprotein (LDL).[5,9,10] In patients with diabetes mellitus, plasma glucose levels should be strictly regulated by lifestyle modification or drug therapy, or both.[5] All PAD patients should be treated with an antiplatelet drug because these agents have been shown to reduce the risk of nonfatal myocardial infarction, nonfatal stroke, and cardiovascular death.[11,12] To relieve symptoms caused by the formation of collateral arteries, supervised walking treatment is recommended in patients with intermittent claudication.[13,14]

Patients without improvement, or whose complaints worsen after supervised exercise, and patients with critical ischemia primarily are scheduled for a revascularization procedure.[5] These patients are treated by use of the same protocol used in our hospitals (UMC Utrecht and St. Antonius Hospital Nieuwegein). Lesions of the common femoral artery (CFA) undergo an endarterectomy. TASC A, B, and C lesions of the superficial femoral artery (SFA) undergo percutaneous intervention. If patients have a suitable greater saphenous vein (>3 mm), TASC C lesions that are assessed as too complicated for percutaneous intervention and TASC D lesions are treated with a venous supragenicular bypass. Patients with extensive TASC C and TASC D SFA lesions who lack a greater saphenous vein suitable for bypass surgery are treated primarily with remote superficial femoral artery endarterectomy (RSFAE). It is usually only when RSFAE fails that patients will undergo prosthetic supragenicular bypass (see **Chapter 8**, Figure 1).

Despite the revascularization techniques to optimize peripheral vascular perfusion, patency rates after revascularization of the femoral artery are still disappointing. The primary patency rate is about 65% for venous bypass grafts after 3 years of follow-up but is worse, at about 50%, for RSFAE and synthetic bypass grafts.[15] A high percentage of restenosis occurs during the first year of follow-up. However, there are no useful predictive markers for restenosis. To improve assisted primary or secondary patency rates, a strict follow-up scheme is necessary. If predictive markers could be used to identify patients at high-risk for restenosis, a follow-up scheme could be tailored to the individual patient, and patients at risk for restenosis could be checked and treated more intensively.

RATIONALE OF THIS THESIS

It would be a big step forward in the attempt to reduce the prevalence of cardiovascular disease if patients at risk for future cardiovascular events could be identified. If patients could be identified before a stroke or myocardial infarction, cardiovascular disease prevalence would be dramatically decreased. For patients with known cardiovascular disease, it is important to predict the chance for a secondary adverse cardiovascular event. Although most patients will already be receiving medical treatment, it is well established that there is room for improvement regarding compliance for medication use and lifestyle modification. In addition, clinically silent atherosclerotic disease could be identified to select patients at high risk so they could receive preventive surgical or medical treatment.

Previous studies have focused on traditional cardiovascular risk factors as predictors for systemic outcome. Clinical characteristics associated with PAD and adverse cardiovascular events are male sex, smoking, diabetes, hypertension, hypercholesterolemia, and renal insufficiency.[16-21] However, the discriminative power of these risk factors is limited because of their high prevalence among vascular patients. Furthermore, circulating markers, like C-reactive protein (CRP), are associated with adverse cardiovascular events,[22] but an increase in CRP is not specific for cardiovascular disease. In secondary manifestations, the predictive value is limited, and there is no consensus regarding clinical application.[23]

Other studies have focused on plaque histology predictive for local outcome. Autopsy studies of patients who died after myocardial infarction showed plaque characteristics that were associated with coronary artery occlusion.[24] These cross-sectional studies showed features of the “vulnerable plaque,” including increased inflammation, a large lipid core, and a thin fibrous cap.[24] However, these vulnerable plaque characteristics can also be observed in plaques from asymptomatic patients.[25] Besides, these cross-sectional studies are not appropriate to conclude if vulnerable plaque characteristics are predictive of future cardiovascular events.

Instead of searching for local plaque characteristics predicting local plaque rupture, or systemic markers predicting systemic events, a third concept was launched: local plaque characteristics predicting systemic cardiovascular events. The focus with this concept was shifted from the vulnerable plaque to the vulnerable patient.[26-29] With the hypothesis that local atherosclerotic plaque composition is associated with systemic cardiovascular outcome,

the "Athero-Express" study (Differential ATHEROsclerotic plaque EXPRESSion of mRNA and Protein in Relation to Cardiovascular Events and Patient Characteristics) was conducted in 2002.[30] This large, longitudinal biobank contains atherosclerotic plaques from carotid and femoral endarterectomies and is linked to 3-year clinical follow-up.

This thesis will focus on patients after femoral endarterectomy. As explained above, restenosis occurs in a large number of patients after femoral endarterectomy; however, there are still no useful markers predicting restenosis. Secondly, the chance for a life-threatening cardiovascular event in the near future after femoral endarterectomy is impressive. Useful predictive markers are urgently needed. The ultimate goal would be to prevent a second life-threatening event in high-risk patients, for example, by stricter follow-up, closer control of reducing atherosclerotic risk factors, and optimal medical or, eventually, invasive treatment.

The main objective of this thesis was to find plaque characteristics predictive for local (restenosis) and systemic adverse cardiovascular events. Extensive research on the predictive value of the carotid plaque has been performed. Histologic carotid plaque markers have been found to be predictive for restenosis and for future adverse cardiovascular events after carotid endarterectomy.[26,31] Validating the concept that a single atherosclerotic plaque could predict local and systemic cardiovascular events in a second vascular territory (femoral artery) would be important for future incorporation in clinical practice.

OUTLINE OF THIS THESIS

In **Chapter 2** we discuss the use of endarterectomy of the common and superficial femoral artery in one procedure: remote superficial femoral artery endarterectomy (RSFAE). RSFAE has patency rates that are equal to prosthetic bypass surgery, the benefits of minimal invasive surgery, and preserves the option of bypass surgery in the future. The pitfall is the high percentage of restenosis; therefore, predictive markers for restenosis are needed. In **Chapter 3** we discuss the benefits of tissue biobank efforts, with the Athero-Express biobank as an example.

In **Chapters 4**, **5**, and **6**, we identify clinical characteristics that are associated with plaque characteristics. In **Chapter 4** we show that alcohol use is associated with carotid and femoral plaque histology and also with the occurrence of adverse cardiovascular events. In **Chapter 5** we describe clinical characteristics correlated with intraplaque hemorrhage (IPH), a vulnerable plaque feature. In **Chapter 6** we describe different IPH types that are correlated with stable or vulnerable plaque characteristics and suggest that not every IPH type is as "vulnerable" as assumed.

In **Chapters 7**, **8** and **9** we report studies on femoral plaque histology in relation with clinical outcome. In **Chapter 7** we report clinical and plaque characteristics associated with restenosis after femoral endarterectomy. In **Chapter 8** we show that the histology of the common and superficial femoral artery is significantly different and that femoral plaque histology is predictive for restenosis after endarterectomy. Differences in histology could be a reason for

different restenosis rates after endarterectomy of the common or superficial femoral artery. In **Chapter 9** we demonstrate that the dissected femoral plaque hides predictive value for adverse systemic cardiovascular events during follow-up after femoral endarterectomy.

Chapters 10 and **11** contain a summary and discussion and a general Dutch discussion of this thesis.

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PART I

Atherosclerotic plaque as a diagnostic tool

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CHAPTER 2

Remote Superficial Femoral Artery Endarterectomy

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ABSTRACT

Remote superficial femoral artery endarterectomy (RSFAE) is an effective minimal invasive treatment modality of TASC C and D atherosclerotic lesions of the superficial femoral artery (SFA) with at least equal patency rates as supragenicular synthetic bypass grafts.

This procedure is performed through a single femoral arteriotomy and the intima core in the SFA is dissected using the Vollmar ring and the Mollring cutter devices respectively. The intimal core distally of the transaction zone is secured by an expandable polytetrafluoroethylene-covered nitinol stent.

By its minimal invasive character, RSFAE will lead to lower rate of postoperative complications and shorter hospital stay compared to supragenicular bypass graft surgery. Additional advantage in comparison with percutaneous procedures is the opportunity of open endarterectomy of the common femoral and/or profunda artery. Synthetic material will be avoided and vein will be preserved for possibly future cardiovascular surgery. Reobstruction of the SFA tends to have, in contrast to bypass grafts, less severe symptoms due to preservation of collaterals and thereby lower amputation rate.

Achilles heel of RSFAE is the relatively high percentage of first year restenosis due to neointimal hyperplasia. Strict follow-up at 3, 6 and 12 months is advised including duplex ultrasound. In case of symptomatic or asymptomatic hemodynamic restenosis (>50%) percutaneous transluminal angioplasty must be performed to improve long-term patency. The majority of reobstructions can be treated by endovascular means. New endovascular techniques, like balloon cryoplasty or drug eluting stents have to be studied in combination with RSFAE to optimize its technique and improve patency rates.

INTRODUCTION

Atherosclerosis is a systemic disease and segmentally distributed [1]. Total prevalence of asymptomatic peripheral arterial disease is in the range of 3% to 10%, increasing to 15% to 20% in persons aged older than 70 years. The prevalence of intermittent claudication increases with age; 3% in patients aged 40 to 6% in patients aged 60 years.

A minority of patients with chronic intermittent claudication will need major lower limb amputation. Clinical stabilization or improvement of ischemic complaints may be due to the development of collaterals, metabolic adaptation of ischemic muscle, or patients altering gait to favor non-ischemic muscle groups. [2]

Patients with peripheral arterial disease (PAD) typically have multiple cardiovascular risk factors, which puts them at markedly increased risk for cardiovascular events. Treatment of these risk factors, like smoking, hyperlipidemia, hypertension and diabetes markedly reduce their risk of cardiovascular events, as well as their risk of progression to amputation and progression of disease. [2]

In 70% of patients with peripheral arterial occlusive disease, the superficial femoral artery (SFA) is affected, primarily at the adductor hiatus and Hunter canal. Possible explanations of the high incidence of occlusions in this region are the location of the distal SFA between the tight adductor muscles, the S-shaped configuration of the artery at this level and the high incidence of arterial branching causing unfavorable haemodynamic circumstances. [1]

Several treatment options are available for atherosclerotic occlusions of the SFA. The traditional approach has been (supervised) exercise treatment combined with medical and lifestyle management for patients with intermittent claudication and bypass surgery for those patients with extensive occlusive disease and limb-threatening ischemia. However, endovascular techniques are rapidly evolving, which increases the arsenal of less invasive treatment options.

Long-term patency rates for percutaneous interventions of long segmental occlusions (> 10 cm) are nevertheless disappointing. Such long lesions, designated as type C and D according to TransAtlantic Inter- Society Consensus (TASC) classification, require surgical intervention.[2] Venous supragenicular bypass grafts are superior to prosthetic grafts; however, almost half of patients lack a sufficient vein or the vein has been used for previous cardiovascular grafting procedures. [3-8] A combination of surgical debulking of the chronic obstructed SFA without the need for venous or prosthetic grafts is combined in the remote superficial femoral artery endarterectomy (RSFAE). [9]

TECHNIQUE

After exposure of the femoral arterial bifurcation including the proximal SFA by a single vertical groin incision, 5000 IU of Heparin are administered to the patient. After three minutes, only the proximal SFA is clamped to provide uninterrupted flow to the deep femoral artery. Then, a longitudinal arteriotomy is made in the proximal SFA. Endarterectomy plane is defined by a meticulous dissection of the intimal core in the right cleavage plane between the lamina elastica interna and the circular fibers of the media or, preferably, between media and the

smooth lamina elastica externa of the adventitia. Then the intima core is dissected using the Vollmar ring stripper (Vollmar Dissector, Aesculap®, San Francisco, CA, USA) until it reaches the distal limit of the atheroma in the SFA, which has to be done under fluoroscopic guidance. The Vollmar dissector will be removed and exchanged for the Mollring cutter (Mollring Cutter®, LeMaître Vascutek, San Jose, CA, USA; see Figure 1 and 2). The Mollringcutter is a modification of the ringstripper. The metal shaft has a double ring construction at the distal end. Both rings have sharpened inner edges thereby mimicking a pair of scissors as the lower ring shears along the upper ring when a trigger is pulled. This allows transaction of the distal intima core (see Figure 2).

After the atheroma is cut at its distal end, the intima core and Mollringcutter are removed, all under fluoroscopic guidance. A 7 Fr sheath can be advanced in the desobstructed proximal SFA for introduction of steerable hydrophilic guide wires ranging between 0.018 and 0.035 inches to pass the transaction zone distally. The distal transection zone is secured by percutaneous transluminal angioplasty (PTA) with additional stent placement. A commonly used stent is the aSpire stent (aSpire stent®, LeMaître Vascutek, San Jose, CA, USA; see Figure 3), an expandable polytetrafluoroethylene (ePTFE), covered, double- spiral nitinol stent that is flexible yet has sufficient high radial strength to withstand torsional stresses proximal to the knee joint. The open helical design of the aSpire stent offers the possibility of preserving major genicular collateral vessels (see Figure 4).

After stent placement, a completion arteriography must be performed to check that no distal thromboembolic complications have occurred and to verify the patency of the desobstructed SFA; additional embolectomy must be peformed when necessary (see Figure 4). If required, a common femoral artery and profunda artery desobstruction can be performed and the arteriotomy may be closed with or without patch.

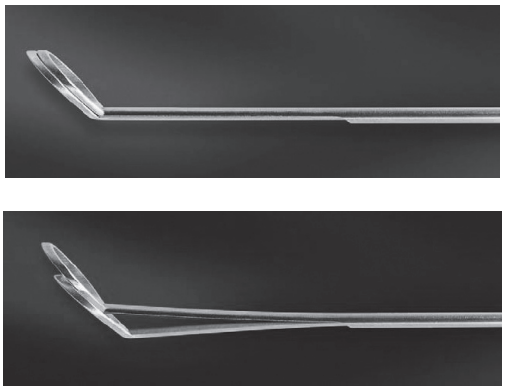

figure 1. *Moll Ringcutter®*

BENEFITS

Remote SFA endarterectomy offers the vascular surgeon a less invasive alternative to bypass graft surgery in patients with chronic long (TASC C and D lesions) segment SFA occlusions. Only a single groin incision is needed, in contrast to a second distal incision that is required

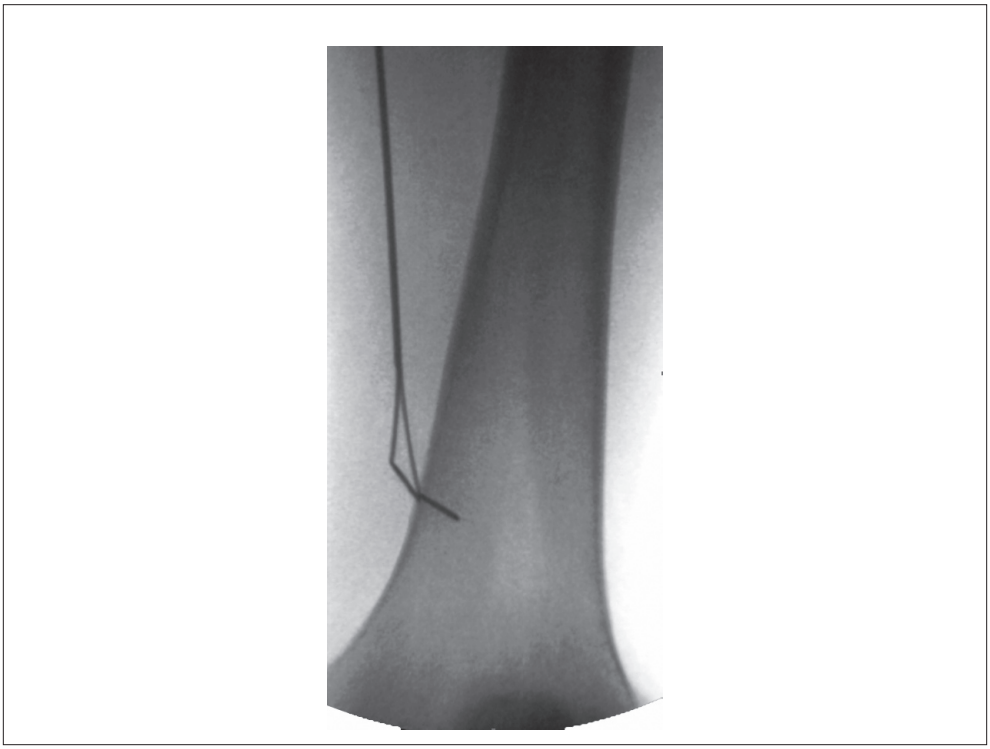

figure 2. *Moll ringcutter under fluoroscopic guidance*

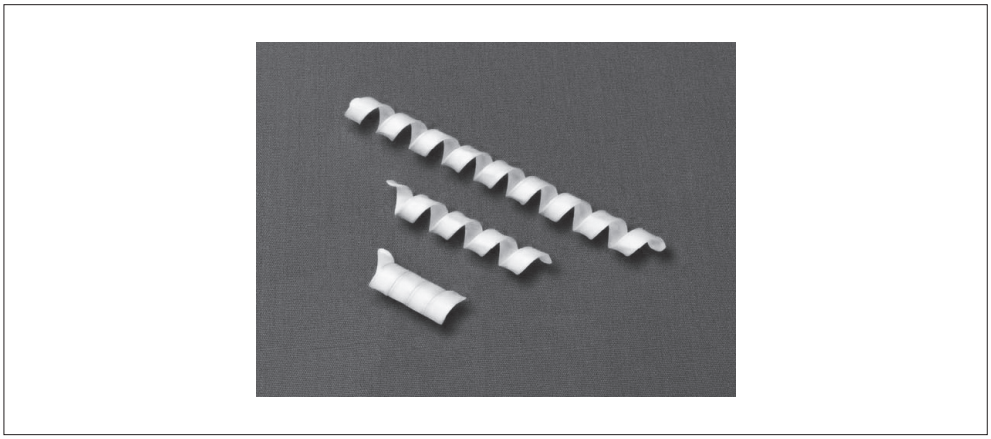

figure 3. *aSpire stent®*

with semi-closed endarterectomy or bypass graft surgery. The potential advantage is a lower rate of postoperative complications related to the surgical wound, such as hematomas, lymphoceles, edema, wound, and graft infections. In addition, RSFAE leads to short-term hospital stay, with more than 50% of patients discharged the second post-operative day, thereby lowering hospital costs. [10]

Other advantages of RSFAE are the possibility of avoiding synthetic material and the use of the ipsilateral greater saphenous vein, which can be preserved for a future cardiovascular grafting procedure. Furthermore, in contrast with (prosthetic) bypass grafting, patients with reocclusions after RSFAE tend to have less severe symptoms compared with patients with supragenicular bypass occlusions, possibly due to the preservation of collaterals during RSFAE.[10-12]

Reobstruction after remote endarterectomy is often preceded by gradual progressive restenosis of the SFA itself, without obstruction of the collateral genicular side branches. This concept was verified in a report by Smeets et al. where 79 reocclusions after remote endarterectomy in 239 patients occurred, but symptoms were mild and only 2 (2,5%) amputations were necessary. [12] This in contrast to occlusion of supragenicalur bypass grafts, which often results in worse symptoms compared with the original operation indication, because of thrombosis of collaterals and runoff vessels and results in a higher amputation ratio. [13] The initial technical success rate of RSFAE is more than 85% in experienced hands.[10, 14] There is a learning curve for this technique. It takes approximately 5-10 operations to master the technique.

Moreover, just because the common femoral artery doesn't lend itself for percutaneous intervention, advantage of RSFAE is the possibility of relatively simple open endarterectomy of the common femoral artery when indicated. Fifty percent of the RSFAE procedures are extended with an additional endarterectomy of the common femoral artery with or without profunda plasty and thereby improving inflow and patency rate of the desobstructed SFA (unpublished data).

RSFAE could also be performed in combination with distal bypass graft surgery. The greater of lesser saphenous veins are the conduits of choice for infrapopliteal limb salvage bypass operations, especially in the presence of a foot ulcer. Unfortunately, the ipsilateral or

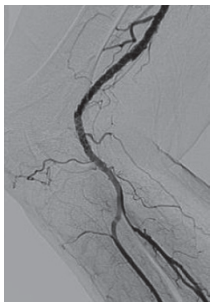

figure 4. *aSpire stent in situ with preservation of collaterals after completion of remote superficial femoral artery endarterectomy*

contralateral saphenous vein has been reported to be of poor quality, previously stripped, or harvested for coronary bypass in up to 50% of patients who require a distal bypass operation. [15, 16] If an infragenicular or femorocrural bypass is necessary and adequate vein is not available, RSFAE and distal bypass with residual sapheneous vein graft is a safe and moderately durable adjunct for limb salvage. The proximal anastomosis of the venous graft is made at the level of the transaction zone in the distal SFA or proximal popliteal artery. [16]

PATENCY

Percutaneous transluminal angioplasty is the preferred choice of treatment in limited disease such as stenoses or occlusions up to 10 cm (TASC A and B lesions). [2] Patency rates for endovascular treatment of long segment occlusions or multiple stenoses of the SFA (TASC type C en D lesions), however, are still disappointing. Published data showed 1-year primary patency rate of 43% and 5-year primary and secondary patency rates of 25% and 41% respectively. [17, 18] Currently, surgical intervention is the appropriate treatment modality for these lesions. [2]

As is already known, venous bypass grafts are superior to synthetic grafts. A recent meta-analysis showed 5-year primary patency rates of 57.4% for supragenicular polytetrafluoroethylene (PTFE) grafts compared with 77.2% for venous grafts. Secondary graft patency was 73.2% for synthetic and 80.1% for venous grafts. All studied patients had intermittent claudication. Patency rates in patients with critical ischemia were approximately 10% lower in both groups. [19]

In the past few years the patency rates of RSFAE have been studied by various authors. In 2004 Rosenthal et all. published the results of a retrospective multicenter trial with primary cumulative patency rate of 68,6% and primary assisted patency rates of 88,5% at 18 months.[14] Two years later Rosenthal et al. reported a 3-year cumulative primary patency rate of 60.6% and an assisted primary patency rate of 70.2% for RSFAE in a population of 210 patients.[11] Martin at al. showed even better results that year, with 3-year primary and assisted primary patency rates of 70% and 76% in more than 100 patients. This study also showed, comparable to peripheral bypass graft trials, that the patency rate was associated with the extent of the disease; patients with claudication did have significantly better patency rates compared to patients with critical limb ischaemia.[10]

Only one prospective study (non-randomized) of the RSFAE was done in the last recent years by Knight et all. Nevertheless, this study reported only short term results with 18-months cumulative patency rates of 60% and assisted primary patency rates of 70%.[20]

So far, randomized trials, comparing RSFAE and femoropopliteal above knee bypass graft surgery, are lacking. Because of that, a multi-centre trial was started at the end of 2005. This recently performed trial in which more than 100 patients were randomized between supragenicular femoropopliteal bypass surgery and RSFAE confirmed that RSFAE is an effective minimally invasive procedure with comparable short-term patency for PTFE grafts, but with significantly shorter hospital stay (unpublished data).

What we may conclude from literature is that remote superficial femoral artery endareterectomy has been shown to be a valuable alternative in the surgical treatment of

long occlusive disease of the SFA. Although venous bypass grafting shows superior patency rates, remote endarterectomy has equal or possibly even better patency rates than prosthetic grafts.

A continuing problem of RSFAE is the relatively high percentage of restenosis during the first year. Ho et al. described that 83% of all restenoses were detected within 1 year and that these lesions were located throughout the entire SFA and not specifically related to the distal part of the artery or within the stent.[21] Another report by the same authors concluded that revision of early (within 1 year) recurrent stenosis significantly improves the mid-term (30 months) patency rates of RSFAE from 60% to 74%. [22] This has been described in graft-surveillance studies as well. Early restenoses will be caused by neointimal hyperplasia, whereas late recurrent stenoses might result from progression of the underlying atherosclerotic disease. These early lesions often lead to failure unless corrected. More than 50% of the non-revised restenoses detected within the first year after RSFAE progressed to reocclusions, whereas late restenoses developing after the first year seem to have a fairly benign course.[22] An intensive duplex surveillance program during the first postoperative year is warranted (3, 6 and 12 months postoperative), with preventive PTA in case of >70% restenoses or symptomatic restenosis >50%. In the near future, research needs to focus on prevention of neointimal hyperplasia in the early postoperative stage, resolving the Achilles heel of remote superficial femoral artery endarterectomy.

FUTURE PERSPECTIVES

Treatment of chronic long-segment occlusions of the SFA remains a difficult task, despite intensive research and newly developed techniques. A distinction must be made between systemic and local future improvements.

The usual medical treatment of patients with peripheral arterial disease includes at least an antithrombotic agent (e.g., aspirin or clopidogrel) and lipid-lowering drugs (e.g., statins) to reduce the risk of cardiovascular events and improve metabolic and endothelial dysregulation. Some studies claim reduced progression of systemic atherosclerotic disease in patients with lipid-lowering therapy.[23] Other studies claim that lowering blood viscosity with pentoxifylline or isovolemic hemodilution will create arteriolar vasodilatation and improvement of endothelial function by inhibition of the sympathic nerve (with L-Arginine) and may add clinical benefits. [2] Unfortunately thus so far, no medicine has the property to stop or improve the progression of atherosclerotic disease.

Testing has begun during the last few years of new-generation stents, including covered nitinol stents or bioabsobable magnesium stents. These stents have the theoretical advantage of removing the long-term stimulus for neointimal hyperplasia from the vessel wall. [24] Expanded PTFE covered stent grafts and drug-eluting stents also show promising patency rates owing to their apparent antineointimal hyperplasia effect. [25] Recent published data show promising results of PTA balloons coated with paclitaxel, a chemotaxis substance, to prevent early restenosis in stenotic or occluded femoral artery disease (mean length of lesion 7.4 ± 6.5 cm). Paclitaxel might inhibit the proliferation of vascular smooth muscle cells due to the exposure of the chemotaxis substance. [26] New endovascular treatment

modalities include cryoplasty, cutting balloon PTA, laser and brachytherapy, or drug-coated balloon PTA, but none of them has proved superiority because of the lack of prospective or randomized studies. [25, 27, 28]

Because of the surgical removal of the intima layer during RSFAE, this technique is preeminently available to combine with the aforementioned new developed techniques because of the direct contact of the proliferative layer (e.g., smooth muscle cells) of the arterial wall with, for example, frost or chemo taxis substance. Studies combining new endovascular techniques with remote endarterectomy of the SFA have been started. Results will be published in the near future.

CONCLUSION

Remote endarterectomy plays a unique role in the treatment of TASC C and D lesions of SFA occlusive disease, combining surgical desobstruction of the common femoral artery and minimal invasive debulking of the entire SFA. Patency rates are at least equivalent with prosthetic bypass grafts. The Achilles heel of this technique remains the occurrence of early restenosis; however, most restenoses can be treated by endovascular means. In addition, consequences of reocclusion are less severe compared with bypass graft surgery. Aggressive follow-up and treatment of restenoses, accordingly, is needed during the first postoperative year to maintain patency. Currently, studies are being performed that combine RSFAE with the latest techniques, such as cryoplasty, to prevent early restenosis and to further improve primary patency rates.

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CHAPTER 3

Tissue biobanks

Principles of Translational Science in Medicine: from bench to bedside.
M. Wehling. 1st edition. Cambridge University. 2010.p101-111

ABSTRACT

Longitudinal studies that include plasma biobanks are being executed on regular basis. Serological protein expression often reflects a disease state and may serve as a surrogate measure for therapeutic efficacy. The combination of well archived and characterized collections of human tissue samples with associated clinical and personal data is less common, but increasingly appreciated for etiological and prognostic studies in specific populations. Biopsies and locally extracted tissue samples contain essential information regarding the pathogenesis of diseases and are considered as the gold standard in diagnostic oncology research, but are rarely collected in the field of cardiovascular medicine. Access to upcoming laboratory technologies and bioinformatics provides ideal circumstances to improve translational research, which makes construction of tissue biobanks an increasingly interesting activity. The combination of genetic, transcript and protein analyses on large numbers of samples will enable study designs that will go far beyond the descriptive level of cross-sectional studies. For instance, imaging studies will benefit from increasing insight in pathological characteristics, which will facilitate the development of new modalities.
The use of tissue biobanks will help to elucidate the function and medical relevance of human genes, proteins and posttranslational protein modifications and will become a central part in the search for the personalized medicinal strategy by integrating the different biological networks in a systems biology approach.

INTRODUCTION

Understanding the aetiology and pathogenesis of human diseases, in order to improve diagnosis and treatment is a major priority for biomedical research. Diseases can be initiated by different stimuli and progress may vary widely among affected individuals. In addition, humans respond heterogeneously when it comes to therapeutic efficacy. The determinants of the wide variations in clinical presentation and response to treatment are multifactorial in origin. Next to many other factors, the knowledge of individuals' molecular content of the affected tissue, the genetic profile, the immunological and neurological responses are also important hallmarks in the pathogenesis of disease. The pharmaceutical industry is struggling with the disease heterogeneity and in search for biomarkers that will help to understand the underlying pathogenesis of disease and its different presentation in subgroups of patients in order to allow personalized medication.

Collaborations between different medical specialties, such as surgery, pathology and epidemiology have inspired the initiation of tissue biobanks. The use of tissue biobanks will be essential to elucidate the function and medical relevance of human mRNAs, proteins and posttranslational protein modifications and will become a central part in the search for the personalized medicinal strategy by integrating the different biological networks in a systems biology approach. [1]

The first biobank studies were based on cross-sectional designs. Characteristics of tissues obtained from diseased patients were associated with those from healthy individuals. Prognostic biobank studies have gained interest, since they provide insights in the pathogenesis of diseases over time. Current upcoming research technologies and bio-informatics consolidate the position of biobanks since they provide knowledge of systems biology and allow high throughput screening of DNA, plasma and tissue samples. [2]

In the field of atherosclerosis, cross-sectional biobank studies on atherosclerotic specimens revealed insights in plaque composition and vulnerability. Atherosclerosis is still the major killer worldwide and is a typical example where insights into progression of the disease are hampered by the cross-sectional design of the existing biobanks. Longitudinal atherosclerotic tissue biobanking studies are needed for this. In this chapter we will discuss the advantages of tissue biobanking and take the dissection and storage of atherosclerotic plaque as an example. Principles and developments of atherosclerotic tissue biobanking and their position in cross-sectional and prognostic translational vascular research will be discussed.

PRINCIPLES AND TYPES OF TISSUE BIOBANKS

Biobank studies are relatively new concepts in medical research. Epidemiologic studies have been initiated to study potential risk factors for cardiovascular diseases for many years. The Framingham Heart Study, founded in 1948, is the longest running multi-generational epidemiologic study on a global scale. [3] The consequences of traditional risk factors on heart disease such as smoking, gender and age have been explored extensively in this study and outcomes have been implemented in guidelines for clinical practice, patient stratification and preventive measures. [4] However, blood biomarker studies may suggest but do not prove the

underlying pathological mechanisms of vascular diseases in the tissue. Biopsies and locally extracted tissue samples hide specific pathological information that may guide the researcher to potential mechanisms and help to elucidate the aetiology and pathogenesis of the disease. Knowledge of disease pathogenesis will facilitate the development of new diagnostic modalities and stimulate drug development and monitoring for therapeutic interference.
Longitudinal tissue biobanks are archived collections of human tissue samples in combination with associated individual information and clinical data. These collections are essential resources to understand the function and medical relevance of human genes and their products as well as to explore biological networks in which they are operating (systems biology). [5] Biobanks can be subdivided, based on type of biological samples, such as DNA, plasma or tissue samples and study design, which could be translational, cross-sectional, retrospective or prospective. The construction of a biobank with plasma or DNA samples with clinical and epidemiological baseline and follow up data is the most common format. [5] Plasma samples enable identification of systemic markers representing progression or regression of the disease and are even more powerful if they can also act as surrogate markers for efficacy of treatment. [6-8] Gene profiles may also serve as fingerprints of the underlying pathophysiology. Differences in DNA profiles have been firmly integrated in the field of oncology for diagnostic and preventive medicine, nowadays.
As mentioned earlier, tissue biobanks are relatively rare regarding cardiovascular diseases. In the field of oncology, tissue biopsies are recognized as the gold standards for disease stage determination and for diagnostic and prognostic purposes. Different tissue phenotypes have been examined in relation to follow up and tumours are scaled on grades of malignancy in a very standardized manner. Oncological tissues are dissected during surgery and always transported to the pathology department for pathophysiological assessments. Due to the pre-existing logistic and operational procedures it is therefore not surprising that most tissue biobanks with a longitudinal study design are of oncological origin. [9] The search for a bioinformatics based systems biology approach that integrates patient characteristics, tissue phenotypes and genetic profiling are common in the oncology research field.

Although tissue biobank research seems a promising initiative to unravel pathologies in the field of oncology and cardiovascular medicine, from an epidemiological perspective, tissue biobanks often suffer from inherent drawbacks. First, as mentioned earlier, the majority of tissue biobanks are cross-sectional, whereas large tissue collections included in longitudinal studies are rare. Cross-sectional studies are predominantly descriptive and do not elucidate the pathology in relation to time. Measurements of drug efficacy on histology and biomarker levels also do not benefit with cross-sectional study designs. Second, often the tissue biobank studies include a selective patient population and control patients are difficult to include for obvious ethical reasons. Since patients may reveal large matches in risk profile and medication use it can be more difficult to correct for confounding and to extrapolate the study outcome to a non-diseased population when biomarker value is suspected. Third, tissues are often dissected in an end stage of the disease, which makes it difficult to draw inferences regarding causality. Fourth, when studies on tissue biobanks are generated in a single centre, indications to operate, referral patterns pre-and post operative medicinal policies and many other factors may hamper the definition of a study cohort. Extrapolation of the study outcome would be

impeded and external validity may therefore be difficult to assess. Finally, serial tissue dissection will often be unethical, which hampers the study on the natural progression of the disease and the test on drug efficacy.

Based on this, the determinants of a successful tissue biobank study are: 1- A longitudinal study design. Longitudinal biobank studies provide better opportunities to study the predictive value of biomarkers for the onset, progression or recurrence of diseases over time. Longitudinal studies are fundamental to get insight in etiological en pathophysiological conditions. However, essential efforts, such as the inclusion of large numbers of patients including case record forms, background medical data and follow up is time consuming and requires a good infrastructure. The inclusion rate and labour to obtain the follow up data with a low "lost to follow up" number are major challenges when tissue biobanks are considered. The incidence of an endpoint may be high since mostly a selected group of diseases patients has been included. In case of oncological or atherosclerotic tissue biobanks, death may regularly occur as an endpoint and patients who are "lost to follow up" therefore merit careful consideration. 2- Complete clinical patient data documentation at entry of the study (baseline) and during follow up. Specifically symptoms reflecting onset and timing of the disease should be clearly documented. Risk factors, family history and detailed monitoring of medication use including dosage and time of prescription (before and after surgery) are of great value, in order to reveal potential confounders. 3- Tissue examination on immunohistochemical, genetic and protein level. A concise protocol including extraction of RNA and protein is a must. Standardized protocols for quantitative assessment of protein and RNA content as well as degradation are necessary for future comparisons and improve the reproducibility. 4- Professional data and tissue management. Modern technical equipment and detailed, well organized (computerized) documentation is essential for comprehensive biobank research. The freezers encompass the full value of the biobank. Poor freezer management and inaccurate sample labelling may be devastating. [10] Professionalized centralized storage of biobanks within an academic centre should therefore be considered. 5- A good and well described informed consent. Well defined informed consents are the basis of confidential tissue biobank research and enlarge the opportunities of biobanks. Unforeseen ethical issues about secondary samples use, data sharing and assignments of ownership and intellectual properties can be avoided when they have been well and clear documented in advance of patients' agreement of participation in the study. [1, 10]

These issues are increasingly relevant with regard to the expanding biobanks. Organized infrastructures and well defined study designs are essential items in this, since biobank research is susceptible and subject to new, advanced technical developments and collaborations, which intend to increase and improve research progression.

DEVELOPMENTS IN BIOBANKING RESEARCH

Upcoming technologies and bio-informatics have enabled high throughput tissue screening and improved possibilities to study biomarkers that are involved in aetiology and prognosis of disease. [11, 12] Improvement in available micro array and proteomic technology in combination with tissue biobanking and data management is a powerful approach for translational studies

and makes it more interesting to consider tissue as a source for larger cohort studies. Tissue is likely to provide accurate and precise molecular fingerprints representing the stage and progression of local disease, whereas systemic plasma values may be influenced by multiple factors like dietary or reflect systemic diseases. In addition, the pharmaceutical industry encourages and benefits from the new technical developments, since basic large tissue screening also allows testing drug efficacy.
Atherosclerosis is a typical example where insights in the progression of the disease are hampered by the lack of longitudinal biobank studies. The underlying pathogenesis should be clarified for the development of diagnostic and prognostic modalities or therapeutic interventions. Longitudinal tissue biobanks may accomplish a fundamental role in this. In the following sections we will focus on atherosclerosis and associated tissue biobanks as an example how systematic collections of pathological tissue and extensive tissue screening may facilitate the research field towards biomarkers and therapeutic targets to monitor, prevent and treat this disease.

ATHEROSCLEROSIS

Atherosclerosis is a systemic disease, which initiates early in life and presents with clinical symptoms generally after 50 years of age. It is still the major cause of morbidity and mortality in the Western world. Despite advances in risk factor management, each year, 12 million patients die world wide because of cardiovascular events such as myocardial infarction or cerebrovascular accident.[13] Intimal thickening or plaque formation in the artery (intra-luminal expansion of the inner layer of the arterial wall) leads to gradual arterial occlusion. Advanced atherosclerotic plaques in arteries that show expansive remodelling, that accommodates the growing plaque and prevents lumen loss, are prone to rupture. Plaque rupture will lead to acute intra-luminal thrombosis and arterial occlusion and clinically presents as myocardial and cerebral infarction or vascular death. Based on the high mortality rates, there is a strong need to unravel the underlying pathology and to identify patients at risk for future cardiovascular events. Atherosclerotic post mortem tissue biobanks have made a significant contribution to the understanding of plaque destabilization, plaque rupture and subsequent plaque thrombosis and luminal occlusion.[14-16]
Research concerning the morphology and development of the atherosclerotic plaque has been done since the time of Hippocrates (469-377 BC).[17] He has described sudden cardiac death and associated symptoms and features from living subjects with features from dead subjects. Over the last century, cross-sectional studies identified different plaque characteristics where plaque rupture occurs. Rudolph Virchow was the first who described cellular inflammatory changes in the atherosclerotic vessel wall as a result of mechanical forces and as part of a consecutive repair mechanism.[18] Virchow suggested that the atheroma is a product of an inflammatory process within the intima and described that fibrous thickening evolved as a consequence of a reactive fibrosis, induced by proliferating connective tissue cells. Among others, Davies extensively studied numerous post mortem obtained atherosclerotic specimen and he described two faces of atherosclerotic plaques; the stable and unstable plaque. A vulnerable (unstable) plaque was characterized by an increased

number of macrophages, increased expression of tissue factor, reduced levels of smooth muscle cells, a lipid core that occupies a high proportion of the overall plaque volume and a thin fibrous cap, lying over the lipid core that can easily rupture, resulting in a thrombus formation. [15] The vulnerable plaque is defined as a plaque that has a high likelihood of rupturing, causing thrombus formation, leading subsequently to ischemic events.
Another biobank study unravelled another pathological substrate for luminal thrombosis. Virmani et al. described one type of luminal thrombus as the disruption of a fibrous cap covering a lipid core, but they also identified superficial erosion as the basis of thrombus formation without plaque rupture or contact with the lipid core. They discovered, in a large tissue biobank study, that 22 out of 50 of their acutely thrombosed coronary artery plaques were superficial erosions that were more prevalent in female patients. [19, 20] These superficial plaques are characterized by irregular and eroded surfaces, lack of endothelial and inflammatory cells and rich of smooth muscle cells, indicating that fibrous plaques and plaques with a thick fibrous cap covering a necrotic core, can still cause acute arterial thrombosis.

Although these pathological observations have made a significant contribution to the understanding of the mechanisms of plaque rupture and luminal thrombosis, conclusions regarding causality and diagnostic value of the described vulnerable plaque characteristics cannot be made. In fact, the natural history of atherosclerotic plaque progression is unknown. The histopathological observations have been made in patients who died of coronary artery disease. This is a highly selective population and other researchers have established that the features that are associated with the phenotype of the vulnerable plaque (thin fibrous cap, inflammation and a large lipid core) are regularly observed throughout the arterial system. In fact, inflammation and large lipid cores are frequently observed at multiple locations in patients who suffer from any form of atherosclerotic disease. Thus, the positive predictive power that a specific so called vulnerable plaque will rupture and lead to thrombotic occlusion is likely to be low. Although follow up studies and imaging modalities, that would allow sequential studies, are lacking, many research programs and animal models share the classical definition of the vulnerable plaque as the gold standard. The earlier defined characteristics of the vulnerable plaque from large autopsy studies have led to a tremendous growth of etiologic transgenic animal models. Moreover, despite lack of evidence that these features have an acceptable predictive value; the current concepts have also been implemented in clinical imaging studies as a surrogate definition of the vulnerable plaque. [21, 22]

IMAGING MODALITIES AND THE PREDICTIVE POWER OF BIOBANKS

Interest to identify the vulnerable atherosclerotic plaque has led to the development of imaging techniques to identify the plaque which is prone to rupture. Tissue biobanks play a significant role in determination of plaque characteristics for imaging studies. Techniques are currently validated and applied that aim detection of one or more of the aforementioned histological characteristics and biomarkers of the rupture-prone plaque (vulnerable plaque). For instance, the presence of atheroma is studied using Raman spectroscopy, Magnetic Resonance Imaging (MRI), or IVUS elastrography. [22-24] Thrombus and lipid-rich plaques can

also be detected by angioscopy that allows visualization of the plaque surface by colour detection. 25 High macrophage density can be detected with thermography that registers temperature rise in the arterial wall and positron emission tomography (PET). [26, 27] Biomarkers may be detected by MRI or photon emission CT 15070807-based plaque imaging. [28, 29]
However, since these images of the vulnerable plaque are based on cross-sectional observations in a selected patient population, it is doubtful whether these visualization techniques will meet the high expectations and whether these techniques will help us detect the lesion at risk for rupture in the general population. 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 occurrence of rupture of the so-called "vulnerable plaque" is unknown. In fact, since prospective tissue biobank studies on predictive value or causality of plaque characteristics have not been performed, we do not know which characteristics contribute to vulnerability. [30] Currently, the phrase "vulnerable plaque" likely raises expectations that may never be met when it comes to risk prediction.
For identification of histopathological characteristics and biomarkers to detect the patient at risk for plaque rupture that could cause clinical ischemic events, we need an atherosclerotic tissue biobank with a longitudinal design. The Athero-Express study introduces a new concept in search for the patient who is at risk for luminal thrombosis due to plaque destabilization.[31]
Due to the multifactorial nature of the disease, it is too simplistic to assume that a single histological feature or systemic biomarker would suffice to identify the vulnerable plaque that will rupture and lead to a clinical event. The longitudinal Athero-Express biobank study investigates the association between local histological and molecular information in the atherosclerotic plaque with clinical outcome.
The combination of prognostic atherosclerotic tissue biobank studies in combination with upcoming technologies and bio-informatics provides a unique format to clarify the underlying pathology and is an important step towards the improvement and development of diagnostic therapeutic modalities with respect to personalized medicine.

ATHERO-EXPRESS BIOBANK

The Dutch Athero-Express study is a vascular tissue biobank including atherosclerotic carotid tissue and associated clinical and personal data. Athero-Express has been established in 2002 by a collaboration of the departments of cardiology and vascular surgery from the University Medical Center in Utrecht and the St. Antonius Hospital in Nieuwegein. In January 2008 more than 1600 patients were included who underwent a carotid or femoral endarterectomy. The tissue biobank has a longitudinal study design with 3 year post-operative follow up. The main objective is to determine the predictive value of local histological plaque characteristics and molecular biomarkers as determinants for local restenosis or future cardiovascular events somewhere else in the body, such as myocardial infarction, stroke or peripheral intervention. This concept is new in the sense that a search is launched for local plaque markers that predict adverse outcome, irrespective of the histopathological description of the plaque. The concept is based on the knowledge that atherosclerosis is a systemic disease. The vulnerable plaque

is not a single local presentation of the atherosclerotic disease within the arterial system. Multiple regions in the body are generally affected and are prone to rupture. The hypothesis is that within each atherosclerotic plaque information is shared about the vulnerability of the entire vascular system.

Before the longitudinal data were analysed the magnitude of the biobank revealed several determinants that influence plaque phenotype. Cross-sectional studies provided the first observations from the Athero-Express biobank and demonstrated differences in plaque phenotype from symptomatic patients compared to asymptomatic patients. Lesions from symptomatic patients (TIA, stroke) demonstrated predominantly atheromatous plaque phenotype in comparison with asymptomatic lesions which showed a more fibrous phenotype.[16] Subsequent studies demonstrated that atherosclerotic lesions from women are associated with a more stable plaque phenotype, indicating that women may benefit less of a carotid endarterectomy. [32] Other parameters that strongly influence plaque characteristics are age, medication use and time between the last clinical event and surgery.

In addition, we have demonstrated that after duration of 5 years the phenotype of restenotic lesions is comparable to the plaque phenotype of primary symptomatic lesions. [33] Hellings and colleagues have demonstrated in another prognostic study that morphological atherosclerotic carotid plaque characteristics are associated with restenosis during four years of follow up. The degree of macrophage infiltration and lipid core size are inversely related to the clinical outcome, which is a remarkable finding since the presence of these morphological characteristics are presumed as high risk markers for local restenosis following endarterectomy. [34] This supports the idea that an inflammatory response in atherosclerotic plaques may be functional. Inflammatory responses and protease activity in all layers of the vascular wall result in expansive arterial remodelling thereby preventing restenosis after surgical intervention. From a clinical point of view, dissection of the inflammatory plaque is relevant due to two reasons; First, this plaque type is more prone to rupture, causing clinical events. Second, patients' prognosis of restenosis is less likely to occur. With respect to the role of biobanks and imaging modalities, these excellent findings would raise opportunities for imaging studies to assess the inflammatory status of the plaque in a non-invasive way, which may have a major influence on clinical practice.

The search for predictive biomarkers in atherosclerotic tissue that predict adverse events in other vascular territories is currently ongoing and the first results look very promising. Comparison of the proteome of plaques from patients that had an event during follow up, revealed several local plaque markers that were associated with cardiovascular events. The ultimate goal is to compose a set of biomarkers that serves as a specific and sensitive fingerprint of the vulnerable plaque to identify the vulnerable patient.

The development of new diagnostic modalities and pharmaceutical drugs would also benefit from the discovery of new biomarkers and ultimately enable identification and treatment of patients who are prone for cardiovascular events. Tissue biobanks fulfil a pivotal role in this first step towards personalized medicine. [35]

SUMMARY

Longitudinal studies that include plasma biobanks are being executed on regular basis. Serological protein expression often reflects a disease state and may serve as a surrogate measure for therapeutic efficacy. The construction of biobanks encompassing human tissues is increasingly appreciated for immunohistochemical expression studies. However, archived collections of human tissue samples in combination with a longitudinal study design are rare for cardiovascular diseases. From a biomarker perspective, tissues are less applicable for screening compared with plasma derived markers and tissue biobanks mostly apply to a selected study cohort suffering from a specific disease which makes detected biomarkers less likely applicable for primary prevention.

Technologies for harvesting and storing tissues have improved significantly as well as high throughput methodologies for studies on differential expression on RNA and protein level. This makes the construction of tissue biobanks an increasingly interesting activity since transcript and protein analyses of large numbers of samples will allow the study design going far beyond the descriptive level of cross-sectional studies.

In this chapter we discussed the advantages of tissue biobanking and took the dissection and storage of atherosclerotic plaque as an example. Principles and developments of atherosclerotic tissue biobank research and their position in cross-sectional and prognostic translational research and the opportunities with respect to systems biology and personal medicine have been discussed.

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PART II

Association of plaque histology with patient characteristics

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CHAPTER 4

The effect of alcohol on atherosclerotic plaque composition and cardiovascular events in patients with arterial occlusive disease

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ABSTRACT

Objectives: To examine the association between alcohol use, the occurrence of cardiovascular events, and plaque phenotype in patients following femoral or carotid endarterectomy for arterial occlusive disease.

Background: Alcohol has been shown to have cardiovascular protective effects, in patients with cardiovascular disease as well as in healthy individuals. Whether alcohol consumption induces changes in atherosclerotic plaque composition, has not been investigated before.
Methods: Consecutive femoral (n = 224) and carotid (n = 693) endarterectomy specimens underwent histologic examination for the presence of collagen, calcifications, smooth muscle cells, macrophages, fat, and intraplaque thrombus. Patients were followed up for 3 years after the initial operation and investigated for the occurrence of cardiovascular events. Primary outcome was the composite end point "major cardiovascular event." Alcohol consumption was categorized as no alcohol use, 1-10 U/wk, or >10 U/wk.

Results: The Kaplan-Meier estimate of the major cardiovascular event rate after 3 years of follow-up in the femoral group was 35% for no alcohol use and 21% for 1-10 U/wk, whereas only 10% of the group >10 U/wk sustained a major cardiovascular event (p = 0.010). The plaques of alcohol consumers in the femoral group contained significantly smaller lipid cores and less macrophage infiltration than in abstainers. In the carotid group the major cardiovascular event rate was similar in all 3 groups and additionally, a difference in plaque composition could not be observed.

Conclusions: This study shows an inverse relationship between alcohol use and major cardiovascular events after endarterectomy for lower extremity arterial occlusive disease, accompanied by a more stable plaque phenotype. However, no such relationship could be observed for patients with cerebrovascular disease.

INTRODUCTION

Moderate alcohol consumption (10-20 U/wk) has been consistently associated with a lower risk for myocardial infarction, stroke, peripheral arterial occlusive disease, and type 2 diabetes.[1-11] In addition to a beneficial effect in patients with known coronary heart disease, a cardioprotective effect of alcohol has been demonstrated in healthy individuals.[1,2,6-10]
The mechanisms behind the cardiovascular protective effects of alcohol are not fully understood, but are thought to be attributable to an anti-inflammatory effect on low-grade inflammatory diseases such as atherosclerosis.[12,13] Alcohol consumption changes the lipid profile, including increases in high-density lipoprotein cholesterol.[14,15] Alcohol also affects thrombus formation by inhibitory effects on platelet aggregation and function combined with increased fibrinolytic activity and lower fibrinogen levels.[16-18] Moreover, alcohol influences antioxidant capacity and insulin sensitivity, thus restraining atherosclerosis.[5,16,19]
Atherosclerosis is a generalized condition and is therefore associated with an increased risk of secondary cardiovascular events when a primary symptomatic lesion has been diagnosed. Whether patients with clinical presentation of lower extremity arterial occlusive disease or cerebrovascular disease would benefit from the beneficiary effects of alcohol consumption remains unknown. Whether alcohol use is associated with plaque composition is also unknown. The objective of this study was to investigate the association between alcohol intake, cardiovascular events, and plaque characteristics in patients undergoing femoral or carotid endarterectomy.

METHODS

Study design of the Athero-Express Biobank

The design of the Athero-Express study has been reported in detail previously.[20] Inclusion for the current substudy was between April 2002 and March 2007. Included were subsequent patients undergoing (1) remote superficial femoral artery endarterectomy or endarterectomy of the femoral bifurcation with or without additional bypass graft for severe intermittent claudication, critical ischemia, or tissue loss (Rutherford category 2-5)[21] with atherosclerotic lesions (TransAtlantic Intersociety Consensus [TASC] A-D)[22] of the femoral artery, and (2) patients undergoing carotid endarterectomy for (a)symptomatic carotid artery stenosis with more than 70% lumen diameter reduction.

Clinical parameters were recorded at baseline. Patients completed a detailed validated questionnaire, including alcohol consumption habits.[20] This questionnaire is based on the validated Rose questionnaire.[23] For the assessment of alcohol intake, a quantity–frequency method was used.[24] Alcohol consumption was a priori categorized as no alcohol use, 1-10 U/wk, or >10 U/wk. Patients consuming > 20 U/wk could not be analyzed separately since this group was too small; it was underpowered to perform statistical analysis. One unit was defined as 12 grams of alcohol. Subgroup analyses were not performed by type of beverage due to lack of such detailed information.

Atherosclerotic Plaque Characterization

During surgery, the atherosclerotic plaques were excised and directly processed in the laboratory for histologic examination. The plaques were evaluated for the presence of collagen, calcifications, smooth muscle cells, macrophages, fat, and thrombus, as described previously, and independent of knowledge on alcohol consumption.[20] The plaque was divided in 5-mm-thick segments along the longitudinal axis. The segment with the greatest plaque burden was subjected to histologic examination. The plaque morphology of this segment has been shown to be relatively good representative and uniform from within each subject.[25] Comparison of the histologic scorings of adjacent segments revealed a mean κ (weighted kappa) of 0.40 (range, 0.33 to 0.60). When the culprit segment was compared with the more distant segment, the mean κ was 0.24; however, in 91% of cases, the difference between the culprit segment and the distal segment was one category or less.[25] Macrophage infiltration (CD-68), smooth muscle cell infiltration (α-actin), the amount of collagen (Picrosirius red), and calcification (hematoxylin and eosin) were semiquantitatively scored as (1) none or minor or (2) moderate or heavy staining. The criteria for classification were defined as follows: for macrophages: (1) absent or minor CD-68 staining with negative or few scattered cells, or (2) moderate or heavy staining, defined as clusters of cells with more than 10 cells present; for smooth muscle cells: (1) minor α-actin staining over the entire circumference with absent staining at parts of the circumference of the arterial wall, or (2) positive cells along the entire circumference of the luminal border; and for collagen staining: (1) none or minor staining along part of the luminal border of the plaque or (2) moderate or heavy staining along the entire luminal border. Luminal thrombus and intraplaque bleeding were examined in hematoxylin and eosin and fibrin stainings (Mallory staining) and rated as being absent or present. The size of the lipid core was visually estimated as the percentage of total plaque area using hematoxylin and eosin and Picrosirius red stainings, with a division in 2 categories of less than 10% and more than 10%. The histologic examination was performed by 2 independent observers who were blinded for the clinical data. The intraobserver and interobserver semiquantitative analysis of atherosclerotic plaque histology is well reproducible.[25] CD68 and α-actin stainings were scored as the percentage of the plaque area using AnalySIS 3.0 software (Olympus, Tokyo, Japan). This computerized analysis revealed an excellent correlation with the semiquantitative observations. The κ values (weighted kappa) for intraobserver variability of fat, macrophages, smooth muscle cells, collagen, calcifications, thrombus, and overall phenotype were 0.83, 0.85, 0.71, 0.63, 0.81, 0.80, and 0.86, respectively, and κ values for interobserver variability were 0.68, 0.74, 0.54, 0.59, 0.82, 0.75, and 0.71, respectively.[25] The difference in κ value between collagen and the other stains is unsubstantial. The Picrosirius red stains are relatively easy to analyze due to good discrimination between areas that are yes/no stained. In addition, collagen often covers a broad percentage of the total plaque area, which is somewhat different for cellular stainings that are expressed for a low percentage of total plaque area.

Follow-up

Surgical intervention in atherosclerotic disease is a sign of disease progression to a severe extend, with a poor prognosis. Patients diagnosed with critical limb ischemia suffer from a 25% mortality rate the first year after presentation due to fatal cardiovascular events.[22]

Accordingly, most events will occur within the first years after surgery. The follow-up of the Athero-Express trial is therefore limited to 3 years, and all patients in this paper are followed up for at least 1 year after the operation. Once a year, patients were asked to complete a questionnaire during the follow-up for 3 years after the initial surgery. The questionnaire inquired about cardiovascular events and hospitalization in the past year. The general practitioner was contacted when patients did not respond. Clinical end points included incidence of cardiovascular and cerebrovascular death, myocardial infarction, coronary artery bypass graft operation, coronary angioplasty, and stroke. When patients revealed an event during follow-up, the correct diagnosis was obtained from discharge summaries and medical records. Two vascular surgeons from different institutions who were blinded for each other's judgment validated cardiovascular events by separately reviewing medical records.
The primary outcome was a composite end point "major cardiovascular event" and included cardiovascular and cerebrovascular death, myocardial infarction, coronary artery bypass grafting, coronary angioplasty, and stroke. Secondary end points were cardiovascular death and a composite end point "all cardiovascular events," including major cardiovascular events and death as described previously, transient ischemic attacks, and peripheral interventions, including peripheral bypasses or desobstruction procedures, aneurysm rupture and repair, renal artery procedures, and leg amputation. The medical ethics boards of the participating hospitals approved the study, and all patients provided written informed consent.

Data analysis

Statistical analysis was performed with SPSS 15.0 software (SPSS Inc, Chicago, IL, USA).
Event rates (the primary and secondary end points) were calculated with Kaplan-Meier life-table estimates in the two cohorts separately. Being interested in the effect on the primary endpoint (major cardiovascular events) of alcohol consumption only, multivariate Cox proportional hazard regression models were used to adjust for possible confounders. Every baseline characteristic (see Table 1) was entered first in the univariate analyses. Only if a baseline characteristic showed an association ($p < 0.1$) with the primary endpoint this characteristic was entered as covariate in multivariate analysis. Univariate and multivariate analysis was performed in the two cohorts apart. Age and sex were included in all multivariate models. Rutherford classification and Ankle-Brachial Index were included in multvariate models of the femoral cohort. Results are presented as hazard ratios with exact 95% confidence interval (CI). A log-rank test, Mann-Whitney U test, χ^2 test, or Spearman's Correlation was used as indicated to compare both groups. A value of $p < 0.05$ was considered statistically significant. The relation with plaque characteristics was studied in a similar manner, using univariable and multivariate models. The latter included adjustments for age, gender, smoking history, diabetes and body mass index.

RESULTS

A total of 1167 consecutive patients in the Athero-Express Biobank were followed up for a minimum of 1 year postoperatively and were assessed for eligibility (Figure 1). Owing to malignant disease, permanent extramural care, or lack of informed consent, 23 of 302 patients

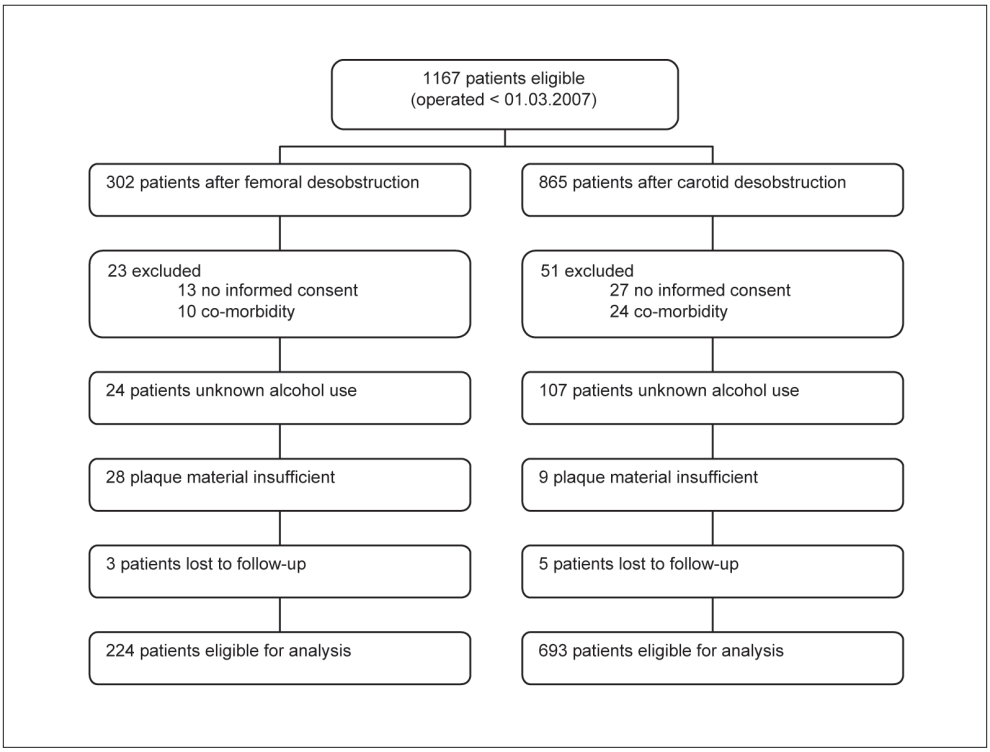


Figure 1. Enrollment of patients

in the femoral group and 51 of 865 patients in the carotid group were excluded. An additional 52 patients in the femoral group and 116 patients in the carotid group were excluded because information on alcohol use was lacking or plaque material was insufficient. Three patients in the femoral group and 5 patients in the carotid group were lost to follow-up. The final analysis therefore included 224 patients operated on for lower extremity arterial occlusive disease and 693 patients operated on for cerebrovascular disease.

Baseline characteristics of the study population are summarized in Table 1a and 1b. In both study groups, patients abstaining from alcohol were significantly older than patients consuming alcohol. Furthermore, significantly more men and more former or current smokers were among the alcohol users in the femoral as well as the carotid group. In the carotid study population, more patients abstaining from alcohol reported a history of coronary artery disease. All shown differences between patients abstaining from alcohol and patients consuming alcohol were linear dose-dependent. In the femoral cohort we observed 35 major cardiovascular events and in the carotid cohort 86 major cardiovascular events during follow-up.

Plaque characteristics in relation to alcohol consumption for both study groups are presented in Table 2. Plaques of the femoral study population revealed larger lipid cores and more macrophage infiltration in patients abstaining from alcohol than in patients consuming alcohol, with a dose-response relationship: 47% of the plaques in the no alcohol group had a lipid core larger than 10% versus 28% for the 1-10 U/wk group and 19% for the >10 U/wk

group (p = 0.002). In the no alcohol group 33% of the plaques showed moderate or heavy macrophage infiltration versus 26% in the 1-10 U/wk group and 17% for >10 U/wk group (p = 0.033). In the carotid study population, such a relationship was not observed. Multivariate analyses did not show materially different results in terms of statistical significance or magnitude of the associations.

Table 1a. Clinical characteristics of the femoral study population in relation to alcohol consumption[a]

	None (n=45)	1-10 U/wk (n=86)	>10 U/wk (n=93)	P value
Age, mean (range), y	69 (48-81)	65 (44-87)	65 (48-84)	.004
Sex				
- Female	17 (37.8)	25 (29.1)	13 (14.0)	.001
- Male	28 (62.2)	61 (70.9)	80 (86.0)	
Body mass index [b], mean (SD)	26 (3.6)	26 (3.4)	25 (3.8)	.364
Current smoker	19 (43.2)	29 (33.7)	32 (34.4)	.441
History of smoking [c]	41 (93.2)	82 (95.3)	92 (100)	.019
Diabetes	9 (20.5)	19 (22.1)	28 (30.8)	.133
Hypertension	32 (71.1)	58 (67.4)	63 (67.7)	.758
Hypercholesterolemia	33 (75.0)	59 (71.1)	64 (68.8)	.473
History of coronary artery disease	21 (46.7)	32 (37.2)	33 (35.5)	.268
History of cerebrovascular disease	22 (48.9)	24 (27.9)	33 (35.5)	.373
History of peripheral artery disease	43 (95.6)	82 (95.3)	89 (95.7)	.944
Indication for operation				
• Rutherford category 3	33 (73.7)	68 (79.1)	67 (72.0)	.423
• Rutherford category 4	10 (22.2)	12 (14.0)	15 (16.1)	
• Rutherford category 5	2 (4.4)	6 (7.0)	11 (11.8)	
TASC classification [d]				
• A - B	14 (31.8)	30 (34.9)	29 (31.9)	.887
• C - D	30 (68.2)	56 (65.1)	62 (68.1)	
Ankle-Brachial Index [e], mean (SD)	0.60 (0.16)	0.58 (0.17)	0.59 (0.19)	.520
Creatinine [f], mean (SD)	120 (86)	100 (44)	95 (30)	.185
HDL [g], mean (SD)	1.08 (0.40)	1.05 (0.29)	1.16 (0.46)	.692
LDL [g], mean (SD)	2.76 (0.98)	2.52 (0.88)	2.56 (0.97)	.799
Statin use	37 (82.2)	63 (73.3)	69 (74.2)	.433
Aspirin use	36 (80.0)	69 (80.2)	75 (80.6)	.923
Oral anticoagulant use	12 (26.7)	22 (25.6)	21 (22.6)	.559
Duration of alcohol use, mean (range), y		44 (19-63)	48 (7-61)	.036
Duration of abstention, mean (range), y	16 (10-38)			

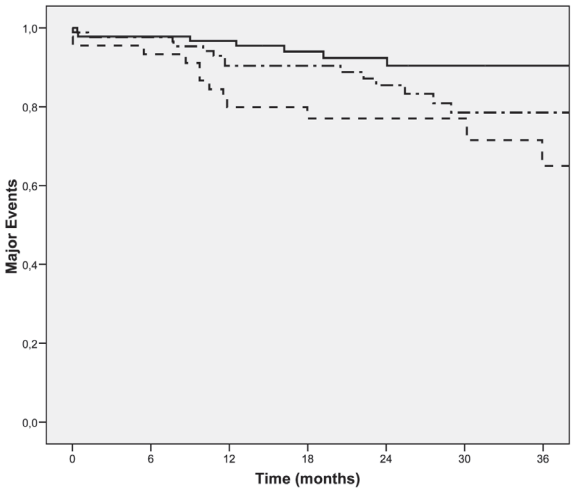
***a**: numbers are presented as No. (%) unless otherwise indicated; **b**: calculated as weight in kilograms divided by height in meters squared; **c**: including current smoking; **d**:TransAtlantic Intersociety Consensus; **e**: calculated as blood pressure at ankle level divided by blood pressure at arm level (mmHg); **f**: expressed in mg/dL; **g**: expressed in mmol/L*

Patients in the femoral group who abstained from alcohol had significantly more major cardiovascular events than those who consumed alcohol after a mean follow-up of 25 months. The Kaplan-Meier estimate of event rate after 3 years of follow-up was 35% for no alcohol and 21% for 1-10 U/wk, whereas only 10% of the >10 U/wk group had sustained a major cardiovascular event (univariate p = 0.010, Table 3a and Figure 2a).

Table 1b. Clinical characteristics of the carotid study population in relation to alcohol consumption[a]

	None (n=210)	1-10 U/wk (n=304)	>10 U/wk (n=186)	P value
Age, mean (range), y	68 (37-88)	67 (43-90)	65 (44-89)	< .001
Sex				
- Female	100 (48.1)	75 (24.9)	28 (15.2)	< .001
- Male	108 (51.9)	226 (75.1)	156 (84.8)	
Body mass index [b], mean (SD)	27 (3.9)	26 (3.8)	27 (3.4)	.186
Current smoker	58 (28.3)	70 (23.3)	54 (29.5)	.853
History of smoking [c]	154 (75.1)	270 (90.9)	168 (91.8)	< .001
Diabetes	48 (23.6)	65 (21.8)	36 (20.2)	.420
Hypertension	150 (73.5)	214 (72.3)	143 (78.6)	.283
Hypercholesterolemia	130 (65.0)	191 (65.9)	127 (70.6)	.261
History of coronary artery disease	76 (36.5)	100 (33.2)	46 (25.0)	.016
History of cerebrovascular disease	204 (98.1)	299 (99.3)	180 (97.8)	.902
History of peripheral artery disease	46 (22.1)	88 (29.2)	38 (20.7)	.840
Duplex stenosis				
• 50-64%	5 (2.4)	11 (3.7)	6 (3.3)	.561
• 65-89%	83 (40.5)	130 (43.3)	79 (43.9)	
• 90-100%	117 (57.1)	159 (53.0	95 (52.8)	
Clinical presentation				
• Symptomatic	168 (80.8)	256 (85)	147 (80.3)	.304
• Asymptomatic	40 (19.2)	45 (15)	36 (19.7)	
Creatinine [d], mean (SD)	90 (30)	103 (55)	98 (30)	.106
HDL [e], mean (SD)	1.12 (0.33)	1.15 (0.35)	1.22 (0.41)	.197
LDL [e], mean (SD)	2.79 (0.93)	2.88 (1.04)	2.89 (1.05)	.808
Statin use	153 (75.7)	221 (74.9)	138 (76.7)	.850
Aspirin use	182 (90.5)	253 (85.8)	164 (91.1)	.945
Oral anticoagulant use	32 (15.9)	41 (13.9)	19 (10.6)	.131
Duration of alcohol use, mean (range), y		49 (8-73)	48 (18-72)	.043
Duration of abstention, mean (range), y	15 (10-55)			

***a**: numbers are presented as No. (%) unless otherwise indicated; **b**: calculated as weight in kilograms divided by height in meters squared; **c**: including current smoking; **d**: expressed in mg/dL; **e**: expressed in mmol/L*

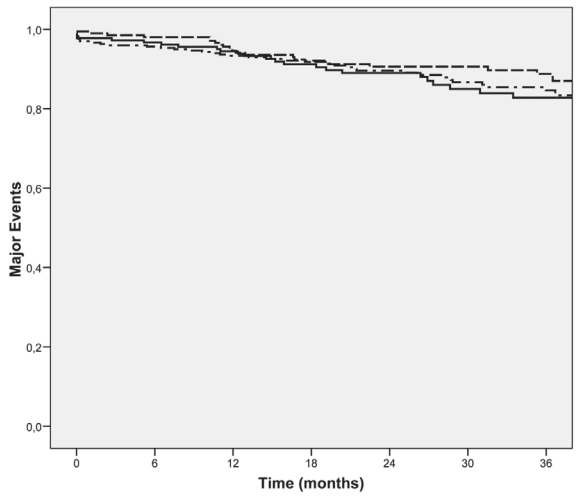


Numbers still at risk	T=0	T=6	T=12	T=18	T=24	T=30	T=36
None (SE)	45	42 (.042)	34 (.060)	27 (.064)	21 (.064)	14 (.080)	10 (.095)
1-10 U/wk (SE)	86	84 (.020)	72 (.032)	58 (.032)	49 (.041)	33 (.054)	20 (.054)
> 10 U/wk (SE)	93	89 (.019)	83 (.019)	60 (.026)	48 (.030)	37 (.036)	20 (.036)

Figure 2a.
Kaplan-Meier survival estimate comparing amount of alcohol consumption with regard to major cardiovascular events in the femoral study population (p = 0.010)

The Kaplan-Meier estimate of event rate after 3 years of follow-up in the carotid group was 11% for no alcohol, 15% for 1-10 U/wk, and 17% for >10 U/wk (univariate p = 0.618, Table 3b and Figure 2b). Adjustment for imbalance in baseline characteristics by use of Cox hazard regression analysis did not alter these results.
The Kaplan-Meier estimate for cardiovascular death rate in the femoral group was 20% for no alcohol, 14% for 1-10 U/wk, and 3% for >10 U/wk at 3 years of follow-up (univariate p = 0.036). Total mortality in the femoral cohort (n = 24) was 25%, 16% and 3% respectively (univariate p = 0.007). In the carotid group, no significant difference was noted in the cardiovascular death rate: 7% for no alcohol, 5% for 1-10 U/wk, and 3% for >10 U/wk (univariate p = 0.235). Total mortality in the carotid cohort (n = 44) was 10%, 7% and 5% respectively (univariate p = 0.281). The event rate for all cardiovascular events by Kaplan-Meier life table analysis in the femoral group was 70% for no alcohol, 57% for 1-10 U/wk, and 53% for >10 U/wk (univariate p = 0.182), and in the carotid group, 21% for no alcohol, 26% for 1-10 U/wk, and 29% for >10 U/wk (univariate p = 0.343).

In the femoral group, with 35 events, univariate analysis showed that age was associated with an increased risk for major cardiovascular events, with a hazard ratio of 1.04 per year of age (95% CI 1.00-1.08; p = 0.039). Other baseline characteristics were not significantly



Numbers still at risk	T=0	T=6	T=12	T=18	T=24	T=30	T=36
None (SE)	210	204 (.010)	183 (.016)	155 (.020)	138 (.021)	105 (.021)	70 (.024)
1-10 U/wk (SE)	304	288 (.012)	269 (.014)	221 (.016)	200 (.018)	141 (.022)	105 (.024)
> 10 U/wk (SE)	186	176 (.013)	159 (.017)	128 (.022)	111 (.025)	79 (.031)	51 (.034)

Figure 2b.
Kaplan-Meier survival estimate comparing amount of alcohol consumption with regard to major cardiovascular events in the carotid study population (p = 0.618)

associated with an altered risk for cardiovascular events.
In multivariate Cox hazard regression analysis, with adjustments for age, gender, smoking, diabetes and body mass index, only alcohol consumption remained independently associated with a decreased risk for major cardiovascular events, with a hazard ratio of 0.52 (95% CI 0.33-0.81; p = 0.004, Table 4).

Table 2. Plaque characteristics versus alcohol consumption in the femoral and carotid group [a]

	Femoral				Carotid			
	Alcohol consumption				Alcohol consumption			
Plaque characteristic	None (n=45)	1-10 U/wk (n=86)	>10 U/wk (n=93)	P value	None (n=210)	1-10 U/wk (n=304)	>10 U/wk (n=186)	P value
Lipid core								
- Lipid core < 10%	24 (53.3)	62 (72.1)	75 (80.6)	.002	46 (22.1)	64 (21.3)	31 (16.8)	.208
- Lipid core > 10%	21 (46.7)	24 (27.9)	19 (19.4)		162 (77.9)	237 (78.7)	153 (83.2)	
Intraplaque thrombus								
- No thrombus	9 (20.0)	30 (34.9)	24 (25.8)	.909	61 (29.3)	79 (26.2)	56 (30.4)	.854
- Thrombus	36 (80.0)	56 (65.1)	69 (74.2)		147 (70.7)	222 (73.8)	128 (69.6)	
Calcifications								
- None or minor	13 (28.9)	36 (41.9)	22 (23.7)	.162	82 (39.4)	137 (45.7)	69 (37.5)	.781
- Moderate or heavy	32 (71.1)	50 (58.1)	71 (76.3)		126 (60.6)	163 (54.3)	115 (62.5)	
Collagen								
- None or minor	6 (14.0)	7 (8.3)	13 (14.6)	.600	39 (18.8)	52 (17.4)	33 (17.9)	.802
- Moderate or heavy	37 (86.0)	77 (91.7)	76 (85.4)		168 (81.2)	247 (82.6)	151 (82.1)	
Smooth muscle cell infiltration								
- None or minor	9 (20.0)	19 (22.1)	22 (23.9)	.604	58 (28.0)	78 (26.0)	61 (33.5)	.264
- Moderate or heavy	36 (80.0)	67 (77.9)	70 (76.1)		149 (72.0)	222 (74.0)	121 (66.5)	
Macrophage infiltration								
- None or minor	30 (66.7)	62 (73.8)	76 (82.6)	.033	81 (39.1)	116 (39.1)	67 (36.4)	.594
- Moderate or heavy	15 (33.3)	22 (26.2)	16 (17.4)		126 (60.9)	181 (60.9)	117 (63.6)	

a: Categoric data are presented as No. (%) unless otherwise indicated

Table 3a. Primary endpoint of the femoral cohort [a]

Femoral cohort	None (n=45)	1-10 U/wk (n=86)	>10 U/wk (n=93)	P value
Major cardiovascular events	35%	21%	10%	.010
• Cardiovascular death	14%	11%	3%	.049
• Cerebrovascular death	7%	3%	0%	.015
• Myocardial infarction	9%	0%	1%	.090
• Coronary artery bypass grafting	3%	0%	2%	.393
• Coronary angioplasty	6%	6%	4%	.869
• Stroke	0%	2%	0%	.201

a: Kaplan-Meier estimate of event rate after 3 years of follow-up

Table 3b. Primary endpoint of the carotid cohort [a]

Carotid cohort	None (n=210)	1-10 U/wk (n=304)	>10 U/wk (n=186)	P value
Major cardiovascular events	11%	15%	17%	.618
• Cardiovascular death	6%	4%	3%	.503
• Cerebrovascular death	1%	0%	0%	.132
• Myocardial infarction	2%	1%	5%	.198
• Coronary artery bypass grafting	0%	2%	3%	.318
• Coronary angioplasty	2%	6%	4%	.361
• Stroke	4%	3%	7%	.148

***a**: Kaplan-Meier estimate of event rate after 3 years of follow-up*

Table 4. Multivariate cox hazard regression analysis for the femoral study population regarding major cardiovascular events

	Hazard Ratio (95% CIa)	P value
Sex	1.18 (0.53-2.65)	.683
Age	1.03 (0.99-1.07)	.126
Rutherford classification	1.04 (0.50-2.18)	.920
Ankle-Brachial Index [a]	1.20 (0.58-8.85)	.743
Alcohol	0.52 (0.33-0.81)	.004

***a**: Calculated as blood pressure at ankle level devided by blood pressure at arm level (mmHg); CI: confidence interval.*

DISCUSSION

This study describes the effects of alcohol consumption in relation to plaque characteristics on the occurrence of future cardiovascular events in patients operated on for lower extremity arterial occlusive disease and in patients operated on for cerebrovascular disease. Our results suggest a protective, dose-related effect of alcohol consumption on major cardiovascular events and death risk in patients with lower extremity arterial occlusive disease. For patients with cerebrovascular disease, such a relationship could not be observed. The fact that no relationship could be demonstrated between alcohol consumption, plaque composition and cardiovascular events could be due to lack of power. However, with 700 patients in the carotid group, and significant variability in plaque types, the carotid cohort was sufficiently powered to detect differences between alcohol groups. The cardiovascular death rate in the carotid group was 7% for > 10 U/wk, 5% for 1-10 U/wk and 3% for no alcohol. This could be interpreted as a trend; however, as stated before, we feel the carotid cohort was sufficiently powered to detect differences between alcohol groups. The atherosclerotic plaques of patients in the femoral group who abstained from alcohol contained more inflammation and larger lipid cores, which are characteristics of vulnerable plaques. A linear relation was distinguished

between histologic markers for plaque stability and alcohol consumption. Again, in the carotid group, such a difference was not observed.

Our results are in concordance with previous studies where alcohol has consistently been associated with a decreased risk of clinical manifestations of atherosclerosis. The effects of alcohol on high-density lipoprotein cholesterol, fibrinogen, thrombogenicity, and insulin sensitivity have been confirmed in randomized trials. These proven anti-atherosclerotic effects of alcohol suggest that the observed inverse association between alcohol use and cardiovascular events is causal.[14-19] Plaque size and intima-media thickness have been studied in relation to alcohol consumption. In most of these previous studies, however, no correlation was observed between alcohol consumption and the atherosclerotic plaque burden.[17, 26-30] In addition, calcification of coronary and aortic plaques determined by computed tomography scan was not associated with alcohol consumption.[31-33] A possible explanation for the difference in observed effects of alcohol consumption on atherosclerotic disease in our study might be that we studied clinical end points and histologic characteristics of atherosclerotic disease, whereas these imaging studies focused on the quantitative measures of atherosclerotic disease. Our observed inverse association between histologic characteristics of vulnerable plaques and alcohol consumption suggests that not plaque amount -but plaque composition- is influenced by the use of alcohol.

Previous studies have demonstrated that symptomatic cerebrovascular disease is related to plaques with a large lipid core and heavy macrophage infiltration.[34,35] Why alcohol is associated with stability of femoral plaques and not with characteristics of carotid plaques remains to be investigated. An explanation could be that lower extremity arterial interventions are generally initiated in a more advanced stage of atherosclerosis than cerebrovascular disease, with a worse prognosis. Patients presenting with chronic critical limb ischemia have a 20% mortality in the first year after presentation, whereas the mortality rate for patients with cerebrovascular disease is 27% at 6 years of follow-up.[22,36] This is supported by the fact that the event rate was much lower in all 3 alcohol groups of the carotid cohort for both the primary and secondary endpoints. Alcohol might influence the stabilization process during aging of the atherosclerotic plaque. We have no data yet to support this. Our results are consistent with recent literature, as in a study by Andersen et al. alcohol consumption did not affect survival in patients suffering from stroke.[37] In addition, the vascular biology of femoral atherosclerosis may not be identical to carotid artery pathophysiology. It remains unknown, if alcohol intake-sensitive pathways potentially might serve a more important role in lower extremity arterial lesions.

Our study has several potential limitations. Although multivariate analysis was used to test many possible confounders, we cannot exclude the possibility of unknown residual confounders. Nonetheless, a beneficiary effect of alcohol has also been demonstrated in experimental trials.[14,15,18,38] We relied on self-reported alcohol consumption. It is difficult to exclude a healthy effect bias, because healthy individuals are more likely to engage in social drinking. However, unhealthy participants might be under-reporting, suggesting that protective effects could occur at even higher intake levels than currently assumed.[24] We did not asses alcohol consumption during the follow-up period of 3 years. Theoretically, changes in drinking habits might have occurred between the carotid and femoral group during follow-up. However, we feel this is very unlikely, because all patients routinely received the same advice regarding

alcohol moderation. Furthermore, for change in alcohol consumption to explain the finding, this change needs to be related to risk of death and needs to have occurred predominantly in the femoral group. Moreover, at baseline we assessed changes in drinking behavior in the past, and there were no differences between groups (data not shown).
Careful interpretation of the data is warranted. This was not a randomized controlled trial and no therapeutic implications can be made from these data. Alcohol consumption also has potential health risks. Alcohol is causally related to cancers of the oral cavity, esophagus, larynx, breast, colon, and liver, and also to hypertension, liver cirrhosis, chronic pancreatitis, injuries and violence, and cardiomyopathy and arrhythmias.[39,40] In this perspective, abstainers should not be encouraged to consume alcohol, and patients consuming moderate amounts of alcohol should not be encouraged to abstain.

CONCLUSION

This study shows an inverse relationship between alcohol use and cardiovascular events in patients who undergo endarterectomy for lower extremity arterial occlusive disease, but not in patients after endarterectomy for cerebrovascular disease. This is the first study to show that a lower risk for adverse events in patients with lower extremity arterial occlusive disease who consume alcohol is accompanied by differences in plaque composition. In the femoral arteries, alcohol abstainers revealed more atheromatous and inflammatory plaque characteristics, known attributes of unstable plaques.

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CHAPTER 5

Age and coumarin-type anticoagulation are associated with the occurrence of intra plaque hemorrhage, while statins are associated less with intraplaque hemorrhage

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ABSTRACT

Introduction: Intraplaque hemorrhage (IPH) is an important determinant of progression and destabilization of atherosclerotic plaque. We recently demonstrated that IPH is an independent predictor of future cardiovascular events after carotid endarterectomy. Thus far, it is unknown whether clinical patient characteristics, such as medication use, are associated with the occurrence of IPH. The purpose of this study was to examine the association of IPH with clinical patient characteristics.

Methods and Results: 1070 consecutive patients who underwent a carotid (n=794) or femoral (n=276) endarterectomy were included. Endarterectomy specimens were subjected to histopathological examination. IPH was observed in 644/794 (81%) carotid and 175/276 (63%) femoral plaques. Carotid IPH was positively correlated with advanced age (69 years [IQR: 62-75] vs. 65 years [IQR: 57-73]; P=0.002) and coumarin-type anticoagulation use prior to operation (104/116 [90%] with coumarin derivatives vs. 540/678 [80%] without coumarin derivatives; P=0.01). Carotid IPH was less frequently observed in patients that used statins prior to endarterectomy (468/595 [79%] with statin vs. 176/199 [88%] without statin; P=0.002). In multivariate analysis, age, coumarin-type anticoagulation use and statin use were independently correlated with carotid IPH. No association was observed between femoral IPH and clinical patient characteristics.

Conclusion: Advanced age and coumarin-type anticoagulation use are associated with the occurrence of IPH, while statin use is associated with less IPH.

INTRODUCTION

Atherosclerosis is a systemic progressive disease and the development of atherosclerotic plaque is a dynamic process. The role of intraplaque hemorrhage (IPH) in the development of atherosclerotic plaque has been recognized for several decades.[1-3] Repeating IPHs contribute to lipid-core expansion through the accumulation of free cholesterol from erythrocyte membranes.[4] In addition, IPH is a potential stimulus for macrophage activation and foam cell formation, thereby increasing plaque inflammation.[4, 5] IPH therefore contributes to both plaque progression and destabilization.

Furthermore, we recently demonstrated that IPH in the dissected carotid endarterectomy specimen is associated with major systemic cardiovascular events, like stroke, myocardial infarction and cardiovascular death, in the first 3 years after carotid endarterectomy.[6]

It is unknown whether patient related characteristics, such as medication use and classical atherosclerotic risk factors, are involved in the occurrence of IPH. Since IPH is a predictor of systemic cardiovascular events and is associated with plaque progression and destabilization, identification of patient characteristics that are associated with IPH may have clinical importance and will give important insight in the process of atherosclerotic disease progression. The purpose of this study was to examine the association of IPH with clinical patient characteristics.

METHODS

Study population

All patients were included in the Athero-Express Biobank study. The design of the Athero-Express study has been described previously.[7] Dissected carotid and femoral plaques, obtained by endarterectomy in two participating Dutch teaching hospitals, were collected and subjected to histopathological examination. In addition, clinical baseline characteristics of all included patients were obtained. The medical ethics boards of both participating hospitals approved the study and all patients provided written informed consent.[7]

1070 consecutive patients who underwent an endarterectomy of the carotid (n=794) or femoral (n=276) artery and were operated between April 2002 and February 2008 were included. The criteria to perform carorid endarterectomy for asymptomatic and symptomatic patients were based on the recommendations published by the Asymptomatic Carotid Surgery Trial (ACST) and the North American Symptomatic Carotid Endarterectomy Trial (NASCET) and European Carotid Surgery Trial (ECST).[8-10] Indications for primary carotid endarterectomy were reviewed in a multidisciplinary vascular team. With respect to femoral endarterectomy, patients underwent a sole endarterectomy of the common femoral artery or remote endarterectomy of the superficial femoral artery to treat severe intermittent claudication, critical ischemia or tissue loss (Fontain classification IIb-IV).[11]

Tissue processing

Processing and examination of the dissected atherosclerotic plaques has been described previously.[7] Directly after excision, the atherosclerotic plaque was transferred to the laboratory.

The segment with the greatest plaque burden (culprit lesion) was fixed in formaldehyde 4%, decalcified in ethylenediaminetetraacetic (EDTA), embedded in paraffin and cut into 4 µm sections for histological and immunohistochemical analysis as described previously.[7]

Histopathological criteria

Plaque characteristics were scored semi-quantitatively as described previously.[7] Plaques were categorized as no/minor staining or moderate/heavy staining for the following stains: HE (calcification), Sirius Red (collagen), CD68 immunostain (macrophages) and alpha-actin immunostain (smooth muscle cells). Using the HE and Sirius Red stains, the size of the lipid core was estimated as percentage of total plaque area and divided into three categories: <10%, 10-40% and >40%. Plaque microvessel density (MVD) was determined by the average number of CD34-immunopositive microvessels of three hotspots within every plaque as described previously.[6] The median MVD of all plaques was calculated and plaques were divided into two groups: average MVD higher or lower than the overall median MVD of all plaques.[6] We based our scoring method for IPH on published and accepted studies.[1, 12] IPH was scored using Hematoxylin and Eosin (HE) and fibrin (Mallory's phosphotungstic acid-hematoxilin) stains and scored as being "present" or "absent." Recently, we have demonstrated that our semi-quantitative analysis of atherosclerotic plaque histology is well reproducible, both intraobserver and interobserver.[13] In addition, alpha-actin and CD68 immunostains were analyzed quantitatively using computerized analyses and results highly correlated with the semi-quantitative analyses.[13]

Statistical analysis

Statistical analysis was performed with SPSS version 15.0 (SPSS Inc, Chicago, Illinois). A Chi- Square test was used to compare categorical variables. The Mann-Whitney U test was used to compare continuous variables with categorized variables. Multivariate binary logistic regression analysis (with backward exclusion of non significant variables using Log Rank test) was used to test the independent association of univariate variables with presence of IPH. Variables showing an association with IPH in univariate analysis (P<0.10) were included in the multivariate analysis. P-values < 0.05 were considered statistically significant.

RESULTS

Patient population

Baseline clinical characteristics are summarized in table 1. Eighty-four percent of the carotid endarterectomy patients presented with symptoms of cerebral ischemia prior to operation (cerebrovascular accident or transient ischemic attack). Seventy-two percent of the patients operated on the femoral artery suffered from disabling claudication (Fontain class IIb) and 28% from critical ischemia or tissue loss (Fontain class III and IV). Femoral endarterectomy patients were more current smokers (P=0.001) and used more coumarin-type anticoagulation (P<0.001) compared to carotid endarterectomy patients. Carotid endarterectomy patients used more anti-platelet agents (P<0.001) than femoral patients.

Table 1. Patient characteristics *

	Carotid (N=794)	Femoral (N=276)
Age; mean (SD)	67 (9.2)	67 (9.0)
Gender		
Female	247 (31)	76 (28)
Male	547 (69)	200 (72)
Symptoms pre-operatively		
Asymptomatic	128 (16)	-
Symptomatic †	666 (84)	-
Fontain class IIb	-	199 (72)
Fontain class III	-	41 (15)
Fontain class IV	-	36 (13)
Diabetes mellitus	184 (23)	79 (29)
Hypercholesterolemia	514 (65)	188 (68)
Hypertension	586 (74)	188 (68)
Current smoking	243 (31)	116 (42)
Anti-platelet agent use §	738 (93)	231 (84)
Coumarine derivative use ‖	116 (15)	67 (24)
Statin use	595 (75)	203 (74)

**Data are presented as No. (%) unless otherwise indicated, † Cerebrovascular accident or transient ischemic attack, § Acetylsalicylic acid and/or dipyridamol and/or clopidogrel, ‖ Acenocoumarol or Fenprocoumon*

Plaque characteristics and intraplaque hemorrhage

IPH was observed in 644/794 (81%) carotid endarterectomy specimens. In femoral endarterectomy specimens, IPH was observed in 175/276 (63%).

As expected according to current insights, we observed an association between IPH and histopathological features of an unstable plaque phenotype. Overall presence of carotid IPH was associated with a large lipid core, no or minor staining of smooth muscle cells, moderate to heavy calcification and high MVD (Table 2). In the femoral plaque, IPH was associated with macrophages (Table 2). No association was observed between overall presence of femoral IPH and other measured plaque characteristics, although there seemed to be a trend with no or minor collagen and high MVD. Thus, predominantly in carotid plaques, IPH was associated with histopathological characteristics of the unstable plaque.

Clinical characteristics and intraplaque hemorrhage

Table 3 shows clinical patient characteristics in relation to presence or absence of carotid IPH. Patients with carotid IPH were significantly older compared to patients without carotid IPH (69 years [IQR: 62-75] *vs*. 65 years [IQR: 57-73] respectively; P=0.002). Plaques from patients using statins prior to carotid endarterectomy revealed less IPH (468/595 [79%]) compared to plaques from patients who did not use statins (176/199 [88%]; P=0.002). In the carotid

Table 2. Carotid and femoral IPH vs. other plaque characteristics *

	Carotid			Femoral		
	No IPH	IPH	P-value	No IPH	IPH	P-value
Lipid core						
no	46 (30)	127 (20)	0.001 †	76 (75)	119 (68)	0.23
<40%	67 (45)	288 (45)		21 (21)	50 (29)	
>40%	37 (25)	229 (35)		4 (4)	6 (3)	
Macrophages						
no/minor	61 (41)	247 (38)	0.60	85 (84)	127 (73)	0.03†
moderate/heavy	89 (59)	397 (62)		16 (16)	48 (27)	
Collagen						
no/minor	23 (15)	117 (18)	0.41	9 (9)	29 (17)	0.08
moderate/heavy	127 (85)	527 (82)		92 (91)	146 (83)	
Smooth muscle cells						
no/minor	26 (17)	196 (30)	0.001 †	22 (22)	39 (22)	0.92
moderate/heavy	124 (83)	448 (70)		79 (78)	136 (78)	
Calcification						
no/minor	76 (51)	240 (37)	0.003 †	36 (36)	54 (31)	0.42
moderate/heavy	74 (49)	404 (63)		65 (64)	121 (69)	
Vessel density						
No/ low density	81 (58)	305 (50)	0.025 †	47 (57)	60 (48)	0.09
Increased density	58 (42)	308 (50)		36 (43)	66 (52)	

*Data are presented as No. (%); † P<0.05

patients, coumarin-type anticoagulation use prior to operation was positively correlated with presence of carotid IPH (104/116 [90%] with coumarin derivatives vs. 540/678 [80%] without coumarin derivatives; P=0.01; table 3). Other medication types, like anti-diabetics and anti-hypertensive's showed no differences (data not shown).

In femoral endarterectomy specimens, no association was found between clinical patient characteristics and overall presence of IPH (Table 3).

Multivariate analysis

To test whether the observed clinical patient characteristics were independently associated with carotid IPH, a multivariate analysis was performed. Age (Odds ratio (OR): 1.03 [1.01-1.05]; P=0.003), coumarin-type anticoagulation use (OR: 1.99 [1.05-3.74]; P=0.03) and statin use (OR: 0.52 [0.32-0.85]; P=0.009) were all independent variables associated with carotid IPH (Table 3). Gender, symptoms prior to operation (e.g. stroke or transient ischemic attack), diabetes mellitus, hypercholesterolemia, hypertension, smoking and anti-platelet agent use were not (independently) associated with carotid IPH.

Table 3. Carotid and femoral IPH vs. clinical characteristics*

	Carotid					Femoral		
	No IPH	IPH	P-value**	Multivariate analysis OR [95%] ‖	P-value #	No IPH	IPH	P-value**
Age; median (SD)	65 (10)	68 (9)	0.002 †	1.03 [1.01-1.05]	0.003 †	67 (10)	68 (8)	0.22
Gender								
Female	54 (36)	193 (30)	0.15	-	-	32 (32)	44 (25)	0.24
Male	96 (64)	451 (70)				69 (68)	131 (75)	
Symptoms pre-operatively					-			
Asymptomatic	26 (17)	102 (16)	0.25	-		-	-	-
Symptomatic	124 (83)	542 (84)				-	-	
Fontain class IIb	-	-	-	-	-	72 (71)	127 (73)	0.77
Fontain class III	-	-				14 (14)	27 (15)	
Fontain class IV	-	-				15 (15)	21 (12)	
Diabetes mellitus								
No	121 (81)	489 (76)	0.22	-	-	75 (74)	122 (70)	0.42
Yes	29 (19)	155 (24)				26 (26)	53 (30)	
Hypercholesterolemia								
No	46 (31)	234 (36)	0.19	-	-	26 (26)	62 (35)	0.10
Yes	104 (69)	410 (64)				75 (74)	113 (65)	
Hypertension								
No	48 (32)	160 (25)	0.07	1.50 [1.01-2.24]	0.05	39 (39)	49 (28)	0.07
Yes	102 (68)	484 (75)				62 (61)	126 (72)	
Current smoking								
No	103 (69)	448 (70)	0.83	-	-	64 (63)	96 (55)	0.17
Yes	47 (31)	196 (30)				37 (37)	79 (45)	
Statin use								
No	23 (15)	176 (27)	0.002 †	0.52 [0.32-0.85]	0.009 †	26 (26)	47 (27)	0.84
Yes	127 (85)	468 (73)				75 (74)	128 (73)	
Anti-platelet agent use ‡								
No	6 (4)	50 (8)	0.11	-	-	15 (15)	30 (17)	0.62
Yes	144 (96)	594 (92)				86 (85)	145 (83)	
Coumarin derivative use §								
No	138 (92)	540 (84)	0.011 †	1.99 [1.05-3.74]	0.03 †	72 (71)	137 (78)	0.19
Yes	12 (8)	104 (16)				29 (29)	38 (22)	

** Data are presented as No. (%) unless otherwise indicated; **P-value calculated by Chi- Square test or Mann-Whitney U test; # P-value calculated by the Binary Logistic Regression Analysis; ‡ Acetylsalicylacid and/or dipyridamol and/or clopidogrel; § Acenocoumarol or Fenprocoumon; † P<0.05; ‖ Variables showing an association with presence of IPH in univariate analysis (P<0.10) were included in the multivariate analysis (Binary Logistic Regression model); OR = Odds Ratio*

DISCUSSION

To the best of our knowledge this is the first large histopathological study in which clinical patient characteristics are related with IPH. IPH is an important determinant of progression and destabilization of the plaque and is a predictor for adverse cardiovascular events in the future.[1-6] From a clinically point of view it therefore is important to determine clinical patient characteristics that are associated with IPH. The results of the present study show that advanced age and the use of coumarin-type anticoagulants are associated with the occurrence of IPH and statin use is associated with less IPH.

We observed IPH in 81% of the carotid and in 63% of the femoral endarterectomy specimens. A highly variable incidence of carotid IPH has been reported in previous studies, ranging from 7.5% to 92.1%.[14] However, previous studies were mostly based on small numbers or on macroscopic plaque examination.[15, 16]

IPH and age

We observed an independent positive correlation between advanced age and overall presence of IPH. With increasing age, carotid atherosclerotic plaques become more atheromatous and the extent of plaque macrophage infiltration and matrix metalloproteinases tend to increase.[17, 18] Our observation indicates that the destabilization of atherosclerotic plaque during aging is accompanied by an increased presence of remnants of IPH, implying that in most plaques IPH is a repetitive ongoing process, rather than a single bleeding event.

IPH and statins

We observed a negative correlation between statin use and IPH, supporting earlier results of Pucci and coworkers.[19] In a mouse model, it has also been demonstrated that statin treatment reduces the frequency of IPH thereby inducing plaque stabilization.[20] Previous studies have already demonstrated that statins reduce the size of the lipid core and increase the amount of smooth muscle cells and collagen.[21, 22] In addition it has been demonstrated that statins reduce plaque inflammation and a decrease in biological activity associated with inflammation (e.g. cytokines, matrix metalloproteinases and cyclooxygenases) has been described after statin use.[23] Furthermore, a decrease in intra plaque neoangiogenesis has also been described after statin treatment. [19] The combination of reduced inflammation with reduced microvessel formation may be a possible explanation for the observed decrease in IPH after statin use.

IPH and coumarin-type anticoagulation

A positive correlation was observed between coumarin-type anticoagulation use and IPH. Patients taking coumarin-type anticoagulants are at risk of bleeding, e.g. cerebral hemorrhage.[24] Our observations suggest that also in the carotid atherosclerotic plaque the risk of bleeding is increased in patients on coumarin-type anticoagulation. We observed a positive correlation between IPH and plaque MVD. Plaque neovessels are usually thin walled without surrounding smooth muscle cells. These fragile vessels have been described to be leaky and are regarded as source of IPH. [4] Animal studies showed that capillary dilatation and

permeability, as well as extravasation off red blood cell, or capillary bleeding are increased by coumarin-type anticoagulants. [25-27] A recent video capillary microscopy study of human nail fold capillaries showed capillary bleedings in 74% of patients on coumarin-type anticoagulation, while only 2% of controls revealed bleedings of the capillaries.[28] A possible explanation for our finding might be that coumarin-type anticoagulants also induce increased hemorrhage from the fragile microvessels in atherosclerotic plaque.

Clinical relevance

Our results may have clinical consequences. The goal of medical treatment of patients with a carotid stenosis is to stabilize the plaque, thereby preventing an ischemic event. The role of statins in patients with advanced atherosclerotic disease is evident since statin use is associated with plaque stabilization, which is underlined by the results of this study.

Although the role of oral anticoagulation therapy in primary and secondary prevention of stroke in patients with atrium fibrillation has been convincingly demonstrated, its role in the prevention of non-cardioembolic stroke and TIA is still a matter of debate.[29] Since IPH is related to plaque progression and destabilization our observed association between coumarin use and IPH suggests that coumarins should not be the first choice of therapy for non-cardioembolic stroke and TIA.

We did not find a relation between clinical patient characteristics and femoral IPH. A possible explanation could be the fact that femoral plaques are mostly dissected from totally occluded arteries, while the carotid arteries are not totally occluded. Therefore femoral plaques are more “end stage” fibrotic plaques, while carotid plaques are dynamic with repetitive IPHs. In recent years it has been demonstrated that high resolution MRI studies can detect IPH, which opens possibilities to explore IPH progression in an earlier stage of the disease.[30]

Limitations

In our study design we investigated only the culprit lesion and not the entire plaque. However, we previously demonstrated that the culprit lesion is representative for the total plaque burden.[13] Secondly, we did not have specific information about the duration of medicine intake prior to operation.

CONCLUSION

Advanced age and coumarin-type anticoagulation are associated with the occurrence of IPH, while statins are associated with less IPH.

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CHAPTER 6

Different stages of intraplaque hemorrhage are associated with different plaque phenotypes: a large histopathological study in 794 carotid and 276 femoral endarterectomy specimens

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ABSTRACT

Background and Purpose: Intraplaque hemorrhage (IPH) is an important determinant of progression and destabilization of atherosclerotic plaque. We recently demonstrated that IPH is an independent predictor of cardiovascular events. IPH has become more clinically relevant since magnetic resonance imaging (MRI) technique is able to visualize IPH in vivo. Different stages of IPH have been described. However, etiology of the different stages is not known and it is unclear if these detected different stages are all associated with the vulnerable plaque phenotype.

Methods and Results: 1070 patients who underwent a carotid (n=794) or femoral (n=276) endarterectomy were included. Histopathological presence of IPH was determined and divided into 3 types: recent, organized and amorphous IPH. Carotid IPH was observed in 644/794 (81%) plaques, divided into 14 (2%) recent, 70 (11%) organized and 560 (87%) amorphous. Femoral IPH was observed in 175/276 (63%) plaques, divided into 2 (1%) recent, 89 (51%) organized and 84 amorphous (48%). Overall presence of carotid IPH was associated with a large lipid core, no or minor staining of smooth muscle cells, no or minor calcification and high microvessel density. Overall presence of femoral IPH was associated with moderate to heavy staining of macrophages. Plaques with organized IPHs revealed more macrophages, a larger lipid core, less smooth muscle cells, less calcification and higher microvessel density than plaques with amorphous IPHs.

Conclusions: IPH is a significant characteristic of carotid and femoral atherosclerotic plaque and can be classified into different types. Organized IPH is associated with unstable and amorphous IPH with stable plaque characteristics.

INTRODUCTION

Intraplaque hemorrhage (IPH) plays an important role in plaque progression and destabilization.[1-5] Atherosclerotic plaque rupture and subsequently symptomatic events are closely related to the presence of plaque neovascularisation and IPH.[6, 7]
We recently demonstrated that the presence of an IPH is an independent predictor of future cardiovascular events in patients that underwent a carotid endarterectomy.[8] In addition we have shown that IPH is associated with coumarin type anticoagulation use and age, while statin use seems to protect against development of an IPH.[9]
IPH has become more clinically relevant since magnetic resonance imaging (MRI) techniques have created the possibility to visualize IPH in vivo.[10] MRI detection of IPH might serve as surrogate marker for the detection of the so called vulnerable plaque. Recent advances in MRI imaging studies have created possibilities to visualize different IPH stages in vivo (fresh, recent and old).[10-12] In earlier histopathological reports about IPH, different stages of IPH have also been described.[1, 13]
Although it has been established that overall presence of IPH is associated with vulnerable plaque components, it is unknown whether indeed all these stages of IPH are associated with a vulnerable plaque phenotype. The purpose of this large histopathological study was to examine different stages of IPH in carotid and femoral plaques in relation to other plaque characteristics.

METHODS

Study population

All studied patients were participating in the Athero-Express Biobank study. The design of this study has been described previously. [9,14] 1070 patients were operated between April 2002 and February 2008 were included. Of these 1070 patients 794 underwent a carotid and 276 a femoral endarterectomy. The clinical patient characteristics of this study population have been published recently.[9]
Indications for primary carotid or femoral endarterectomy were reviewed in a multidisciplinary vascular team. The criteria to perform carorid endarterectomy for asymptomatic and symptomatic patients were based on the recommendations published by the Asymptomatic Carotid Surgery Trial (ACST) and the North American Symptomatic Carotid Endarterectomy Trial (NASCET) and European Carotid Surgery Trial (ECST).[15-17] Patients that underwent a femoral endareterctomy were suffering from severe intermittent claudication, critical ischemia or tissue loss (Fontain classification IIb-IV) and treated with a sole endarterectomy of the common femoral artery or remote endarterectomy of the superficial femoral.[18]

Tissue processing

Directly after excision, the atherosclerotic plaque specimen was taken to the laboratory for processing. According to a standardized protocol the atherosclerotic lesions were dissected into 5 millimetre segments along the longitudinal axis.[14] The segment having the greatest plaque burden was defined as the culprit lesion and was subjected to histological analyses

as described previously.[14] The adjacent segments were immediately frozen in liquid nitrogen and stored in -80 degrees Celcius. The culprit lesion is fixed in formaldehyde 4% and embedded in paraffin. Of each paraffin embedded segment consecutive sections were cut on a microtome and used for histological staining.

Histopathological criteria

Different histopathological types of IPH's have been described previously.[1,13] We based our scoring method for IPH on these published and accepted studies.
The histological parameters in the "Athero-Express Biobank study" are semi- quantitatively scored by two independent observers, which are blinded for the clinical data. When interpretations differ between the two observers, a third observer is consulted.[14] For the present study, IPH was analyzed in more detail. Therefore, two independent observers (WD and AV) analyzed all specimens for IPH in more detail, blinded for the results of the other plaque characteristics.

IPH was scored using Hematoxylin and Eosin (HE), fibrin (Mallory's phosphotungstic acid-hematoxilin) and anti smooth muscle actin immunostains. Four histopathologic categories of IPH were defined: (1) recent: composed of predominantly intact and some degenerated erythrocytes and fibrin located in between the tissue of the plaque without signs of organization; (2) organized: hemorrhagic debris consisting of a mixture of intact and degenerating erythrocytes and fibrin with peripheral ingrowth of capillary vessels and/or of smooth muscle actin positive cells; (3) amorphous: amorphous material consisting of usually sharply demarcated cell deficient material with fibrin and with no or only a few recognizable erythrocytes; (4) amorphous with calcification: amorphous material containing calcified areas (Figure 1). Sparse intact erythrocytes were considered operation artefact and left out of the IPH analysis. IPH with a heterogeneous composition was graded according to the category that occupied the largest area in the plaque. Heterogeneity of IPH in one plaque was rarely. Only 3.9% (32 of the 819) of the plaques with IPH contained different IPH types. In cases of heterogeneity, it was in all cases obvious which of the IPH types was in majority. However, to rule out all potential bias in cases with different IPH types in one plaque, we additionally measured quantitatively the areas of the IPH types to determine which IPH type was in majority. Aperio ImageScope software was used to define and measure quantitatively surface areas of the different IPH types on the HE slides. This method of digitalizing slides in high resolution in our department has recently been described.[19] For each plaque, the area with IPH was calculated as percentage of the total plaque area.

To further characterize the amorphous material in an amorphous IPH, we performed an additional immunohistochemical CD42b staining in a subset of 10 plaques with an amorphous type IPH and we studied these plaques with transmission electron microscopy.
The CD42b gene (also known as GP1BA) is a platelet surface membrane glycoprotein and it functions as a receptor for Von Willebrand factor.[20] With this additional staining we studied whether (remnants of) platelets are present in the amorphous material. Slides were incubated with a monoclonal mouse anti-human CD42b antibody (Abcam, Cambridge, MA, USA) followed by incubation with poly AP-Anti-Mouse IgG (Immunologic, Duiven, The Netherlands).

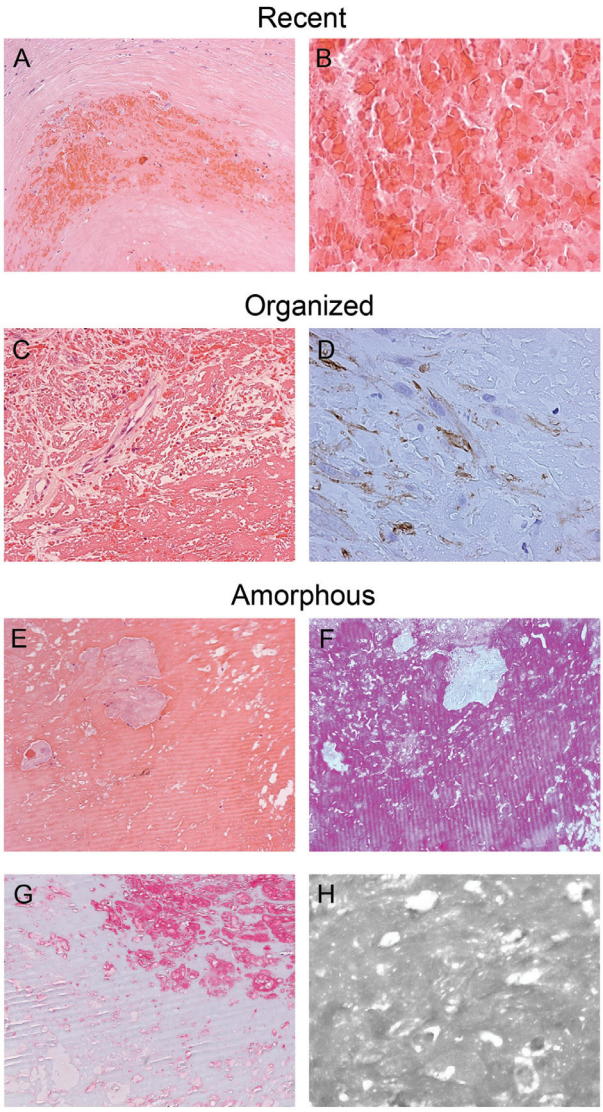

Figure 1. Histological spectrum of intraplaque hemorrhae (IPH)
Histological spectrum of intra plaque hemorrhage (IPH). ***A****; Low power view of recent IPH with predominantly intact erytrocytes and some degenerated erytrocytes in between the tissue of the plaque (hematoxylin and eosin [H&E] stain, x100).* ***B****; detail of panel A (x600).* ***C****; Overview of organized IPH with hemorrhagic debris consisting of a mixture of fibrin and intact and degenerated erythrocytes and with peripheral angiogenesis and infiltration of fibroblasts (H&E stain, x200).* ***D****; detail of organized IPH stained for alpha actin showing infiltration of myofibroblasts (brown staining; x400).* ***E****; Overview of amorphous IPH showing amorphous cell deficient material with calcification.* ***F****; fibrin stain (purple) of amorphous IPH showing presence of fibrin (x100).* ***G****. CD42b immunostaining revealing remnants of platelets in amorphous IPH.* ***H****. Transmission electron microscopy of amorphous IPH showing amorphous material in which no cells, only remnants of cells, were recognized.*

The signal was visualized using Liquid Permanent Red (Dako, Glostrup, Denmarkt).
In addition, we performed transmission electron microscopy to study the amorphous material at higher magnification. Plaques were embedded in Epon and 70 nm sections were cut. The sections were mounted onto formvar-coated copper grids (Stork Veco, Eerbeek, the Netherlands) and stained with a 5% solution of uranyl acetate, followed by Reynold's lead citrate. Sections were viewed in a JEOL JEM-1010 transmission electron microscope operating at 60 kV (JEOL, Tokyo, Japan).

Plaque characteristics were scored semi-quantitatively as described previously.[9,14] Plaques were categorized as no/minor staining or moderate/heavy staining for the following stains: HE (calcification), Pico Sirius Red (PSR, total collagen), anti-CD68 immunostain (for macrophages) and anti alpha actin immunostain (SMA-1, for vascular smooth muscle cells). Using the HE and PSR stains, the size of the lipid core was estimated as percentage of total plaque area with a division in three categories: <10%, 10-40% and >40%.[21] Using a magnification of ×100, anti-CD34-immunopositive microvessels were counted in three areas of the plaque with the highest microvessel density (MVD) as described previously.[22] Subsequently, the average MVD per square millimeter of these areas was calculated for each plaque. The median MVD of all plaques was calculated and plaques were divided into two groups: average MVD higher or lower than the overall median MVD of all plaques. Recently, we have demonstrated that our semi-quantitative analysis of atherosclerotic plaque histology is well reproducible, both intraobserver and interobserver.[23] In addition, SMA-1 and CD68 stains were also analyzed quantitatively using computerized analyses and results highly correlated with the semi-quantitative analyses.[23]

Statistical analysis

Statistical analysis was performed with SPSS version 15.0 (SPSS Inc, Chicago, Illinois). A Chi- Square test was used to compare categorical variables. The Mann-Whitney U test was used to compare continuous variables with categorized variables. P-values < 0.05 were considered statistically significant.

RESULTS

IPH was observed in 644/794 (81%) carotid endarterectomy specimens. Of these 644 IPHs, 14 (2%) were recent, 70 (11%) were organized, 505 (78%) were amorphous and 55 (9%) were amorphous with calcification (Figure 2A). The median percentage carotid plaque cross-sectional area that was occupied by IPH was 5% [inter quartile range (IQR) 3-10] (Figure 2B).

IPH was observed in 175/276 (63%) femoral endarterectomy specimens. Of these 175 IPHs, 2 (1%) were recent, 89 (51%) were organized, 42 (24%) were amorphous and 42 (24%) were amorphous with calcifications (Figure 2A). The median percentage of femoral cross-sectional area that was occupied by IPH was 20% [IQR 8-65] (Figure 2B).

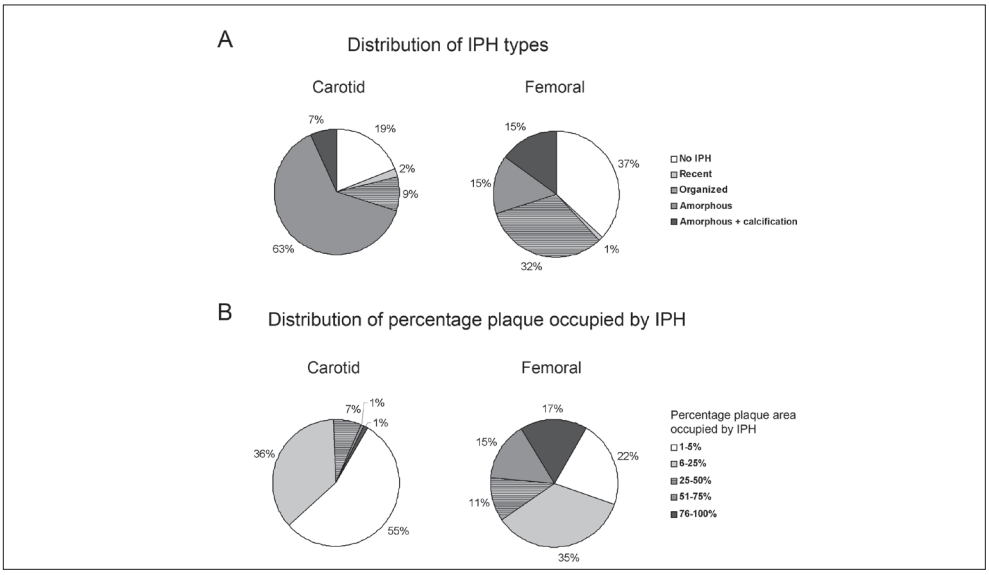

Figure 2. Types of intraplaque hemorrhage

***A**; Discrimination of different types of IPH in carotid and femoral specimens and the percentage of each type.* ***B**; Percentage of the total plaque cross-sectional area occupied by IPH divided in different groups (1-5%, 6-25%, 26-50%, 51-75% and 76-100% of the total plaque area).*

Plaque characteristics and intraplaque hemorrhage

An association was observed between IPH and histopathological features of an unstable plaque phenotype. Overall presence of carotid IPH was associated with a large lipid core, no or minor staining of smooth muscle cells, no or minor calcification and high MVD (Table 1, Figure 3). In the carotid specimens, no association was observed between overall presence of IPH and the amount of macrophages and collagen. In the femoral plaque, IPH was associated with moderate to heavy staining of macrophages (Table 2, Figure 4). No association was observed between overall presence of femoral IPH and other measured plaque characteristics, although there seemed to be a trend with no or minor collagen and high MVD. Thus, overall presence of carotid and femoral IPH was associated with histopathological characteristics of the unstable plaque. The association between overall presence of IPH and plaque characteristics in this study population has recently been published as part of a study that elucidated the association between clinical patient characteristics and IPH.[9]

We additionally studied the association between different stages of IPH and plaque phenotype. The observed number of recent IPHs was too low to perform reliable statistics and we therefore decided to focus on organized and amorphous IPH. In the carotid artery, plaques with organized IPH revealed more macrophage staining, a larger lipid core and higher MVD compared to plaques with amorphous IPH (Table 1; Figure 3). Carotid plaques containing an amorphous IPH revealed more smooth muscle cell staining and calcification than plaques with organized IPH (Table 1, Figure 3). Femoral plaques with organized IPH revealed more macrophage staining and higher MVD than plaques with amorphous IPH (Table 2, Figure 4).

Femoral plaques containing an amorphous IPH revealed more calcification than plaques with organized IPH (Table 2, Figure 4). Thus, organized IPHs revealed more unstable plaque characteristics (macrophages, lipid core and high MVD), whereas amorphous IPHs revealed more stable plaque characteristics (smooth muscle cells and calcification).

To further characterize the amorphous material in an amorphous IPH, we performed an additional immunohistochemical CD42b staining and studied these plaques with transmission electron microscopy in a subset of plaques. In 2/10 amorphous IPHs CD42b was observed, which confirms the presence of (remnants of) platelets in the amorphous material (Figure 1). In addition, transmission electron microscopy of amorphous IPH revealed amorphous material in which no cells, only remnants of cells, were recognized (Figure 1). Thus, amorphous material appeared to be cell deficient, fibrin rich material with evidence for (remnants) of platelets.

Table 1. Carotid plaque *

	None (1)	Recent (2)	Organized (3)	Amorphous +/- calcifications (4)	P-value No IPH vs IPH (=1 *vs.*2+3+4)	P-value 3 *vs.*4
Lipid core						
no	46 (30)	4 (28)	13 (19)	110 (20)	0.001†	0.04†
<40%	67 (45)	5 (36)	22 (31)	261 (46)		
>40%	37 (25)	5 (36)	35 (50)	189 (34)		
Macrophages						
no/minor	61 (41)	4 (29)	9 (13)	234 (42)	0.60	<0.001†
moderate/heavy	89 (59)	10 (71)	61 (87)	326 (58)		
Collagen						
no/minor	23 (15)	4 (29)	13 (19)	100 (18)	0.41	0.72
moderate/heavy	127 (85)	10 (71)	57 (81)	460 (82)		
Smooth muscle cells						
no/minor	26 (17)	3 (21)	28 (40)	165 (29)	0.001†	0.02†
moderate/heavy	124 (83)	11 (79)	42 (60)	395 (71)		
Calcification						
no/minor	76 (51)	6 (43)	33 (47)	201 (36)	0.003†	0.03†
moderate/heavy	74 (49)	8 (57)	37 (53)	359 (64)		
Vessel density						
No/ low density	81 (58)	2 (15)	22 (32)	281 (53)	0.025†	<0.001†
Increased density	58 (42)	11 (85)	47 (68)	250 (47)		

** Data are presented as No. (%); † P<0.05*

Table 2. Femoral plaque *

	No IPH (1)	Recent (2)	Organized (3)	Amorphous +/- calcifications (4)	P-value No IPH vs IPH (=1 *vs.*2+3+4)	P-value 3 *vs.*4
Lipid core						
no	76 (75)	0 (0)	60 (68)	59 (70)	0.23	0.67
<40%	21 (21)	1 (50)	26 (29)	23 (28)		
>40%	4 (4)	1 (50)	3 (3)	2 (2)		
Macrophages						
no/minor	85 (84)	1 (50)	56 (63)	70 (83)	0.03†	0.004†
moderate/heavy	16 (16)	1 (50)	33 (37)	14 (17)		
Collagen						
no/minor	9 (9)	1 (50)	11 (12)	17 (20)	0.08	0.11
moderate/heavy	92 (91)	1 (50)	78 (88)	67 (80)		
Smooth muscle cells						
no/minor	22 (22)	1 (50)	14 (16)	24 (29)	0.92	0.08
moderate/heavy	79 (78)	1 (50)	75 (84)	60 (71)		
Calcification						
no/minor	36 (36)	0 (0)	31 (35)	23 (27)	0.42	0.01†
moderate/heavy	65 (64)	2 (100)	58 (65)	61 (73)		
Vessel density						
No/ low density	47 (57)	0 (0)	27 (42)	33 (55)	0.09	0.005†
Increased density	36 (43)	2 (100)	37 (58)	27 (45)		

** Data are presented as No. (%); † P<0.05*

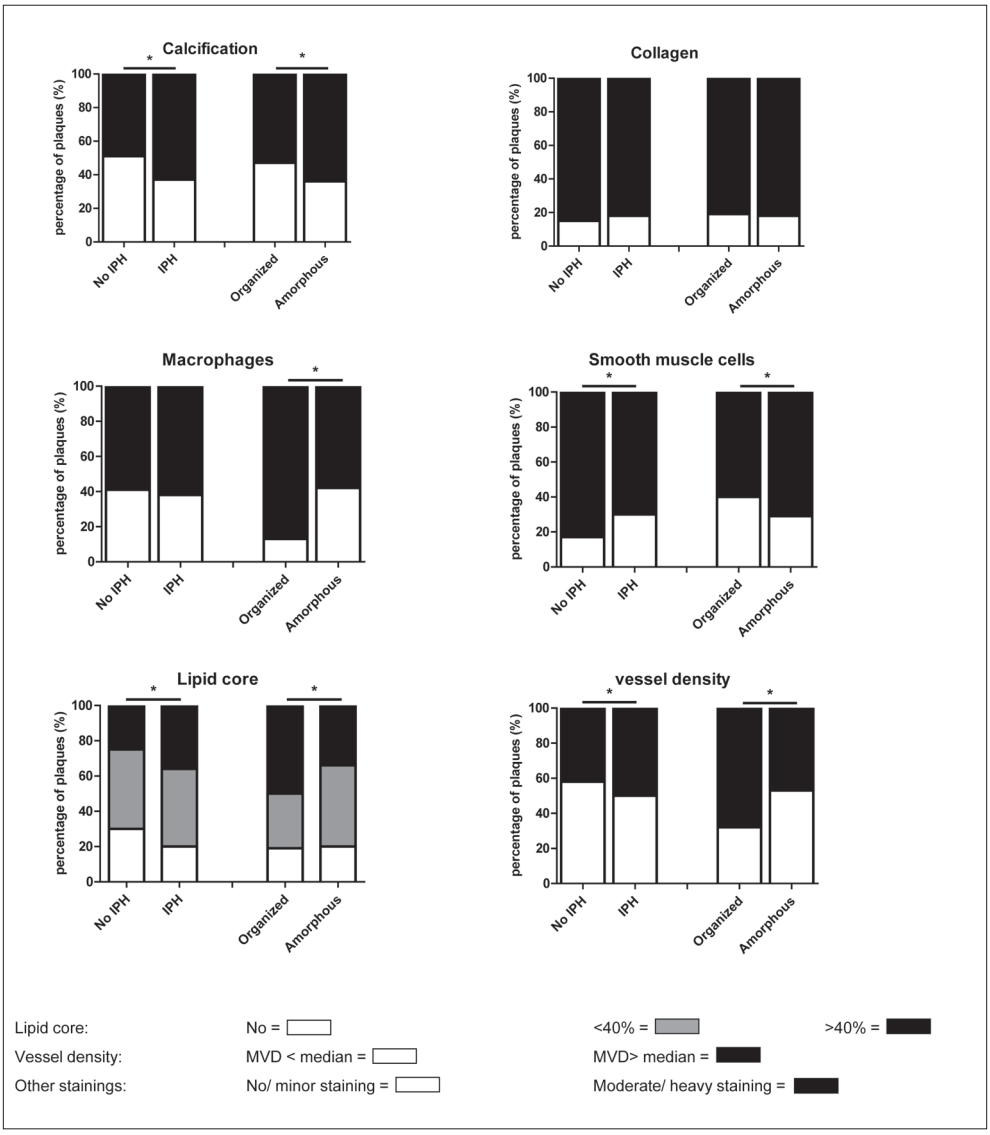

Figure 3. IPH versus other plaque characteristics in the carotid plaque

*Each graph illustrates the relation of a single plaque characteristic with IPH/ No IPH and organized/ amorphous IPH. * = p<0.05; MVD = microvessel density*

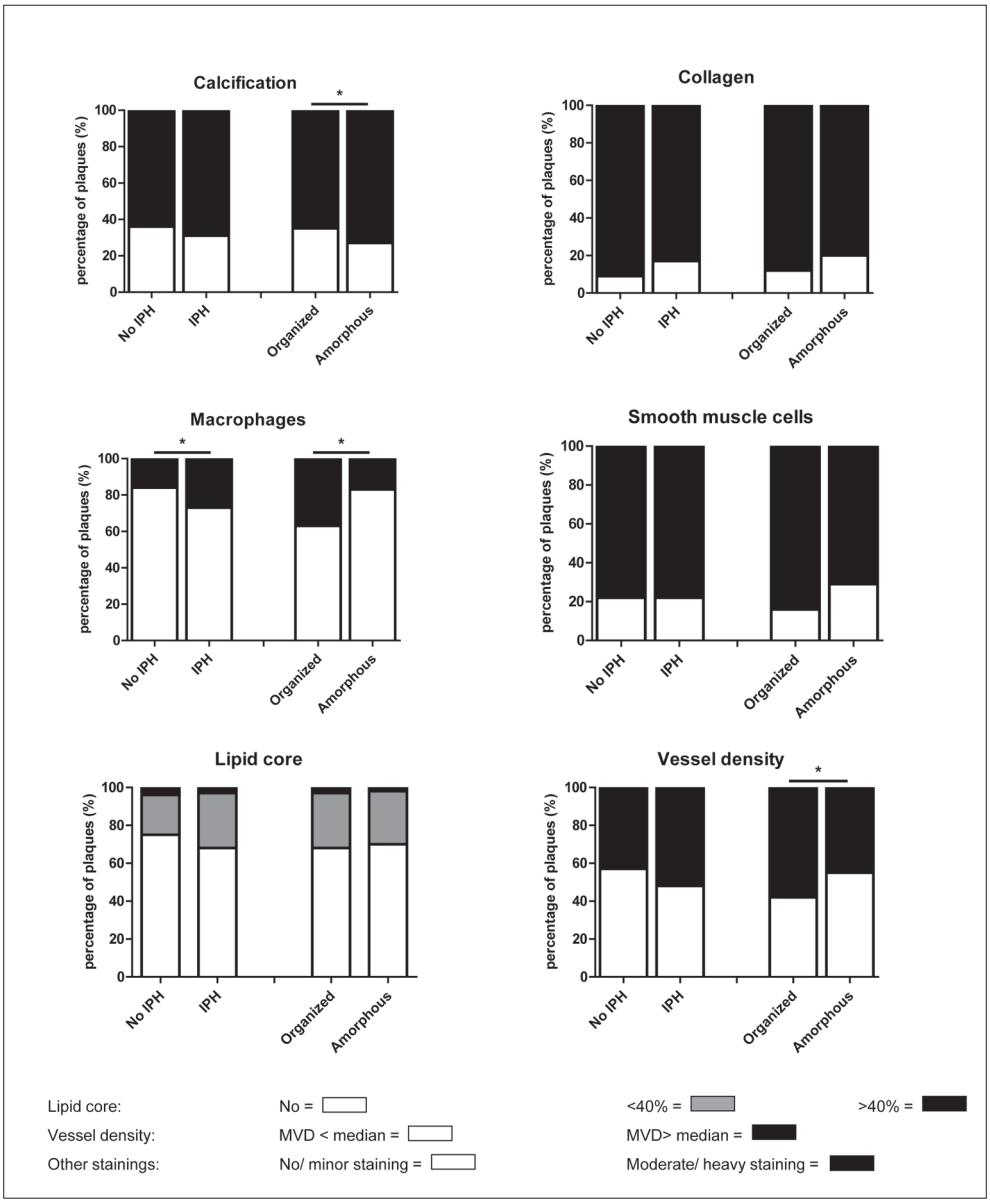

Figure 4. IPH versus other plaque characteristics in the femoral plaque

*Each graph illustrates the relation of a single plaque characteristic with IPH/ No IPH and organized/ amorphous IPH. * = p<0.05; MVD = microvessel density*

DISCUSSION

To the best of our knowledge this is the first large histopathological study in which different stages of IPH have been systematically studied in relation to plaque characteristics. We show that IPH is an important plaque characteristic of symptomatic carotid and femoral atherosclerotic plaques that can be classified into different stages. Second, we show that these different stages of IPH are associated with different plaque phenotypes.

We observed a high IPH percentage: 81% of the carotid and 63% of the femoral endarterectomy specimens contained IPH. According to literature, observed IPH varies from 7.5% to 92%, however these studies were mostly based on small numbers or on macroscopic plaque examination.[24-26] Our results show that on average 5% of the total plaque area of carotid plaques and 20% of femoral plaques consists of IPH. Previously it has been demonstrated that IPH significantly contributes to the lipid core.[4] In the present study we choose to only include IPH's that can be recognized with histology and from which knew for certain that this was IPH. Since we did not count the lipid core as IPH, we probably have underestimated the real percentage of plaque consisting of IPH.

It is unclear whether different IPH types represent different stages of the same process over time, are different tissue processes after an initial bleeding or have different bleeding origins. In previous literature it is suggested that IPH can be divided into different phases: recent (<1 week old), organized (2-6 weeks old) and amorphous (> 6 weeks old).[1, 13] It is beyond any doubt that fresh IPH evolve into organized IPH through a process of fibrovascular ingrowth of the haematoma, basically similar to the process of lumen thrombus organization which has been studied in more detail.[27, 28] However, no experimental studies have been performed to prove that organized IPH types evolve into an amorphous stage, and if so, whether this is true for all cases. Nonetheless, when the observed different IPH types are considered different phases of incorporation of extravasated blood into the plaque mass, our observation indicates, in accordance with recent literature, that an atherosclerotic plaque becomes more stable over time after an IPH.[29]

Another possibility is that the different organized and amorphous types of IPH represent the morphologic substrates of different types of repair response to the plaque hemorrhage. Pathways of plaque destabilization (leading to increased plaque vulnerability), but also of plaque stabilization have been described after a hemorrhage. IPH is able to contribute to plaque destabilization by its contribution to the lipid core and by the induction of an inflammatory response with infiltration of inflammatory cells and the development of new vessels.[1, 4, 5] On the other hand, it has also been reported that IPH can induce a healing response at the site of the hemorrhage with the formation of fibrous and calcified tissue which ultimately leads to plaque stabilization.[30]

A third possibility is that different IPH types arise from a different origin. Most intraplaque neovessels arise from the adventitial vasa vasorum. Adventitial vasa vasorum demonstrate complete maturation, whereas newer branches that transverse the medial layer into the intima are more fragile and leaky.[31] These leaky vessels are characterized by defects between endothelial junctions resulting in diffuse perivascular expression of von Willebrand factor (vWF) as a sign of less intact or leaky endothelium.[4, 5, 32] Hypothetically, insudation of the plaque matrix by large amounts of plasma proteins (fibrinogen, albumin, vWF and others)

derived from the leaky fragile vessels could lead to a cell poor amorphous appearance. On the other hand, organized IPH could originate from rupture of microvessels that allows extravasation of red blood cells (RBC's) and is followed by ingrowth of repair tissue (as is also the case for lumen thrombus).[27] Additionally, extracellular lipids derived from cholesterol rich RBC membranes are responsible for rapid atheroma progression and influx of inflammatory cells.[4] A lack of RBC's in amorphous IPH could be an explanation of a more stable plaque phenotype in plaques containing an amorphous IPH. However, it is important to note that such mechanisms are by no means mutually exclusive, since we frequently observed combinations of them in our series.

From a clinical perspective our results are important, because it suggest that an organized IPH is a better surrogate marker for vulnerable plaque, whereas an amorphous IPH in most cases represents a more stable plaque. This is even more relevant, since recent advances in MRI imaging studies have created possibilities to visualize different IPH stages in vivo.[10, 12, 33]

Carotid and femoral arteries are different anatomical sites with divergent pathophysiological features with respect to plaque generation. Instead of carotid lesions, the femoral plaque is dissected after years of ischemic complaints and is often occlusive resulting in a fibrotic lesion, rich in smooth muscle cells and collagen, with less inflammatory cells, which could be considered as end-stage expression of atherosclerosis. Next to the fact that the number of femoral lesions was smaller compared to the carotids, the differences in stage of disease and anatomical location between both artery types might be a possible explanation for the observed differences in plaque composition and IPH between carotid and femoral arteries.

The present study has some limitations. Due to the cross-sectional study design no conclusions could be made about the evolution of the IPH. Secondly, in our study design we investigated only the culprit lesion. Underestimation of the amount of IPH could be a possibility. However, we previously demonstrated that the culprit lesion is representative for the total plaque burden.[23]

In conclusion, IPH is a significant characteristic of carotid and femoral atherosclerotic plaque and can be classified into different types. Organized IPH is associated with unstable and amorphous IPH with stable plaque characteristics.

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PART III

Histological femoral plaque analysis to predict clinical outcome

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CHAPTER 7

Predictive risk factors for restenosis after remote superficial femoral artery endarterectomy

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ABSTRACT

Objectives: Restenosis following remote superficial femoral artery endarterectomy (RSFAE) remains a challenging problem. Determinants predicting failure are lacking. This study investigated patient characteristics with predictive value for restenosis during the first year after RSFAE.

Design: prospective cohort study

Materials and methods: Ninety patients post-RSFAE were studied for the occurrence of restenosis in the first 12 months postoperatively. At baseline, clinical parameters were recorded. Vessel size was measured on the basis of plaque perimeter in the culprit lesion and lumen diameter on peroperative digital subtraction angiography.

Results: In 57 patients (63%) a restenotic lesion was diagnosed within 12 months following surgery. Patients with longer time interval between start of ischemic walking complaints and RSFAE revealed a significantly higher incidence of restenosis (Hazard Ratio = 1.3 [1.05-1.52] per 4 years). Small plaque perimeter and small SFA diameter on angiography, were significantly associated with restenosis (HR = 0.54 [0.34-0.88] per 10mm and HR = 0.46 [0.27-0.78] per 1.5mm, respectively). In multivariate analysis, age, duration of ischemic walking complaints and lumen diameter were independently associated with increased risk of restenosis after RSFAE.

Conclusions: This study provides evidence that age, vessel size, and duration of ischemic walking complaints before RSFAE are predictive values for restenosis after RSFAE.

INTRODUCTION

Remote superficial femoral artery endarterectomy (RSFAE) is established as a minimaly invasive treatment option for long occlusions, defined as TransAtlantic Inter-Society Consensus [TASC][1] C and D lesions of the superficial femoral artery (SFA). RSFAE has comparable primary assisted and secondary patency rates to prosthetic supragenicular bypass surgery.[2] Besides, hospital stay is shorter and consequences of possible reobstructions are less severe in patients treated with RSFAE.[2,3]

A drawback of RSFAE is the restenosis rate in the first year postoperatively caused by neointimal hyperplasia, with more than 80% of all restenoses occuring in the first year after surgery. Restenosis within the first year has been associated with a higher risk for occlusion. The restenotic lesions are equally distributed in the endarterectomized SFA, including the distal part of the SFA with the stented transection zone.[4]

Determinants predicting failure after RSFAE are lacking. General risk factors for cardiovascular disease are not successful in discriminating the risk for restenosis. The extent and severity of the treated lesion and technical considerations are determinants of failure after percutaneous interventions or bypass surgery.[5] However, these clinical characteristics have not yet been proven to be of value for predicting restenosis after RSFAE. The objective of this study was to investigate patient characteristics that have a predictive value for restenosis during the first year after RSFAE.

METHODS

Study population

All patients in the current study were included in the Athero-Express Biobank an ongoing vascular biobank with a longitudinal study design that has been described previously.[6] Dissected femoral plaques, obtained by endarterectomy in two participating Dutch teaching hospitals, are collected and examined histopathologically. In addition, clinical baseline characteristics of all included patients are obtained. The medical ethics boards of both participating hospitals approved the study, and all patients provided written informed consent.[6] In both hospitals patients suffering from SFA obstructions are being treated by use of the same protocol. Patients without improvement or worsening of their complaints after supervised exercise, and patients with critical ischemia primarily, are discussed in a multidisciplinary meeting. If the patients do have a suitable greater saphenous vein (> 3 mm), TASC C lesions, assessed as to complicated for percutaneous intervention, and TASC D lesions are treated with a venous supragenicular bypass. If the patients with extensive TASC C and TASC D SFA lesions do lack a suitable greater saphenous vein, they are treated primarily with RSFAE. Only when RSFAE fails, patients get a prosthetic supragenicular bypass.

Ninety consecutive patients who underwent RSFAE between February 2003 and October 2007 were selected. All patients underwent unilateral RSFAE with or without an additional open endarterectomy of the common femoral artery. All patients presented with intermittent claudication, critical ischemia or tissue loss (Rutherford category 3-5)[7] due to long-segment occlusion (TASC C and D lesions) of the SFA.

At baseline, clinical preoperative, perioperative and postoperative parameters were obtained from the Athero-Express medical database. Missing data were obtained from medical files or referral letters. The preoperative evaluation included a magnetic resonance angiography (MRA).

RSFAE Technique

This minimally invasive debulking technique has been described previously.[8] In summary, the SFA is exposed through a small groin incision. After anticoagulation with heparin, the proximal SFA is clamped, and a longitudinal arteriotomy is made in the proximal SFA. The intima core is dissected, between the lamina elastica interna and the circular fibers of the media, using the Vollmar dissector (Vollmar Dissector, Aesculap®, San Francisco, CA, USA), until it reaches the distal limit of the atheroma in the SFA. The Vollmar dissector is then exchanged for the Moll ringcutter (Mollring Cutter®, LeMaître Vascutek, San Jose, CA, USA). This device can transect and remove the entire desobstructed intimal core, all under fluoroscopic guidance. After the SFA is debulked, the distal transaction zone is secured by a stent and a completion angiography is performed to check the patency of the SFA and outflow arteries.

Atherosclerotic plaque

The excised plaques were directly transferred to the laboratory, processed, and examined as described previously.[6] The atherosclerotic lesions were dissected into 5-mm segments, and the segment with the greatest plaque area was defined as the culprit lesion. This segment was fixed in formaldehyde 4%, decalcified for 1 week in ethylenediaminetetraacetic acid, and embedded in paraffin. Segments adjacent to the culprit lesion were snapfrozen in liquid nitrogen and stored at -80˚C for future analysis.[6]

Arterial size

Cross-sections of the elastin von Gieson staining of the harvested atherosclerotic plaques were captured by digital image microscopy (AnalySiS version 3.2, Soft Imaging GmbH, Munster, Germany), and the perimeter of the plaque was measured in each cross-section by tracing the internal elastic lamina (Figure 1). Because the studied femoral atherosclerotic plaques are dissected between the internal elastic lamina and the circular fibers of the media, we assumed that the perimeter of the dissected atherosclerotic plaque (the perimeter of the internal elastic lamina) is a measure of preoperative artery size.

The diameter of the arterial lumen was also measured on angiography performed at the end of the procedure as a measure for residual lumen size. All angiographies were stored in an electronic database. All patients received an aSpire stent (aSpire stent®, LeMaitre Vascular, San Jose, CA, USA) to secure the transaction zone in the distal SFA. The distance between two nitinol frames of the double helix configuration of the aSpire stent is a standard distance. This distance between the two nitinol frames at four helices of one aSpire stent was measured and averaged and used for calibration. Next, the SFA lumen diameter was measured at 3 standardized levels: 1, 3 and 5 cm proximal of the stent in the distal SFA. The measurement outcomes at the 3 fixed points were then averaged. An interventional radiologist supervised the execution of all angiographic measurements and measurements were performed with

the computer systems of our radiologists (Picture Archiving and Communications System [PACS] from Agfa-Gevaert Group).

Follow-up

Restenosis after initially successful RSFAE most often occurs in the first year postoperatively. These restenotic lesions have to be treated at an early stage to maintain patency whether symptomatic or not.[9] Follow-up, including history, physical examination and duplex ultrasound scanning, was scheduled at 3, 6 and 12 months and annually thereafter. Duplex ultrasound scanning to detect restenotic lesions was performed according to protocol. The entire common femoral artery, the proximal profunda femoral artery, the entire SFA (from origin till popliteal artery, including distal stent), and the entire popliteal artery, were scanned in every patient. Obstructions are classified on the base of the peak systolic velocity (PSV) within the obstruction (numerator of the PSV-ratio) and distally of the obstruction (denominator of the PSV-ratio). If the stenotic lesion is at the distal end of the artery, the PSV of the denominator will be measured proximal of the lesion. A stenosis of 50% is considered if the PSV-ratio is 2.5. We considered a restenotic lesion if there was a lumen reduction of 50% or more (= PSV- ratio ≥ 2.5). Additional MRA may be performed in case of restenosis at the preference of the treating vascular surgeon.

Data analysis

Statistical analysis was performed with SPSS version 15.0 software (SPSS Inc, Chicago, IL, USA). In univariate analysis, the association between baseline data and restenosis was tested for significance with the Cox regression analysis. Hazard ratios (HR) were calculated. The 95% confidence intervals (CI) not containing 1 or values of $P < 0.05$ were considered statistically significant. To test independency of the univariate variables, multivariate Cox regression analysis (with backward exclusion of nonsignificant variables using likelihood ratio test) was

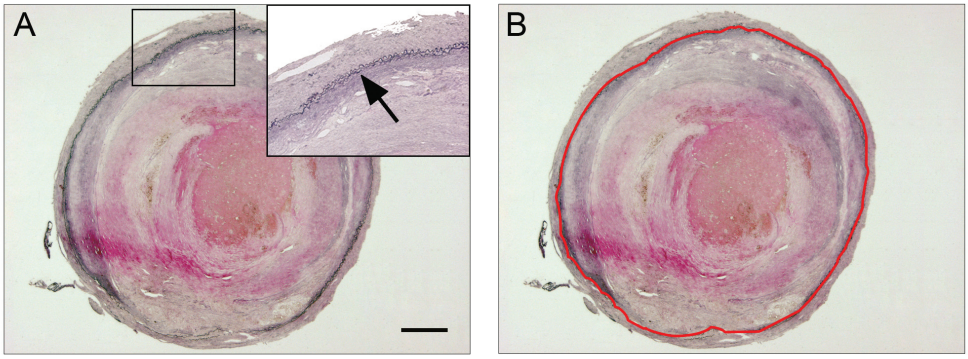

Figure 1. Measurement of the perimeter of the plaque in the culprit lesion of the occluded superficial femoral artery

***A**: Elastin van Gieson staining of the atherosclerotic plaque. Inlay: magnification of the marked area showing the internal elastic lamina in black (arrow). Bar = 1mm. **B**: Measurement of the perimeter of plaque by tracing the internal elastic lamina (in red) using digital image microscopy).*

performed. Variables showing an association with restenosis (P < 0.1) in univariate analysis were included in the multivariate analysis. Furthermore, age, sex, and operation indication were always included in the multivariate analysis models.

RESULTS

The study included 90 consecutive patients (74% men) undergoing unilateral RSFAE between February 2003 and October 2007. The baseline patient characteristics are summarized in Table 1. Mean patient age was 67 years. In 72 patients (80%) Rutherford category 3 was the indication for operation. Median duration of ischemic walking complaints due to SFA occlusion before surgery was 56 months (range: 4-303 months).

Table 1. Clinical characteristics [a]

Characteristics	All patients (n=90)	Restenosis (n=57)	No restenosis (n=33)	Hazard ratio [b] (95% CI)	P-value
Age, mean (range), years	67 (50-84)	67 (50-83)	67 (52-84)	1.18 (0.85-1.65)	0.33
Sex					
Male	67 (74)	38 (67)	29 (88)	0.39 (0.22-0.69)	0.001*
Female	23 (26)	19 (33)	4 (12)	-	
Current smoker	39 (43)	23 (40)	16 (50)	0.76 (0.45-1.30)	0.32
Hypertension	59 (66)	38 (67)	21 (64)	0.99 (0.99-1.01)	0.62
Diabetes Mellitus	27 (30)	17 (30)	10 (30)	1.51 (0.84-2.73)	0.17
Hypercholesterolemia	63 (70)	42 (74)	21 (64)	1.40 (0.77-2.52)	0.27
Body mass index, mean (range)	26 (17-35)	26 (21-35)	27 (17-32)	0.98 (0.90-1.08)	0.73
Statin use	73 (81)	26 (36)	47 (64)	1.27 (0.43-3.72)	0.67
Acetylsalicylic acid use	75 (83)	26 (35)	49 (65)	1.65 (0.54-5.06)	0.38
Clopidogrel use	24 (27)	8 (33)	16 (67)	1.22 (0.46-3.26)	0.69
Clinical presentation [c]					
Rutherford class III	72 (80)	50 (88)	22 (67)	2.46 (0.34-17.92)	0.38
Rutherford class IV	12 (13)	6 (11)	6 (18)	1.99 (0.24-16.66)	0.53
Rutherford class V	6 (7)	1 (1)	5 (15)	reference	
Duration of ischemic walking complaints, median (range), months	56 (4-303)	65 (3-303)	28 (3-248)	1.27 (1.05-1.52)	0.01*
Patent runoff arteries					
1 artery	9 (10)	6 (11)	3 (10)	1.43 (0.59-3.48)	0.44
2 arteries	32 (36)	23 (40)	9 (29)	1.46 (0.84-2.53)	0.19
3 arteries	47 (52)	28 (49)	19 (61)	reference	
Missing	2 (2)	0 (0)	2 (6)		

*[a] Data are presented as No.(%) unless otherwise indicated, [b] Cox regression analysis, [c] Comparison of Rutherford class II-III and Rutherford class IV vs. reference, * P < 0.05*

A restenotic lesion was diagnosed in 57 patients (63%) within 12 months after RSFAE, including one patient with an early restenosis (< 30 days). Of the 57 patients with restenosis, 47 (82%) were symptomatic. 34 patients (72%) had claudication complaints (Rutherford category 2 or 3).
8 (17%) presented with critical leg ischemia (Rutherford category 4), and 3 (6%) had tissue loss (Rutherford category 5). In addition, 2 patients (4%) were readmitted with acute critical leg ischemia.
Twenty patients were asymptomatic or had Rutherford class 2 ischemia and were treated conservatively. Twenty-four patients were treated with percuteneous transluminal angioplasty (PTA), 11 patients received a bypass graft and two patients an open re-endarterectomy of the common femoral artery/ proximal SFA with proximal patch plasty.
In univariate analysis, gender was associated with restenosis. A restenotic lesion was found in 19 of 23 women (83%) and in 38 of 67 men (57%) within 12 months (P = 0.001; Table 1).
Patients with longer time interval between the start of ischemic complaints and RSFAE revealed higher incidence of restenosis. Median duration of ischemic walking complaints before surgery was 65 months (range, 3-303 months) for patients with restenosis and 28 months (range, 3-248 months) in patients without restenosis (Figure 2). Risk of restenosis increased with 30% (HR: 1.3 [95% CI 1.05-1.52]) per 4 years of ischemic walking complaints (P = 0.012; Table 1).
Histopathologic analysis of the excised atherosclerotic plaques revealed that all femoral arteries were totally occluded at the time of RSFAE and the atherosclerotic plaque was dissected between intima and media as a circular core (Figure 1).
A small perimeter of the plaque was significantly associated with restenosis. Median plaque perimeters were 16.9 +/- 4.9 mm in patients with restenosis and 20.2 +/- 4.7 mm in those without restenosis (P = 0.01; Table 2). Patients with a plaque perimeter smaller than the median (<17.6mm) had a significantly higher risk of restenosis than patients with a larger perimeter (Figure 3). The risk of restenosis decreased by 46% per 10mm increase of plaque perimeter (HR: 0.54 [95% CI 0.34-0.88]; P = 0.01).
Consistent with the results of the plaque perimeter, median diameters of the SFA lumen, as measured on perioperative angiography, were significantly smaller in patients with restenosis

Table 2. Per-operative results

Characteristics	All patients (n=90)	Restenosis (n=57)	No restenosis (n=33)	Hazard ratio [a] (95% CI)	P-value
Operation time, mean (range), minutes	124 (70-190)	121 (70-187)	129 (70-190)	0.80 (0.62-1.05)	0.10
Blood loss, mean (range), ml	246 (50-1200)	228 (50-500)	280 (50-1200)	0.68 (0.43-1.07)	0.96
Length of dissected intima core, median (SD), cm	27 (5.6)	25 (6)	28 (4)	0.97 (0.92-1.02)	0.24
Lumen diameter on peri-operative angiography, median (SD), mm	5.7 (1.0)	5.6 (0.9)	6.4 (1.0)	0.46 (0.27-0.78)	0.004*
Perimeter of the plaque, median (SD), mm	17.6 (5.1)	16.9 (4.9)	20.2 (4.7)	(0.54) (0.34-0.88	0.01*

*[a] Cox regression analysis, * P < 0.05*

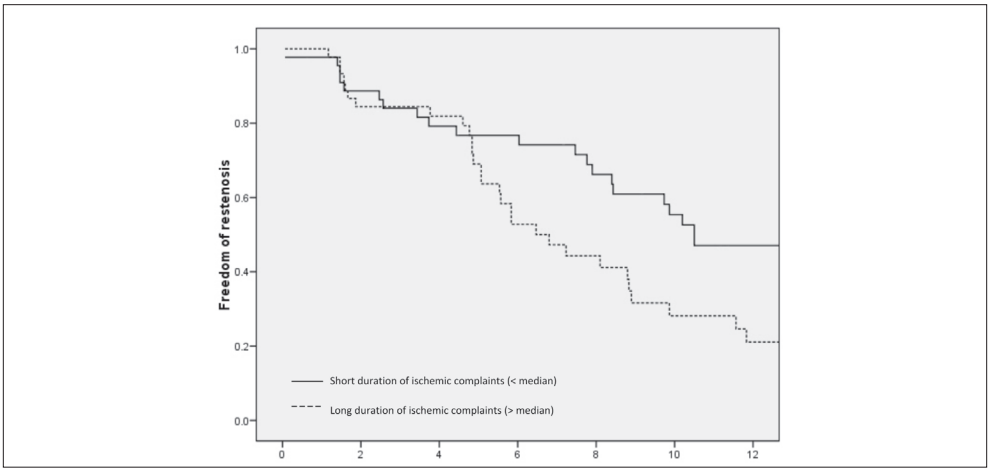

Figure 2. Restenosis in relation to duration of ischemic walking complaints
Patients with longer duration of ischemic walking complaints (>median) due to superficial femoral artery occlusion revealed a significantly (P = 0.01) higher incidence of restenosis in the first 12 months postoperatively than patients with shorter duration of ischemic walking complaints (<median).

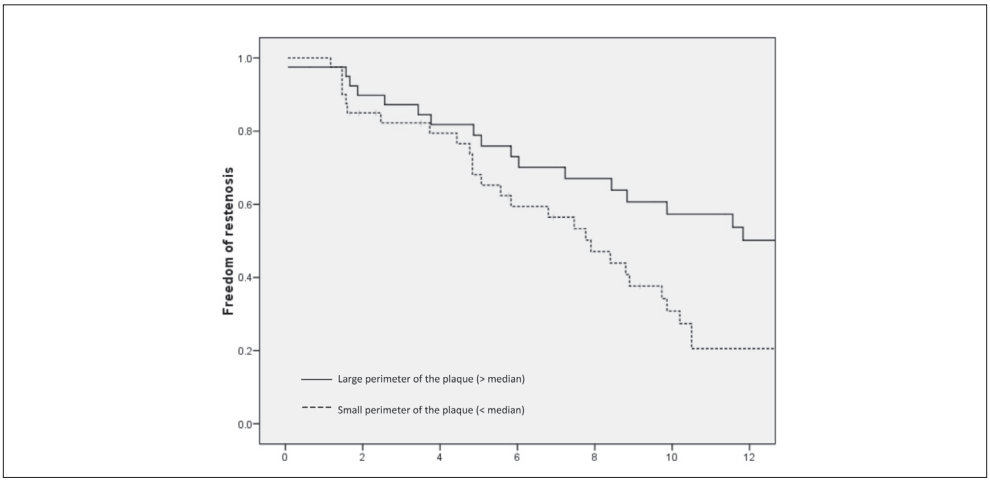

Figure 3. Restenosis in relation to plaque perimeter
Patients with a smaller perimeter of the plaque (<median) revealed significantly (P = 0.01) higher incidence of restenosis in the first 12 months postoperatively than patients with a larger perimeter of the plaque (>median).

(5.6mm) compared with patients without restenosis (6.4mm; Table 2). The risk of restenosis decreases by 54% per 1.5mm increase of lumen diameter as measured on the perioperative angiography (HR: 0.46 [95% CI 0.27-0.78]; P = 0.004).

Multivariate analysis

Because sex, time interval between ischemic complaints and RSFAE, plaque perimeter, and lumen diameter on peroperative angiography were associated with 1-year restenosis, these variables were consequently entered in multivariate Cox hazard regression analysis. Age and

operation indication (Rutherford category) were always included in the multivariate analysis models. In multivariate analysis, age (HR: 1.61 [95% CI: 1.03-2.53]; P=0.04), duration of ischemic walking complaints (HR 1.29 [95% CI: 1.03-1.62]; P=0.03), and lumen diameter (HR: 0.37 [95% CI: 0.19-0.72]; P<0.01) were independently associated with increased risk of restenosis after RSFAE (Table 3).

Table 3. Multivariate cox regression analysis [a]

Characteristics	Hazard ratio (95% CI)	P-value
Age, per 10 years	1.61 (1.03-2.53)	0.04*
Sex		ns
Male	-	
Female	-	ns
Clinical presentation		
Rutherford class II-III	-	
Rutherford class IV	-	0.03*
Rutherford class V-VI	-	ns
Duration of ischemic walking complaints, per 4 years	1.29 (1.03-1.62)	< 0.01*
Perimeter of the plaque, per 10mm		
Lumen diameter, per 1.5mm	0.37 (0.19-0.72)	

Abbreviations: CI, confidence interval; ns, not significant (removed from the multivariate model based on likelyhood ratio), [a] Cox regression analysis with backward exclusion of non-significant variables using likelihood ratio test
** P < 0.05*

DISCUSSION

Restenosis is a drawback in the first year after RSFAE; thus far, predictive clinical variables for restenosis are not available. This study shows that SFA diameter, age, and interval between occurrence of ischemic walking complaints and the RSFAE procedure are predictive for restenosis after RSFAE. These findings may have an effect in clinical practice. RSFAE should be reconsidered in older patients, in patients with a long history of ischemic complaints, and in patients with small SFA diameter. Follow-up and treatment of restenosis may need to be more aggressive for these subgroups.

A recent published randomized trial, comparing RSFAE and supragenicular bypass surgery for long occlusions of the SFA, concluded that venous bypass is superior to RSFAE.[2] However, RSFAE has comparable secondary patency rates (61%) to prosthetic bypass grafts (63%) with the advantage of avoiding prosthetic material and shorter hospital stay.[2] The difference between the RSFAE patency rates mentioned in the trial and the 12 months restenosis rate in our study can be explained by the following. Published studies on RSFAE reported primary patency rates, which is defined as uninterrupted patency without any procedures performed on the target lesion. We reported the “freedom of restenosis rate” and scored all patients with a lumen reduction of 50% or more as restenosis, although there was a substantial

number of asymptomatic patients which were treated conservatively (n=20). Our primary patency rate, according to the definitions is 59% and comparable with first year patency rates of RSFAE (and prosthetic bypass grafts) described in literature.

The reason to focus on all patients (including asymptomatic patients without reintervention) with ≥ 50% restenosis, is that we wanted to determine the whole spectrum of patients with significant lumen reduction after RSFAE, and determine which factors are of influence on restenosis.

In research considering peripheral artery disease, little is known about arterial size in relation to restenosis, although, a recent study of bypass surgery showed an increase of graft restenosis as graft diameter diminished.[10] In cardiology, vessel size is well established as an important determinant of an adverse outcome after revascularization.[11-13] It is biologically plausible that a reduction in luminal diameter by a constant amount of neointimal hyperplasia results in a proportionally higher-grade of restenosis in small compared with large vessels. Revascularization results in arterial injury, initiating a proliferative vascular cascade that causes smooth muscle cell proliferation and migration resulting in neointimal hyperplasia.[13] The amount of neointimal hyperplasia is largely independent of vessel size, and thus late luminal loss, an angiographic measure of neointimal hyperplasia, is similar across a wide range of vessel diameters.[14] Accordingly, small vessels are more prone to restenosis than larger vessels because they are less able to accommodate neointimal tissue without compromising blood flow.[13]

This study found gender was not an independent predictive variable for restenosis, although in univariate analysis women showed significant more restenosis than men. Published reports indicate gender differences in the risk of restenosis can be explained by the physical size of the patient. Although coronary artery diameter is highly related to body size, women have smaller coronary arteries than men after accounting for differences in body size. These findings further support the hypothesis that smaller coronary arteries explain higher perioperative mortality with coronary artery bypass grafting and poorer outcomes with other treatments for coronary disease in women and smaller people.[11,15,16]

In univariate analysis, age seemed to have no relation with restenosis. However, in the multivariate model age seemed to be an independent predicitor for restenosis. The current way statistical analysis is performed can make this happen. Usually, only variables showing a relation in the univariate analysis (typically $P < 0.1$) are included in the multivariate analysis. However, these variables could also be related with each other (confounding). Therefore, multivariate analysis will discriminate if these variables are dependent or independent of each other. We stated that we always included age (and gender, and operation indication) in the multivariate analysis.

In univariate analysis, age seemed to be confounded to the null (i.e. showed no relation). However, age was an independent variable in the multivariate analysis. This can only be explained if age has been associated with the other independent variables or a combination of them. In our study, the “older” patients can not be compared with the “younger” patients in relation to the other predictive variables.

We can only speculate about the predictive value of the duration of ischemic walking complaints due to SFA occlusion for restenosis. In most cases, the SFA is occluded for a long period and will be fibrous. Fibrous plaques have previously been associated with arterial

shrinkage resulting in smaller vessel size.[17-18] Our results suggest that longer occlusion time and subsequent arterial fibrotic shrinkage may result in a smaller residual lumen after intervention which makes the artery more prone to develop restenosis.
This study may have important implications for the care and treatment of patients with arterial obstructive disease in the SFA. Structured exercise and medical treatment are the initial approach to the treatment of intermittent claudication. Failure to respond to this would lead to limb revascularization.[1] Accordingly, almost all patients undergo operations after a long period of ischemic complaints. The median duration of ischemic walking complaints in our study was 56 months before surgery. As was explained, a likely assumption is that the occluded fibrous femoral artery will shrink over time. Subsequently, patients with longer duration of ischemic complaints will have smaller arteries with a higher restenosis rate. Therefore, our findings lead to a recommendation of a more aggressive treatment strategy in a subgroup of patients. The major challenge however, is to identify the subgroup of patients who would benefit from this aggressive treatment; consequently, a longitudinal study is required to support this concept.
Known factors influencing outcomes after percutaneous intervention include the extent of the disease, use of a stent, amount of calcification, and runoff below the knee.[5,19-21] Factors influencing the outcome of bypass surgery focus on the quality of the bypass (graft diameter, graft length and type of bypass) instead of the extent and severity of the lesion.[5,10] Similar to percutaneous interventions, bypass surgery for tissue loss has worse outcomes compared with bypass for claudication.[22,23] The reason for this is not fully understood and is not simply explained by inflow and outflow levels, because distal origin grafts as well as pedal bypass grafts have durable results.[5,24] In this study, runoff, TASC classification and operation indication were not associated with restenosis after RSFAE. It is notable that recent reports concerning RSFAE also show no relation between these variables and restenosis.[2,25]

Our study has several potential limitations. Our data are prospectively obtained but are retrospectively analyzed. Findings of this study have to be confirmed in a larger randomized study. Our findings are based on a relatively small patient group, and confirmation in a larger cohort is required.

This study is the first study to provide clinical characteristics that are predictive for RSFAE restenosis in the first year. Clinicians should reconsider RSFAE in older patients, patients with a longer history of ischemic complaints and in patients with small SFA diameter. Follow-up and treatment of restenosis may need to be more aggressive for these subgroups.

CONCLUSION

This study provides evidence that age, vessel size, and duration of ischemic walking complaints before RSFAE are predictive values for restenosis after RSFAE.

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CHAPTER 8

Histologic atherosclerotic plaque characteristics are associated with restenosis rates after endarterectomy of the common and superficial femoral arteries

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ABSTRACT

Objectives: This study assessed the predictive value of histologic plaque characteristics for the occurrence of restenosis after femoral artery endarterectomy.

Background: It would be advantageous if patients at increased risk for restenosis after arterial endarterectomy could be identified by histologic plaque characteristics of the dissected plaque. Differences in atherosclerotic plaque composition of the carotid artery have been associated with restenosis rates after surgical endarterectomy. However, whether atherosclerotic plaque characteristics are also predictive for restenosis in other vascular territories is unknown.

Methods: Atherosclerotic plaques of 217 patients who underwent an endarterectomy of the common femoral artery (CFAE, n = 124) or remote endarterectomy of the SFA (RSFAE, n = 93) were examined and scored microscopically for the presence of collagen, macrophages, smooth muscle cells, lipid core, intraplaque hemorrhage, and calcifications. The 12-month restenosis rate was assessed using duplex ultrasound imaging (peak systolic velocity ratio ≥2.5).

Results: The 1-year restenosis rate was 66% (61 of 93) after RSFAE compared with 21% (26 of 124) after CFAE. Plaques with characteristics of high collagen and smooth muscle cell content were positively associated with the occurrence of restenosis, with odds ratios of 2.90 (95% confidence interval, 1.82-4.68) and 2.20 (1.50-3.20) for SFA and CFA, respectively. SFA plaques showed significantly heavier staining for collagen (69% vs 31% for CFA; P < .001) and smooth muscle cells (64% vs 36% for CFA; P < .001). After multivariate analysis, the operation type (CFAE or RSFAE), gender, and the presence of collagen were independent predictive variables for restenosis after endarterectomy of the CFA and SFA.

Conclusion: Plaque composition of the CFA and SFA differs. Furthermore, the dissection of a fibrous collagen-rich plaque is an independent predictive variable for restenosis after endarterectomy of the CFA and SFA.

INTRODUCTION

Prevention and risk management of restenosis after endarterectomy of the peripheral arteries represents one of the major challenges in peripheral revascularization. Extent of the disease, number of run-off arteries, and diabetes mellitus are known risk factors after peripheral vascular intervention.[1,2] In contrast with established risk factors, the relation between local atherosclerotic plaque characteristics and development of restenosis after femoral artery endarterectomy has never been studied. It would be advantageous if patients at risk for restenosis could be identified directly after surgery by examining the dissected plaque.
Atherosclerotic plaque composition of the carotid artery has been associated with restenosis after surgical endarterectomy. Macrophage infiltration and a large lipid core in the dissected carotid plaque were independent predictive parameters for lower restenosis rates after carotid endarterectomy.[3] However, whether atherosclerotic plaque characteristics are also predictive for restenosis in other vascular territories is unknown.
This study was conducted to determine whether histologic plaque characteristics of the femoral artery are predictive for the occurrence of restenosis after endarterectomy.

METHODS

Patient population

The studied patient population is a subgroup of patients who are included in the Athero-Express Biobank. The subgroup consists of all patients who underwent a sole endarterectomy of the common femoral artery (CFAE) or remote endarterectomy of the superficial femoral artery (RSFAE). The design of the Athero-Express Biobank has been described previously.[4] Briefly, Athero-Express is an ongoing vascular biobank study with a longitudinal study design. The main objective is to determine the predictive value of local atherosclerotic plaque characteristics for the occurrence of future local and systemic cardiovascular events. Dissected femoral plaques, freshly obtained during endarterectomy in two participating Dutch hospitals, are collected and undergo histologic examination. In addition, clinical follow-up is obtained 1 to 3 years after the surgical intervention.
In both hospitals, patients with peripheral leg ischemia are being treated by use of the same protocol (Figure 1). First, patients with intermittent claudication are treated conservatively with supervised walking exercise. Patients without improvement and disabling complaints or whose complaints worsen after supervised exercise, and patients with critical ischemia primarily, are discussed in a multidisciplinary meeting with interventional radiologists and vascular surgeons. Patients with a sole lesion (TransAtlantic Inter-Society Consensus [TASC] A and B lesions) of the CFA are treated with endarterectomy and closed with patch plasty (Figure 1).
TASC C lesions of the SFA, assessed as not treatable by percutaneous intervention, and TASC D lesions of the SFA are treated with a venous supragenicular bypass. A patient who does not have a suitable great saphenous vein will be treated primarily with remote superficial femoral artery endarterectomy (RSFAE; Figure 1). If the RSFAE fails, patients undergo prosthetic supragenicular bypass.

The current study selected the 238 patients who underwent a CFAE (TASC A and B lesions) or RSFAE (TASC C and D lesions) to treat severe intermittent claudication, critical ischemia, or tissue loss (Rutherford classification II-VI) from June 2002 until October 2007. Surgical procedures were performed in a standardized way, as described previously.[5,6]

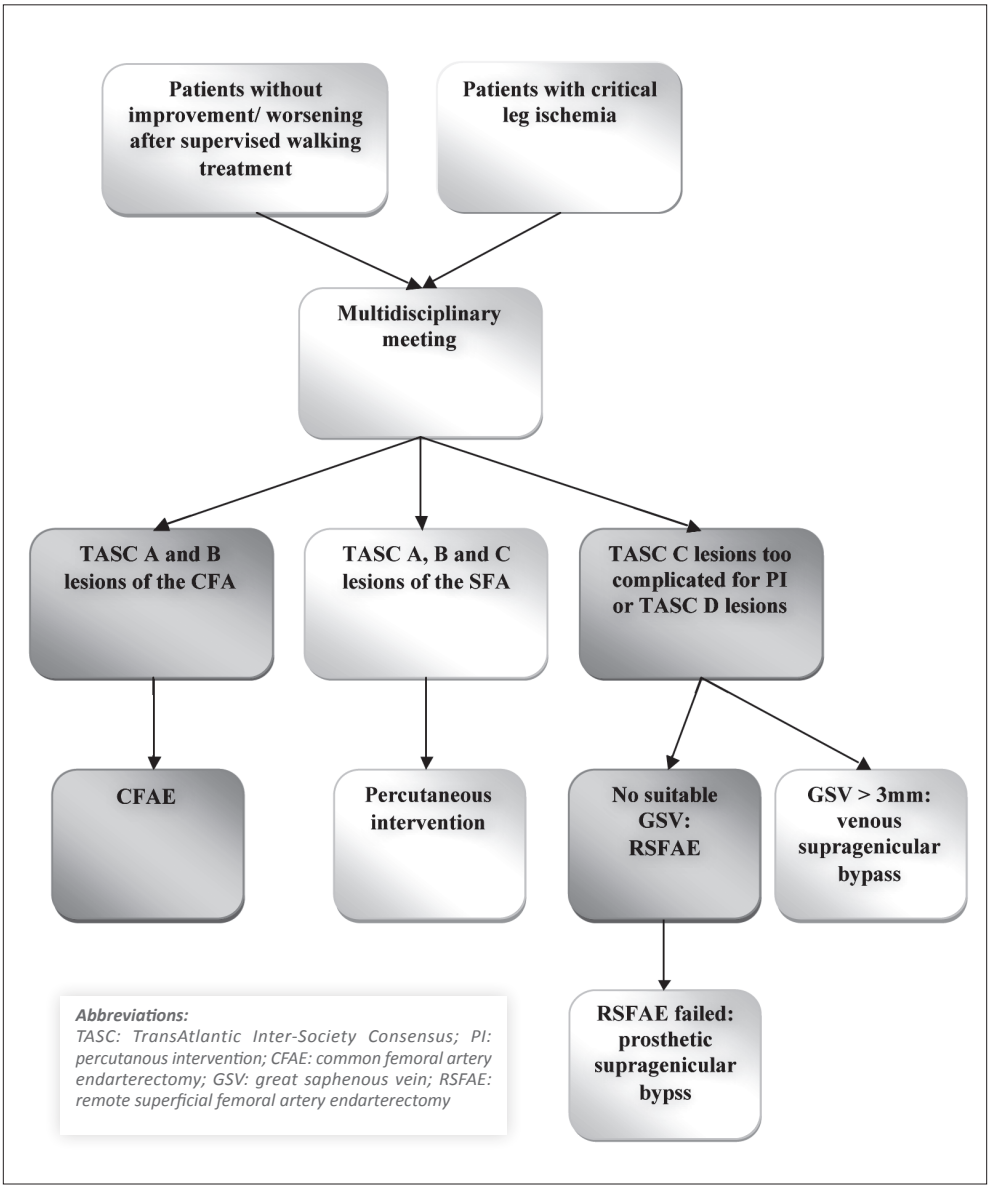

Figure 1. Treatment protocol of patients with peripheral leg ischemia when conservative treatment failed

In both hospitals, patients with peripheral leg ischemia are being treated according the same protocol. The gray boxes in the diagram are the studied patients.

Clinical characteristics of all patients were prospectively recorded at baseline, medical records were studied, and patients completed a detailed validated questionnaire. The medical ethics boards of both participating hospitals approved the study, and all patients provided written informed consent.[4]

Plaque characteristics

The procedures for processing and examination of the dissected atherosclerotic plaques have been described previously.[4] Directly after excision, the atherosclerotic plaque was transferred to the laboratory. According to literature, we chose the segment with the greatest plaque burden as the culprit lesion. When the femoral plaque was totally occluded, we chose the most voluminous and intact part of the plaque.[4] The culprit lesion was fixed in 4% formaldehyde, decalcified in ethylenediaminetetraacetic acid, and embedded in paraffin. Segments adjacent to the culprit lesion were snap frozen in liquid nitrogen and stored at -80˚C for future analysis.

The paraffin segment of the culprit lesion was cut on a microtome into sections of 5 µm for histologic and immunohistochemical staining and the following stainings were performed to characterize the plaque: Picrosirius red (collagen and lipid core), CD 68 (macrophages), α-actin (smooth muscle cells), hematoxylin and eosin (calcification, lipid core, and intraplaque hemorrhage), and fibrin (intraplaque hemorrhage). All stainings were examined microscopically, and plaque characteristics were scored semi-quantitatively (no/ minor vs. moderate/heavy). The definitions of each staining category have been published previously.[4] All scorings are based on visual estimates and are rated on ordinal scales. Two observers independently scored all stainings. A third independent observer was consulted when interpretations differed between the first 2 observers. Briefly, no or minor represent absent or minimal staining with a few clustered cells, whereas moderate and heavy represent larger areas of positive staining.[4]

Before determination of the different histologic characteristics, we differentiated between the intima and media in the hematoxylin and eosin (H&E) and elastin von Gieson (EvG) staining, by deciding were the internal elastic lamina was. The studied histologic characteristics are only scored in the intimal layer.[4,7]

No or minor macrophage and smooth muscle cell infiltration is shown by absent or minimal staining with few clustered cells, whereas moderate and heavy infiltration is shown by moderate or heavy staining with larger areas of clustered cells. No or minor collagen content is shown by absent or minimal staining for collagen with a thin fibrous cap, whereas moderate or heavy collagen is shown by moderate staining or heavy staining for collagen, covering any lipid in the atherosclerotic lesion, as visualized with and without polarized light. No or minor calcifications are indicated by absent or nonconfluent speckled area of plaque calcification, whereas moderate or heavy represent nodules of calcified plaque or confluent area of calcified plaque, respectively.

Intraplaque hemorrhage was scored as present or absent. The size of the lipid core was estimated as a percentage of total plaque area with a division in three categories: none, <40%, and ≥40%. We have recently demonstrated that our semi-quantitative analysis of atherosclerotic plaque histology is well reproducible, both intraobserver and interobserver variability.[7] CD-68 and α-actin stains were also analyzed quantitatively, and these values revealed excellent correlations with our semi-quantitatively analyses.[7]

Follow-up

Follow-up was scheduled at 3, 6, and 12 months, and annually thereafter, and included history, physical examination, and duplex ultrasound scanning. Duplex ultrasound scanning to detect restenotic lesions was performed according to protocol. The entire CFA, the proximal profunda femoral artery, the entire SFA (from origin to the popliteal artery, including the distal stent after RSFAE), and the entire popliteal artery were scanned in every patient. Obstructions were classified on the basis of the peak systolic velocity (PSV) within the obstruction (numerator of the PSV ratio) and proximally of the obstruction (denominator of the PSV ratio). A stenosis of 50% was considered if the PSV ratio was 2.5. The end point of this study was restenosis 12 months postoperatively, which was assessed by duplex ultrasound imaging. Lumen reduction of ≥50% using duplex ultrasound imaging (peak systolic velocity ratio ≥ 2.5) was considered as the presence of significant postoperative restenosis.

Data analysis

Statistical analysis was performed with SPSS 15.0 software (SPSS Inc, Chicago, Ill). Univariate analysis was used to test baseline characteristics for association with restenosis. Categoric variables were tested using cross tables (2 × 2), and the accompanying *P* value was calculated with the χ^2 statistic. Continuous variables were tested nonparametrically by using the Mann-Whitney U test. *P* values <.05 were considered statistically significant. To test independency of the univariate variables, multivariate binary logistic regression analysis (with backward exclusion of nonsignificant variables using likelyhood ratio test) was performed. Variables showing a significant association with restenosis in univariate analysis were included in the multivariate analysis. Age, sex, and operation indication were always included in the multivariate analysis models.

RESULTS

A total of 238 patients underwent a CFAE or RSFAE. Excluded from the study were two patients who died in the first postoperative month of coronary artery disease and 19 patients whose atherosclerotic plaque material was insufficient for histologic analyses. Therefore, a total 217 patients were examined for the current study.
CFAE was done in 124 patients (57%) and RSFAE in 93 patients (43%). Of the 217 patients, 168 (77%) were operated on for intermittent claudication (Rutherford class III). The other patients had critical ischemia or tissue loss (Rutherford class IV-VI). Men comprised 73% of the operated-on patients, and most patients were affected by risk factors for atherosclerotic disease, as summarized in Table I.

Most of the atherosclerotic plaques were fibrotic, 85% contained moderate (n = 114) to heavy (n = 74) staining for collagen, 75% had moderate (n = 90) to heavy (n = 73) staining for smooth muscle cells, and 69% had moderate (n = 46) to heavy (n = 104) staining for calcifications. Macrophage staining was only moderate (n = 36) to heavy (n = 7) in 20%, and a lipid core was present in 25% of the plaques (n = 162). Intraplaque hemorrhage was present in 65% of all plaques (n = 141).

Duplex ultrasound examinations revealed a restenosis (≥50% lumen reduction) in 87 patients (40%) 12 months post-operatively. Restenosis was present in 56% of women (33 of 59) compared with 34% of men (54 of 158), which was significant. Current smoking was also significantly associated with restenosis (Table I). Restenosis rates were significantly higher in patients who underwent a RSFAE compared with CFAE (61 of 93 [66%] vs 26 of 124 [21%], respectively; Table I). Restenosis occurred after RSFAE in 61 patients. Of these, 38 (62%) were symptomatic and needed a reintervention, which consisted of percutaneous transluminal angioplasty (PTA) in 25, a bypass graft in 11, and open repeat endarterectomy of the proximal SFA with proximal patch plasty in 2. Of the 26 patients with restenosis after CFAE, 4 (15%) needed reintervention, 3 were treated with a repeat endarterectomy of the CFA with proximal patch plasty, and 1 patient was treated with a renewed bypass graft.

In univariate analyses, the presence of collagen and smooth muscle cells were significantly ($P < .001$) associated with restenosis (Figure 2). The risk that stenosis would develop was higher in patients with moderate or heavy collagen or smooth muscle cell staining in their plaque with odds ratios (OR) of 2.90 (95% confidence interval [CI], 1.82-4.68) and 2.20 (95% CI, 1.50-3.20), respectively than in patients who showed no or minor staining of collagen or smooth muscle cells in their femoral plaque. In addition, heavy staining for calcification was inversely related ($P = .001$) with restenosis rates (OR, 0.67; 95% CI, 0.53-0.86). The presence of macrophages, lipid core, or intraplaque hemorrhage was not related with restenosis development (Figure 2).

Of the total of 87 patients with restenosis (≥50% lumen reduction with duplex ultrasound) 12 months post-operatively, 5 patients were detected with a restenosis by ultrasound duplex at 3 months, another 36 patients at 6 months, and another 46 patients at 12 months follow-up. We could not show a significant difference in plaque histology between patients suffering from early restenosis (<6 months) compared to patients suffering from late (>6 months) restenosis (data not shown).

When the histologic plaque characteristics between the CFA and SFA were compared, staining for calcification, collagen, and smooth muscle cells differed significantly between the two femoral arteries (Figure 3). Moderate to heavy staining for collagen was significantly more prevalent in the SFA (89 of 93 [96%]) compared with the CFA (99 of 124 [80%]; $P < .001$). Moderate to heavy staining for smooth muscle cells also appeared more prevalent in the SFA (83 of 93 [89%]) compared with the CFA (80 of 124 [65%]; $P < .001$). Moderate to heavy calcifications were significantly more present in the CFA (94/124 [76%]) than in the SFA (56 of 93 [60%]; $P < .001$). Intraplaque hemorrhage, lipid core, and macrophages did not differ significantly between the CFA and SFA plaques (Figure 3).

Collagen, calcification or smooth muscle cell content in the plaque did not correlate with the presence or absence of diabetes, use of statins or use of aspirins (data not shown).

To determine if histologic plaque characteristics were independent predictors for restenosis, we performed a multivariate analysis. The initial variable set consisted of sex, age, severity of ischemia, operation (CFAE or RSFAE), collagen, smooth muscle cells, heavy staining for calcification, and current smoker. Type of operation (CFAE or RSFAE), collagen staining, and sex were independent predictive parameters for restenosis development after femoral endarterectomy (Table II). In our cohort, there was no interaction between collagen and type of artery or between collagen and sex.

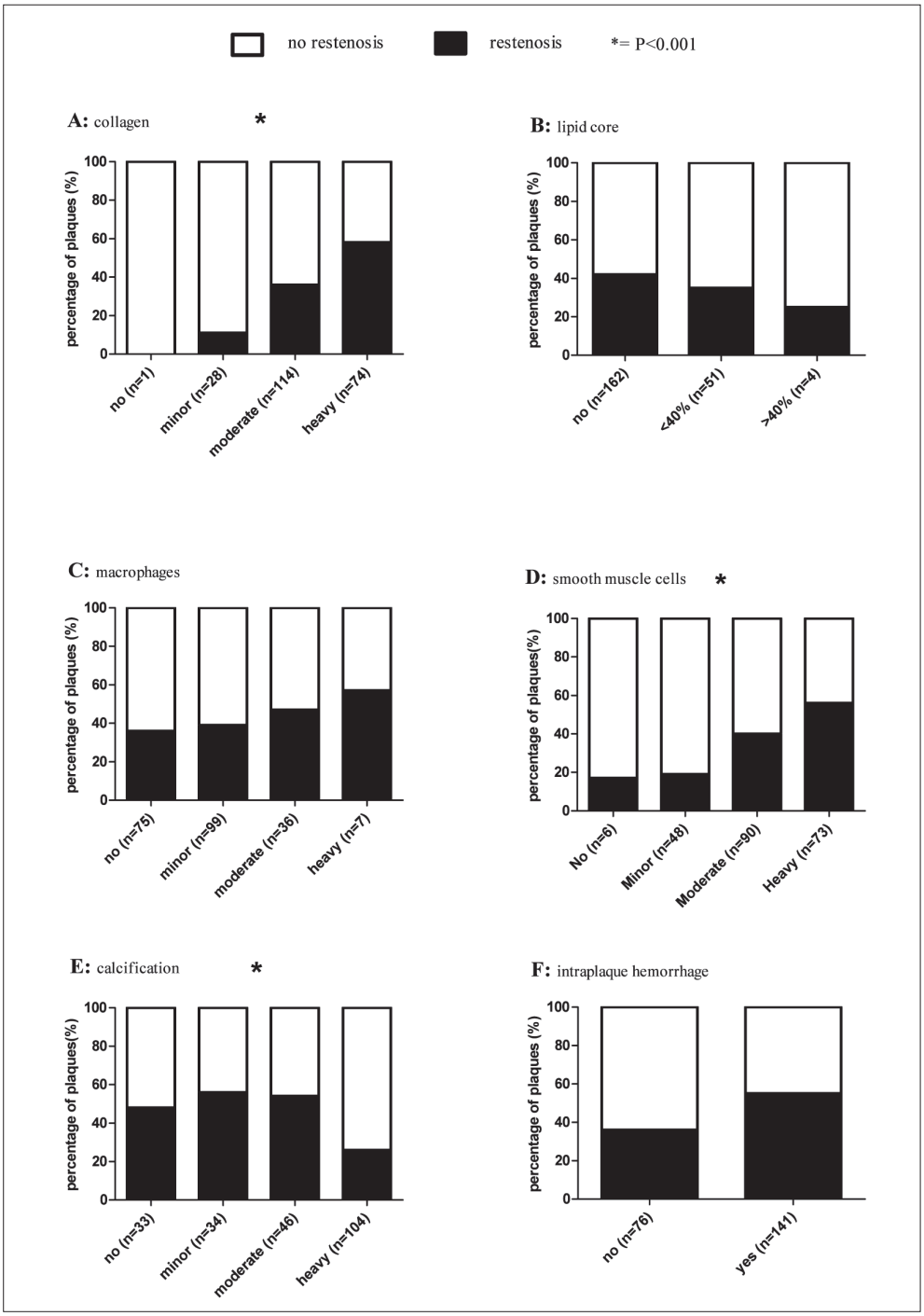

Figure 2. Plaque characteristics in relation to restenosis.
*Different plaque characteristics inrelation to restenosis after endarterectomy of the femoral arteries. **A**: collagen; **B**: lipid core; **C**: macrophages; **D**: smooth muscle cells; **E**: calcification; **F**: intraplaque hemorrhage. *P < .001.*

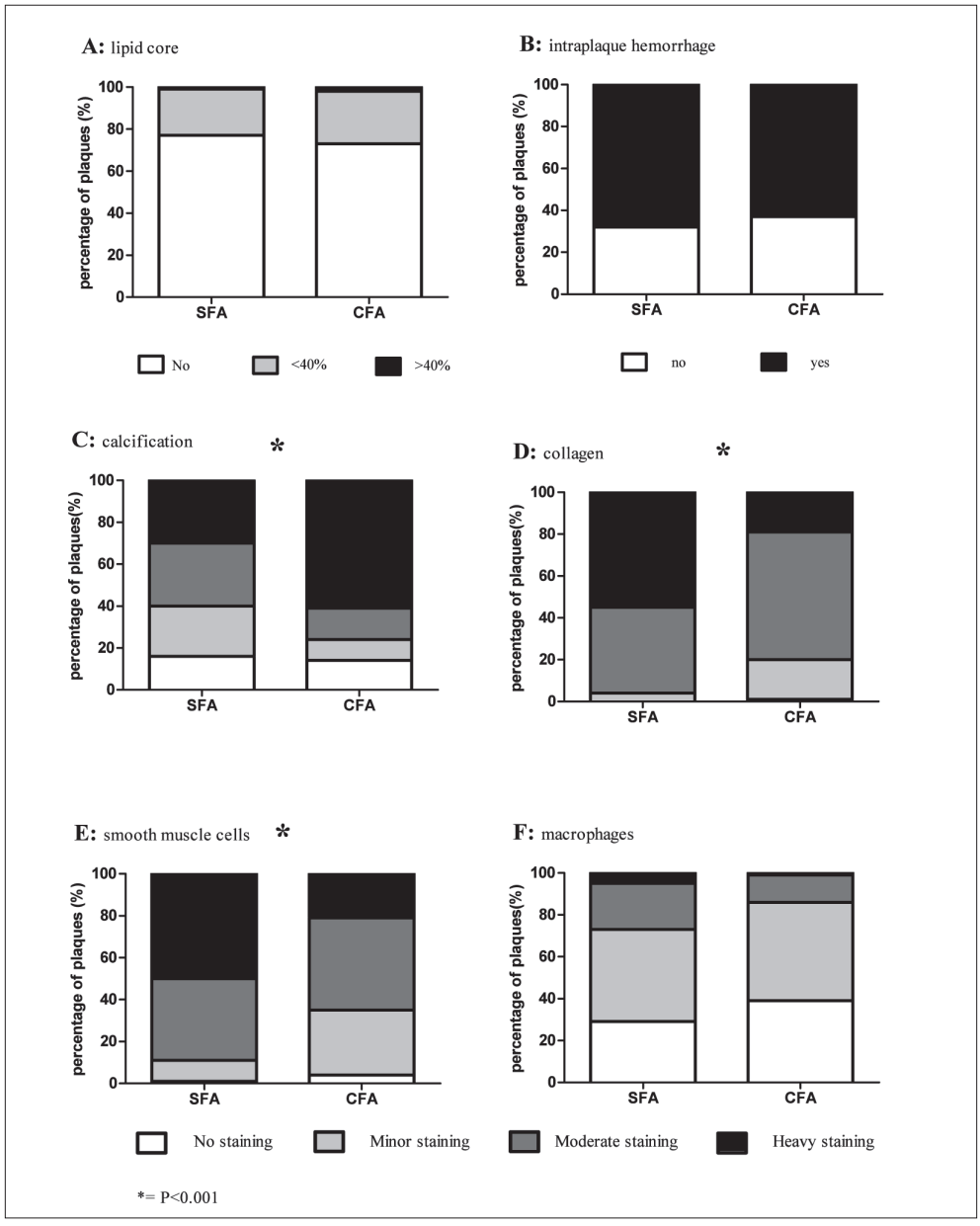

Figure 3. Plaque characteristics in relation to operation (CFAE or RSFAE)

Different plaque characteristics for the common femoral artery (CFA) and the superficial femoral artery (SFA). ***A****: lipid core;* ***B****: intraplaque hemorrhage;* ***C****: calcification;* ***D****: collagen;* ***E****: smooth muscle cells;* ***F****: macrophages. *P < .001.*

DISCUSSION

This is the first study, to our knowledge, that provides evidence that histology of the dissected femoral plaque hides predictive value for risk of restenosis after CFAE and RSFAE. Logically, the operation type is a strong independent predictor for restenosis: RSFAE is performed for TASC C/D lesions and CFAE for TASC A/B, and CFAE and RSFAE are different operation techniques. However, when we corrected for the operation type and inherent to this, for the different TASC lesions, moderate to heavy staining of collagen in the dissected plaque seemed to be an independent variable for restenosis. Furthermore, we observed that the histologic plaque characteristics differ between the CFA and SFA.

Reported restenosis rates seem high in this study compared to literature.[5,8] Published studies on CFAE or RSFAE reported primary patency rates, which is defined as uninterrupted patency without any procedures performed on the target lesion. We reported restenosis rates and scored all patients with a lumen reduction of 50% or more as restenotic lesion, although a substantial number of asymptomatic patients were treated conservatively.

A recent published article of our group showed that the inflammatory unstable carotid plaques with high content of macrophages and a large lipid core were associated with less restenosis, probably due to inflammatory activity in the vascular wall, which elicits expansive remodeling.[3] In this study, we determined that the fibrotic (stable plaque) features of femoral artery plaques, like collagen and smooth muscle cells, were associated with a higher incidence of restenosis whereas the inflammatory plaque characteristics were not. Although these results may suggest conflicting observations between the carotid and femoral artery with respect to restenosis prediction, the observation supports the underlying concept that stable plaques are associated with a higher restenosis rate. The different plaque characteristics (eg, low content of macrophages and absence of a lipid core vs high collagen and smooth muscle cell content) are considered determinants of the same plaque type: the stable fibrous plaque.

It has been demonstrated that inflammatory carotid plaque characteristics are less prevalent in patients with a longer time interval between the clinical onset of symptoms (eg, stroke) and carotid endarterectomy.[9] Owing to the current treatment policy of supervised walking exercise after the start of intermittent claudication, patients are often operated on several years after the initiation of ischemic complaints, whereas carotid endarterectomy generally occurs within 1 month after the first ischemic event. This may partly explain why inflammatory characteristics in the femoral plaque were much less prevalent than fibrous characteristics.

We can only hypothesize about the mechanism for the increased restenosis rates when fibrous, collagen-rich plaques have been dissected. Previously, fibrotic characteristics of the femoral plaque have been associated with constrictive remodeling (arterial shrinkage or negative remodeling). Postmortem studies have demonstrated that constrictive remodeling is an important determinant of lumen decrease in the femoral artery.[10] In addition, femoral and coronary arteries with the smallest vessel area encompass plaques that show fewer inflammatory cells and are more fibrous.[11-13] Other studies described that collagen accumulation results in arterial shrinkage analogous to scar constriction.[14] A likely assumption is that the occluded femoral artery will shrink over time due to fibrotic remodeling, subsequently resulting in a smaller lumen area after intervention and increased restenosis rates.

To our knowledge, this is the first study showing differences in plaque phenotype between the CFA and SFA. The SFA plaques contained significantly more collagen compared with the CFA plaques, and this characteristic was associated with different restenosis rates between these arteries. Our results may also have implications for other vascular territories. For instance, it is appreciated that the left anterior descending coronary artery is more prone for restenosis after intervention than the other coronary arteries.[15] The reason for this remarkable difference is not clear. One hypothesis is that like the femoral and carotid arteries, the difference in patency rates after intervention of other vascular territories may also be associated with differences in plaque characteristics.

It is known from literature that race is of influence on atherosclerotic disease. For example, blacks have larger coronary lumen and area enclosed by internal elastic lamina compared to whites and Indian Asians have less lower limb atherosclerosis than Europeans, unexplained by established risk factors.[16,17] Because only Caucasian Europeans were included in this study we can only speculate that race will be of influence on histological femoral plaque findings.

Study limitations

This study has several limitations. First, we can only speculate about the possibility of arterial shrinkage (negative remodeling) as an explanation for the current observations. Future research has to confirm our hypothesis that constrictive remodeling and subsequently reduced vessel size and residual lumen are a reason for lower patency rates after endarterectomy. Second, we included patients operated on with two different techniques (CFAE and RSFAE), and inherent to these operations, the TASC lesions that were operated on were also different. However, this is intrinsic to the different arteries that were operated on, and besides, we corrected for this in the multivariate analysis.

Third, according to our study design we defined the lesion with the greatest plaque burden as the culprit lesion.[4] We assume that this is the most representative lesion of the total plaque. We did not examine different lesions in a single patient. The assumption that the histology of the culprit lesion is consistent in the total plaque, might be a study limitation.

This study has multiple clinical applications. To our knowledge, this is the first study to prove that femoral plaque histology could predict increased risk of restenosis after endarterectomy. Furthermore, it has been determined that plaque histology differs between the common and superficial femoral artery. This may point to new clues for the understanding of the mechanisms of predicting and preventing restenosis in both arterial territories.

Besides, individual risk assessment for restenosis is needed to improve patency rates after femoral endarterectomy. The new concept that plaque histology can predict restenosis rate, will likely contribute to this. Third, our results can be helpful for developing strategies for incorporating imaging of atherosclerotic plaques into clinical trials.

The authors are convinced that structural histologic analysis of the dissected femoral plaque could be an important factor for predicting restenosis after endarterectomy. Restenosis after femoral endarterectomy is still a major complication. Using histologic plaque characteristics for the prediction of restenosis, is a new concept. Together with other risk factors, plaque histology could be of influence on the intensity of the follow-up scheme of the individual patient.

This study will certainly influence the indication for RSFAE. We may conclude that women with collagen rich plaques should be considered as high risk-patients for restenosis post-RSFAE, and venous bypass grafting is the preferred operation for this subgroup. When venous material is lacking, we think that women can be treated by RSFAE, but they require more stringent follow-up to identify early restenosis.

CONCLUSION

Plaque composition of the CFA and SFA differs. Furthermore, the presence of a fibrous collagen-rich atherosclerotic plaque is an independent predictive variable for restenosis after CFAE and RSFAE.

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CHAPTER 9

Intraplaque hemorrhage is a predictor for adverse cardiovascular events after femoral endarterectomy

Submitted

ABSTRACT

Objectives: to examine the association between plaque phenotype and adverse cardiovascular events following femoral endarterectomy.

Background: Established cardiovascular risk factors can only partly explain the increased risk for life threatening secondary manifestations of atherosclerotic disease in patients with peripheral arterial disease. It is unknown whether dissected femoral plaques contain histological markers that are predictive for secondary events.

Methods: Consecutive femoral (n = 320) specimens underwent histologic examination for the presence of collagen, macrophages, smooth muscle cells, lipid core, vessels, intraplaque hemorrhage, and calcifications. Patients were followed up for 3 years after endarterectomy and investigated for the occurrence of cardiovascular events. Primary outcome was defined as any major adverse cardiovascular event (cardiovascular death, non-fatal myocardial infarction, non fatal stroke) or cardiovascular intervention, which was not planned at the time of inclusion and not related to the target femoral vessel.

Results: During a mean time follow-up of 2.5 years, 128 patients of the 320 included patients (40%) reached the primary outcome. Patients with an intra-plaque hemorrhage in the dissected plaque demonstrated an increased risk of primary outcome (risk difference 49% vs. 36%, HR with [95%CI] = 1.56 [1.06-2.29]). Besides plaque calcification (HR: 1.50 [1.01-2.21]), other plaque characteristics showed no significant associations with clinical follow-up in univariate analysis. After correction for the established cardiovascular risk factors and medication use through multivariate analysis, intraplaque hemorrhage revealed to be an independent predictor for major cardiovascular events during follow-up.

Conclusion: Intraplaque hemorrhage in the dissected femoral plaque is an independent predictor for future adverse cardiovascular events.

INTRODUCTION

Patients undergoing revascularization procedures for peripheral arterial disease (PAD) suffer from increased cardiovascular morbidity, which is mainly related to coronary artery disease with a mortality rate of 30% after 5 years.[1, 2] This increased risk is only partly explained by the well recognized cardiovascular risk factors.[3]
There has been a major interest in levels of circulating parameters of inflammation, in patients suffering from PAD, to assess the individual risk profile for cardiovascular morbidity. Increased levels of tumor necrosis factor-alpha, C-reactive protein (CRP) and interleukin-6 are associated with PAD[4] and with adverse outcome after revascularization, including restenosis and mortality.[5] Increased levels of parameters reflecting a systemic inflammatory component, like CRP, have also been found to be associated with increased atherosclerotic plaque macrophage content, greater lipid core and thinner fibrous cap.[6] However, CRP is an aspecific marker for inflammation and more specific markers for atherosclerotic events are necessary.
Recently we showed that local atherosclerotic carotid plaques hide characteristics that reflect the systemic progression of the disease. A high concentration of Osteopontin (OPN), collagenase matrix metalloproteinase -8 (MMP- 8) and adipocyte-fatty acid-binding-protein (FABP-4) in a dissected carotid plaque have all been associated with an increased incidence of myocardial infarction, stroke and peripheral arterial disease.[7-9] Furthermore, it has been demonstrated that carotid intraplaque hemorrhage is a predictive marker for systemic adverse secondary cardiovascular events in other vascular territories.[10] These aforementioned observations raise the hypothesis that specific plaque features, like intra plaque hemorrhage, reflect a pathogenetic destabilizing process that is also present in other vascular territories at risk. Therefore, we hypothesized that the femoral plaque contains histological plaque characteristics which hide predictive value for occurrence of secondary manifestations of atherosclerotic disease.

METHODS

Patient population

Patients participating in this study underwent an endarterectomy of the common and superficial femoral artery between January 2002 and January 2009, to treat intermittent claudication (Fontain class IIb), critical ischemia (Fontain class III), or tissue loss (Fontain class IV). All patients were included in the Athero Express biobank. The study design has been reported earlier.[11] Briefly, dissected femoral plaques, obtained in two Dutch teaching hospitals (St. Antonius Hospital Nieuwegein and University Medical Center Utrecht), were collected and subjected to histopathological examination according to protocol. All patients agreed with participation in the study and provided written informed consent and both medical ethic boards of the hospitals approved the study. Baseline clinical data were recorded from questionnaires filled in by the patient, and encompass cardiovascular risk factors, medical history and medication use. Missing data were collected from the patient charts from the participating hospitals and general practitioners.[11] The definitions of hypertension, hypercholesterolemia and diabetes were restricted to those cases requiring medical treatment.

Femoral endarterectomy

In both participating hospitals, patients with peripheral leg ischemia were treated according to the same protocol which has been reported earlier.[12] First, patients with intermittent claudication were treated conservatively with supervised walking exercise. Patients without improvement and disabling complaints or whose complaints worsen after supervised exercise, and patients with critical ischemia primarily, are discussed in a multidisciplinary meeting with interventional radiologists and vascular surgeons. Patients with a sole lesion (TransAtlantic Inter-Society Consensus [TASC] A and B lesions) of the common femoral artery (CFA) were treated with endarterectomy and closed with patch plasty. TASC C lesions of the superficial femoral artery (SFA), assessed as not treatable by percutaneous intervention, and TASC D lesions of the SFA were treated with a venous supragenicular bypass. A patient who does not have a suitable great saphenous vein was treated primarily with remote superficial femoral artery endarterectomy (RSFAE).[12]

Histopathological examination

The processing and examination of the atherosclerotic plaque was executed according to a standardized protocol and has been described previously.[11] Directly after dissection, the plaque was divided into segments of 5 mm thickness along the longitudinal axis. The segment with the greatest plaque burden (culprit lesion) was fixed in formaldehyde 4%, decalcified in ethylenediaminetetraacetic (EDTA), embedded in paraffin and cut into 4 µm sections for histological and immunohistochemical analysis.[11]

Specimens were stained with CD68 for macrophages, alpha actin for smooth muscle cells, Picro Sirius red for collagen, Hematoxylin eosin (HE) for general overview including calcifications, CD34 for microvessels and HE, fibrin and alpha actin for intraplaque hemorrhages. Macrophages and smooth muscle cell infiltration were quantitatively scored (average percentile positively stained area of total plaque area in three hotspots) using computerized analyses, as well as semi-quantitatively as no/minor or moderate/ heavy staining. Plaque microvessel density (MVD) was determined quantitatively and reported as the average number of CD34-immunopositive microvessels of three hotspots within every single plaque.[10] A hotspot was defined as one high power field at 40x magnification. For vessel quantification, we used a grid (100x 100µm) overlying every hotspot to improve the reproducibility and to avoid counting vessels twice. The vessel density was determined at by counting the number of vessels crossed by a bar of the grid within the selected hotspots. Increased vessel density was defined as an average vessel count per hotspot higher than the median of the cohort.

Next to the macrophage and smooth muscle cell content, also collagen, calcification, lipid core and intraplaque hemorrhage were scored semi-quantitatively as described previously.[11] Macrophages: no or minor CD68 staining: negative or clusters with less than 10 cells present; moderate or heavy CD68 staining: cell clusters with >10 cells present or abundance of positive cells. Smooth muscle cells: no or minor alpha actin staining over the entire circumference with absent staining at parts of the circumference of the arterial wall; positive cells along the circumference of the luminal border, with locally at least few scattering cells. Collagen: no or minor staining along part of the luminal border of the plaque; moderate or heavy staining along the entire luminal border. Calcification: no or minor staining along part of the luminial

border of the plaque or a few scattered spots within the lesion; moderate or heavy staining along the entire luminal border or evident parts within the lesion. Using the HE and Sirius Red stains, the size of the lipid core was estimated as percentage of total plaque area and divided into three categories: <10%, 10-40% and >40%. The majority of the plaques was occlusive and revealed fibrous components. Subsequently because of the very low percentage of femoral plaques with a lipid core of > 40%, we decided to combine the groups 10-40% en >40% lipid core. Intraplaque hemorrhage was scored using Hematoxylin and Eosin (HE), fibrin (Mallory's phosphotungstic acid-hematoxilin) stains and anti smooth muscle actin immunostains and scored as being "present" or "absent" (figure 1). Sparse intact erythrocytes were considered to be operation artefacts and left out of the intraplaque hemorrhage analysis. The applied semi-quantitative analyses of atherosclerotic plaque histology are well reproducible, both intraobserver and interobserver.[13] In addition, alpha-actin and CD68 immunostains were analyzed quantitatively using computerized analyses and results showed strong correlations with the semi-quantitative analyses.[13]

Follow-up

All patients underwent clinical follow-up after operation. Adverse events during initial hospital stay were recorded from the admission charts. After discharge from the hospital, all patients received a questionnaire at 1, 2 and 3 years postoperatively.[11] Patients were asked if they had suffered from any vascular adverse event and whether they had been hospitalized that past year. If one of these questions was answered positively, medical information was obtained from the concerning hospital, including additional investigations, like electrocardiogram, lab results, imaging studies or operation report for example, to verify the potential outcome event. If the patient did not respond, the general practitioner was contacted. All information per potential event was studied by two independent observers of the outcome assessment committee, who were blinded to the results of the current study. If the two observers disagreed about the judgment of a vascular event, a third opinion was obtained.

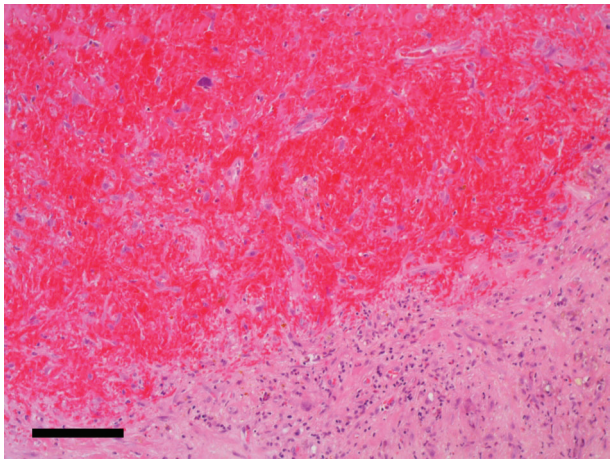

Figure 1. Intraplaque hemorrhage
Hematoxylin and eosin staining of intra plaque hemorrhage in femoral atherosclerotic plaque. Bar = 100 µm.

Definition clinical outcome

The primary outcome of the Athero Express study is a composite endpoint,[10, 11] which is defined as any systemic cardiovascular event or intervention, that was not planned at the time of inclusion. It includes cardiovascular mortality (fatal stroke, fatal myocardial infarction, sudden death, and other vascular death), non-fatal stroke, non-fatal myocardial infarction, and any arterial vascular intervention (e.g. coronary angioplasty/ stenting, coronary bypass, mesenteric angioplasty, peripheral vascular surgery/ percutaneous intervention that was not related with the initial target femoral vessel).

Statistical analysis

SPSS 15.0 software (SPSS Inc, Chicago, Ill) was used for all statistical analysis. Associations between the primary outcome and clinical characteristics, and plaque parameters were examined with univariate analyses. The Kaplan- Meier estimate of cumulative event rate at 3 year after femoral endarterectomy and the corresponding risk difference according to plaque characteristics at baseline were calculated. To correct for confounding factors, and to assess the independence of associations multivariate analysis was performed including all parameters that showed an association ($P< 0.1$) with the primary outcome (Multivariate Cox regression analysis, Method: backward stepwise likelihood ratio). Age, gender and smoking status were always included in the multivariate model. Associations between primary outcome and the clinical or plaque characteristics with a $P< 0.05$ and a 95% confidence interval not including 1 were considered as statistically significant.

RESULTS

In total 385 consecutive patients who underwent endarterectomy of the common or superficial femoral artery, between 2002 and 2009, were included in the Athero-Express study. Sixty five patients were excluded for the following reasons: not sufficient histologic material in 37 patients and 28 patients had no eligible long term follow-up. Our final cohort consisted of 320 patients.

The majority of all patients were men (72% [232/320]) and 73% (234/320) of the included patients suffered from disabling claudication pre-operatively. 27% suffered from critical ischemia. Baseline characteristics are summarized in Table 1. The majority of the dissected plaques were fibrous, with moderate to heavy staining for collagen, smooth muscle cells and calcifications and with no/minor staining for macrophages with a small lipid core. 65% (207/320) of the plaques revealed an intraplaque hemorrhage (Table 2).

During mean follow-up of 2.5 years (±0.8), 128 patients of the 320 included patients (40%) reached the primary outcome. 20/320 patients (16%) died from an adverse cardiovascular event, 14/320 patients (11%) suffered from a non fatal myocardial infarction, 4/320 patients (3%) suffered from a non fatal stroke and 90/320 patients (70%) underwent a cardiovascular intervention.

Table 1 shows the associations between the clinical baseline characteristics and primary outcome during follow-up. In univariate analysis, diabetes mellitus (Hazard Ratio (HR) 1.83 [1.28-2.62]; p=0.001), hypertension (HR: 1.90 [1.23-2.92]; p=0.004) and hypercholesterolemia

(HR: 1.86 [1.22-2.83]; p=0.004), a history of myocardial infarction (HR: 1.59 [1.11-2.29]; p=0.01), serum creatinin (HR: 1.00 [1.00-1.01]; p=0.03) and critical ischemia prior to endarterectomy (HR: 1.55 [1.06-2.24]; p=0.02) were all significantly associated with primary outcome during follow-up. The use of statins or anti-platelet agents was not associated with primary outcome (Table 1).

The histopathological examination of the femoral plaques in relation to the clinical follow-up revealed that plaques containing an intraplaque hemorrhage (207/320 [65%]) or moderate to heavy staining for calcification 214/ 320 (67%) were associated with a higher adverse cardiovascular event rate during 3 year follow-up after femoral endarterectomy. The Kaplan-Meier estimate of 3 year risk was 49% in patients whose plaque contained intraplaque hemorrhage compared to 36% in patients whose plaque did not contain intraplaque hemorrhage, with a risk difference of 13% (HR: 1.56 [1.06-2.29]; p=0.02; table 2, figure 1). Patients with moderate to heavy staining for plaque calcification showed a 3 year risk difference of 13% for adverse cardiovascular events (HR: 1.50 [1.01-2.21]; p=0.04; table 2). None of the other plaque characteristics showed a significant relation with clinical follow-up (table 2).

Table 1. Clinical characteristics and relation to primary outcome*

Patient Characteristics	All patients (N=320)	Number of patient with event [A]	Risk of primary outcome HR [95% CI] [#]	P-value
Age (mean,SD)	67 (9.1)	68 (8.2)	1.02 [1.00-1.04]	0.22
Sex				
female	88 (28%)	33/ 88 (38%)	- ref -	
male	232 (72%)	95/ 232 (41%)	1.05 [0.71-1.56]	0.82
Current smoker	120 (38%)	53/ 120 (44%)	1.20 [0.84-1.70]	0.32
Diabetes	92 (29%)	48/ 92 (52%)	1.83 [1.28-2.62]	0.001[†]
Hypertension	224 (70%)	102/ 224 (46%)	1.90 [1.23-2.92]	0.004[†]
hypercholesterolemia	220 (70%)	100/ 220 (46%)	1.86 [1.22-2.83]	0.004[†]
History: myocardial infarction	93 (29%)	46/ 93 (50%)	1.59 [1.11-2.29]	0.01[†]
History: leg amputation	9 (3%)	6/ 9 (67%)	2.15 [0.95-4.90]	0.07
Body mass index (mean, SD)	26 (3.7)	26 (3.2)	1.02 [0.97-1.07]	0.47
Serum Creatinin (mean, SD)	101 (59)	107 (60)	1.00 [1.00-1.01]	0.03[†]
Statin use	241 (76%)	101/ 241 (42%)	1.27 [0.83-1.94]	0.28
Anti-platelet agent use [‡]	273 (85%)	108/ 273 (40%)	0.86 [0.53-1.39]	0.54
Coumarin derivative use [§]	77 (24%)	30/ 77 (39%)	0.90 [0.60-1.37]	0.62
Clinical presentation				
Fontain class IIb	234 (73%)	87/ 234 (37%)	-	
Critical ischemi a (Fontain class III-IV)	86 (27%)	41/ 86 (48%)	1.55 [1.06-2.24]	0.02[†]

** Data are presented as No. (%) unless otherwise indicated; [A] primary outcome; [#]Cox regression analysis; [‡] Acetylsalicylacid and/or dipyridamol and/or clopidogrel; [§] Acenocoumarol or Fenprocoumon; [†] P<0.05*

Multivariate analysis

To proof independency for the prediction of primary outcome, we performed a multivariate analysis (Table 3). The initial variable set consisted of age, gender, current smoker, diabetes mellitus, hypertension, hypercholesterolemia, a history of myocardial infarction or leg amputation, serum creatinin, critical ischemia pre-operative, moderate to heavy staining for plaque calcification and intra-plaque hemorrhage.

After backward stepwise removal, intra-plaque hemorrhage seemed to be an independent predictor for primary outcome after femoral endarterectomy (HR: 1.61 [1.07-2.42]). Furthermore, cardiovascular risk factors including smoking (HR: 1.52 [1.04-2.23]), hypertension (HR: 1.63 [1.04-2.55]), diabetes mellitus (HR: 1.59 [1.08-2.35]]) and hypercholesterolemia (HR: 1.87 [1.20-2.91]) were independently associated with primary outcome (table 3).

Table 2. Plaque characteristics and relation with primary outcome*

Plaque Characteristics	All patients N=320	Number of patients with event [A]	3 years cumulative risk (KM estimate)	3 years risk differ-ence	Risk of primary outcome HR [95% CI][#]	P-value
Lipid core						
Absent	235 (73%)	88/ 235 (37%)	41%	-		
Present	85 (27%)	40/ 85 (47%)	49%	+8%	1.29 [0.89-1.88]	0.18
Macrophages						
no/ minor	246 (77%)	97/ 246 (40%)	43%	-		
moderate/heavy	74 (23%)	31/ 74 (42%)	45%	+2 %	1.09 [0.73-1.64]	0.67
Macrophages (QA)	0.6 (1.4)	0.6 (1.2)	-	-	1.03 [0.91-1.17]	0.64
Collagen						
no/ minor	45 (14%)	17/ 45 (38%)	43%	-		
moderate/heavy	275 (86%)	111/ 275 (40%)	43%	+0%	1.02 [0.61-1.71]	0.93
Smooth muscle cells						
no/ minor	74 (23%)	32/ 74 (43%)	49%	-		
moderate/heavy	246 (77%)	96/ 246 (39%)	42%	-7%	0.80 [0.54-1.20]	0.28
Smooth muscle cells (QA)	3 (3.9)	2.9 (3.2)	-	-	0.97 [0.93-1.02]	0.26
Calcification						
no/ minor	106 (33%)	35/ 106 (33%)	35%	-		
moderate/heavy	214 (67%)	93/ 214 (44%)	48%	+13%	1.50 [1.01-2.21]	0.04 [†]
Intraplaque hemorrhage						
Absent	113 (35%)	36/ 113 (32%)	36%	-		
Present	207 (65%)	92/ 207 (44%)	49%	+13%	1.56 [1.06-2.29]	0.02 [†]
Vessel density						
no/low density	98 (52%)	45/ 98 (46%)	49%	-		
increased density	92 (48%)	31/ 92 (34%)	36%	-13%	0.83 [0.52-1.31]	0.42

** Data are presented as No. (%); [A] primary outcome; [#] Cox regression analysis; [†] P<0.05*
Macropahes and smooth muscle cells also presented with quantitative analysis (QA) as mean (standard deviation)

Table 3. Multivariate analysis

	Risk of primary outcome HR [95% CI]	P-value
Age	N.S.	-
Gender: male	N.S.	-
Current smoker	1.52 [1.04-2.23]	0.03
Diabetes Mellitus	1.77 [1.21-2.57]	0.03
Hypertension	1.63 [1.04-2.55]	0.03
Hypercholesterolemia	1.83 [1.18-2.83]	0.007
History: myocardial infarction	N.S.	-
History: leg amputation	N.S.	-
Serum Creatinin	N.S.	-
Critical ischemia (Fontain class III-IV)	1.50 [1.00-2.21]	0.049
Calcification	N.S.	-
Intra- plaque hemorrhage	1.67 [1.12-2.49]	0.01

N.S.= removed from the multivariate model based on the backward stepwise likelihood ratio including 1 in the confidence interval.

DISCUSSION

Patients with PAD are at great risk for cardiovascular morbidity and mortality. A better assessment of their individual risk profile is of great clinical importance. This study shows that the presence of intraplaque hemorrhage in the dissected femoral plaque is predictive for systemic adverse cardiovascular events, independent of established cardiovascular risk factors.

We report that intraplaque hemorrhage was observed in 65% of the femoral plaques (207/320). Besides our study group, we could not find other authors reporting the incidence of femoral intraplaque hemorrhage which makes comparison difficult. A highly variable incidence of carotid intraplaque hemorrhage has been reported in previous studies, ranging from 7.5% to 92.1%. However, previous studies were mostly based on small numbers or on macroscopic plaque examination.[14]

With a mean follow-up of 2.5 years, the primary outcome was reached in 40% (128/of 320) of the studied patients. 16% of the patients died from an adverse cardiovascular event. According to literature, mortality rate of patients with PAD is up to 30%.[1, 2] Improvements in best medical treatment and risk factor management over time might explain our lower mortality rate. According to literature, the high percentage of patients that underwent a cardiovascular intervention during follow-up is in line of prediction in patients with PAD.[2]

As expected in this study population with PAD, the prevalence of cardiovascular risk factors was high. In line with known literature, classic cardiovascular risk factors were independent predictors for future secondary adverse events.[15] However, due to the higher prevalence of these risk factors among vascular patients, their discriminative power is limited. Inflammatory

markers, such as CRP and interleukin 6 are also associated with systemic cardiovascular events, however their predictive value is limited and clinical application is lacking so far. Therefore we focused on markers specific for cardiovascular vulnerability, like intraplaque hemorrhage.

Intraplaque hemorrhage plays an important role in plaque progression and destabilization.[16-19] Repeating IPHs contribute to lipid-core expansion through the accumulation of free cholesterol from erythrocyte membranes.[18] In addition, IPH is a potential stimulus for macrophage activation and foam cell formation, thereby increasing plaque inflammation.[18, 19] Atherosclerotic plaque rupture and subsequently symptomatic events are closely related to the presence of plaque neovascularisation and IPH.[20, 21]

Since atherosclerosis is a systemic disease, it is likely to assume that markers of disease progression may be widely distributed in all diseased vessels. Furthermore, atherosclerotic disease in the femoral artery indicates a generalized susceptibility to atherosclerosis with more advanced disease in other arteries and vulnerability to coronary death.[22]

Recently we showed the first evidence for this new concept, that carotid plaque histology (intra-plaque hemorrhage) is predictive for adverse cardiovascular events in the future.[10] Furthermore, also protein markers withdrawn from the carotid plaque, like OPN, MMP-8 and FABP-4 were found to be predictive for adverse cardiovascular events originating in all vascular territories during follow-up.[7-9] These findings support our assumption that specific local plaque characteristics reflect the status of plaque destabilization in other vascular territories. With this study we show that this concept also applies for femoral artery plaques.

This observation is relevant since plaque phenotypes of carotid and femoral plaques obtained during endarterectomy differ substantially. Compared with the carotid plaque, the femoral plaque is dissected years after onset of ischemic complaints and is often occlusive resulting in a fibrotic lesion, rich in smooth muscle cells and collagen. Although the significant differences in gross plaque composition, we observed that the same histological plaque characteristic, e.g. plaque hemorrhage, was associated with secondary manifestations of the disease. Adversely, we could not confirm a relation between the presence of intraplaque vessels and primary outcome despite this positive correlation in carotid plaques.[10] Intraplaque hemorrhages are related to angiogenesis from the adventitia towards the plaque and angiogenesis is related to lipid overload and takes place early in atheroma development.[23,24] The density of neovessel formation is positively correlated with the extent of necrotic core formation and inflammatory infiltrates, suggesting that vessel formation appears to be linked to the evolution of atherosclerosis from early stage to a complicated lesion.[25] Femoral plaques are fibrotic with less inflammatory cells, thereby presenting an end-stage expression of atherosclerosis following occlusion. Possibly, neo-cappilaries which developed in early atherosclerotic stage are becoming fibrotic and dysfunctional in end-stage lesions. Small immature neo-vessels who are retreating in fibrotic lesions are probably less visible, while remainders of repetitive hemorrhages stay more apparent.

The authors want to emphasize that this study was not conducted to explain the mechanism behind the predictive value of intraplaque hemorrhage. According to literature, intraplaque hemorrhage is an important determinant of progression and destabilization of the plaque.[18, 19, 21] Although speculative, it might be conceivable that repetitive bleedings in femoral plaques resemble the instability of plaques elsewhere in the body.

Limitations

In our study design we investigated only the culprit lesion and not the entire plaque. However, we previously demonstrated that the culprit lesion shares similar characteristics as the total plaque burden.[11] Secondly, we do not have plaques of different vascular territories in one patient to compare plaque histology in different territories and relate this to clinical outcome. A positive correlation of the presence of IPH between two vascular territories would have further strengthened the concept. Third, we used a combined endpoint because this study is not powered to analyze the predictive value of IPH for all subgroups separately.

Our study may have some clinical implications. IPH has become relevant since recent advances in magnetic resonance imaging (MRI) techniques have created the possibility to visualize IPH in vivo.[25-27] IPH might serve as surrogate marker for the detection of the so called vulnerable patient with MRI, thereby serve for primary or secondary prevention of cardiovascular events in the future. Furthermore, the visualization of the atherosclerotic plaque is not used for risk assessment of secondary events in daily clinical practice yet.

CONCLUSION

Intraplaque hemorrhage in the dissected femoral plaque is a predictor for adverse cardiovascular events, independent of known cardiovascular risk factors. Dissected plaques can be examined post- operatively to predict the risk profile for secondary events of the individual patient.

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PART IV

CHAPTER 10

Summary and General discussion

Although adverse cardiovascular events are a major cause of morbidity and mortality in patients with peripheral arterial disease (PAD), there are still no clinically applicable features to predict the patient at risk. Restenosis will occur in almost 50% of these patients in the first 3 years after revascularization of the femoral artery, and 20% of patients with claudication intermittent will have died of an adverse cardiovascular event after 5 years.[1,2] The ultimate goal would be to predict which patients are at risk for restenosis and for life-threatening secondary cardiovascular events. A stricter follow-up scheme, closer control of reducing atherosclerotic risk factors, and optimal medical, or eventually, invasive treatment could lead to a reduction of newly developed or progressive cardiovascular disease.
Traditional atherosclerotic risk factors and systemic inflammatory markers, such as C-reactive protein, are not sufficient to accurately predict patients at risk for cardiovascular events.[3] Furthermore, autopsy studies of patients who died after myocardial infarction revealed plaque characteristic that were associated with plaque rupture, the so-called vulnerable plaque characteristics.[4] However, these vulnerable plaque characteristics can also be observed in plaques from asymptomatic patients,[5] and these cross-sectional studies are not designed to conclude if vulnerable plaque characteristics are predictive for future cardiovascular events.
At the UMCU, we have generated a new concept that focuses on local plaque characteristics predicting systemic cardiovascular events.[6-9] Because atherosclerosis is a systemic disease and plaque composition correlates between different arterial segments within individuals,[5,10] a single atherosclerotic plaque could provide information on systemic cardiovascular outcome. Focus is thereby from the vulnerable plaque to the vulnerable patient. The main objective of this thesis was to find plaque characteristics predictive for local (restenosis) and systemic adverse cardiovascular events.

ATHEROSCLEROTIC PLAQUES AS A DIAGNOSTIC TOOL

The atherosclerotic specimens that are stored in the Athero-Express biobank are obtained by endarterectomy. One of the surgical techniques is remote superficial femoral artery (SFA) endarterectomy (RSFAE), as discussed in **Chapter 2**. The benefit of this technique is its minimally invasive character, with only one groin incision, with the advantage of optional additional endarterectomy of the common femoral artery. The Achilles' heel of RSFAE is the relatively high percentage of first-year restenosis. Because dissected plaques contain predictive value for adverse cardiovascular events, plaques should be routinely analyzed after RSFAE. After harvesting the plaque, a tissue biobank is needed to process the plaque and combine the discovered characteristics with clinical characteristics and follow-up.

In **Chapter 3** the benefits and downsides of biobank initiatives are discussed. Biobanks can be discriminated by the type of biologic samples and study design. Most atherosclerotic tissue biobanks have a cross-sectional study design. By definition, these studies are descriptive and do not elucidate the pathogenesis of the progression of the disease in relation to time. Biobanks with a longitudinal study design, like the Athero-Express, provide better opportunities to study the predictive value of tissue biomarkers for onset and progression of the disease.

Longitudinal biobanks also have some pitfalls, however: tissues are often dissected in an end stage of the disease, which makes it difficult to draw inferences regarding causality, and tissue biobanks are often generated by one institution, with more bias opportunity. The Athero-Express is generated by two institutions; however, the possible effect of bias is not excluded. Tissue biobanks also play an important role in the assessment of plaque characteristics for imaging studies and validating imaging studies. Predictive plaque characteristics found in our studies have to be extrapolated to imaging studies that could lead to secondary or primary prevention of cardiovascular events in the future.

PLAQUE HISTOLOGY, CLINICAL CHARACTERISTICS, AND CLINICAL OUTCOME

We studied the correlation of clinical patient characteristics with femoral plaque histology to identify clinical risk factors for the vulnerable femoral plaque. According to the literature, moderate alcohol use revealed a protective role for cardiovascular events.[11-16] Alcohol seemed to have a favorable effect on high-density lipoprotein cholesterol, fibrinogen, thrombogenicity, and insulin sensitivity and, therefore, an antiatherosclerotic effect.[17-20] However, it was unclear if patients with proven PAD or cerebrovascular disease would benefit from alcohol consumption and if alcohol is associated with plaque histology. In **Chapter 4** we describe an inverse relationship between alcohol use and major adverse cardiovascular events in patients after femoral endarterectomy. A possible explanation for this is a more stable femoral plaque phenotype, with smaller lipid core and less macrophage infiltration, in moderate alcohol consumers compared with abstainers.

It is remarkable that we could not demonstrate these observations for patients after carotid endarterectomy, but the carotid cohort was sufficiently powered to detect possible differences in plaque phenotype between patient groups. The reason for the observed differences between carotid and femoral plaques in relation to alcohol use remains unclear. A possible speculative explanation could be that a femoral endarterectomy is generally initiated in a more advanced stage of atherosclerosis. Alcohol might influence the stabilization process during aging of the atherosclerotic plaque. Our results are consistent with recent literature, as in a study by Andersen et al., that found alcohol consumption did not affect survival in patients with stroke.[21] In addition, the vascular biology of femoral atherosclerosis may not be identical to carotid artery pathophysiology. It remains unknown if alcohol intake-sensitive pathways might serve a more important role in lower extremity arterial lesions.

Intraplaque hemorrhage (IPH) is an important determinant of progression and destabilization of the atherosclerotic plaque (see "Intraplaque hemorrhage: atherosclerosis a bleeding disease?"). However, it was unclear if there were clinical patient characteristics related to IPH. For future treatment options of patients with advanced atherosclerotic disease, it could be advantageous if clinical risk factors associated with IPH, and thereby the vulnerable plaque, could be identified. As reported in **Chapter 5**, we observed a positive correlation between advanced age and coumarin-type anticoagulation use and carotid IPH. A negative correlation was found between statin use and carotid IPH. These findings underline the stabilizing effect of statins and support the view not to subscribe coumarin derivatives for patients with cerebrovascular disease.[22-24] Surprisingly, we could not demonstrate an association between

femoral IPH and clinical patient characteristics. A possible explanation is the smaller femoral cohort compared with the carotid cohort. Secondly, femoral atherosclerotic plaques could be end-stage fibrotic plaques, whereas carotid plaques are undergoing different phases of remodelling with repetitive bleeding.
After identifying patient characteristics related with plaque histology, we studied patient and femoral plaque characteristics that were associated with clinical outcome after femoral endarterectomy. In **Chapter 7** we identified increasing age and a long duration of ischemic walking complaints before operation as independent clinical characteristics for worse local outcome. Older patients and patients with a long history of ischemic walking complaints before endarterectomy suffered significantly more from restenosis. When relating plaque histology to clinical outcome, we demonstrated that independent predictors for restenosis were target vessel size, measured as the perimeter of the intima in the plaque, and the diameter of the angiographic lumen. A smaller SFA diameter was associated with worse outcome (**Chapter 7**). According to these results, clinicians should reconsider RSFAE in older patients, patients with a longer history of ischemic complaints, and in patients with small SFA diameter. Furthermore, follow-up and treatment of restenosis may need to be more aggressive for these subgroups.

In **Chapter 8** we demonstrated a significant difference in plaque histology between the common femoral artery (CFA) and SFA. The SFA plaque contained significantly more collagen and smooth muscle cells compared with the CFA plaque. Furthermore, the dissection of a fibrous collagen rich plaque was associated with restenosis after femoral endarterectomy, independent of other risk factors. Higher restenosis rates after endarterectomy of the SFA compared with the CFA might be partly explained by the more fibrous appearance of the SFA. Our observation that fibrotic (stable) plaque characteristics are associated with restenosis after endarterectomy is in accordance with observations of the carotid plaques. Inflammatory (unstable) carotid plaques with high content of macrophages and a large lipid core were associated with less restenosis, probably due to inflammatory activity in the vascular wall, that elicits expansive remodeling.[25] We can only hypothesize about the mechanism for the increased restenosis rates when fibrous, collagen-rich femoral plaques have been dissected.
Fibrotic characteristics of the femoral plaque have been associated with constrictive remodeling (arterial shrinkage or negative remodeling),[26] and collagen accumulation results in arterial shrinkage, analogous to scar constriction.[27] Possibly, the occluded femoral artery will shrink over time due to fibrotic remodeling, subsequently resulting in a smaller lumen area after intervention and increased restenosis rates. These findings have clinical implications. Although restenosis rates after femoral endarterectomy are high, there are still no useful features predicting patients at risk for restenosis. Using plaque histology of the dissected plaque as prognostic factor would be a new feature in clinical practice. Structural histologic analysis of the dissected femoral plaque could be incorporated to identify the patient at risk for restenosis. Patients with collagen-rich plaque could be treated by RSFAE; however, more stringent follow-up to identify early restenosis is required. Besides discovering predictive features for restenosis after femoral endarterectomy, another objective of this thesis was to study femoral plaque characteristics predictive for systemic cardiovascular outcome.

Recently, the presence of IPH and intraplaque vessels was associated with cardiovascular outcome after carotid endarterectomy.[6] In **Chapter 9** we showed that femoral IPH is also an independent predictor for systemic adverse cardiovascular events after femoral endarterectomy. Although femoral plaques are mostly occluded and fibrous, compared with carotid plaques, the predictive value for cardiovascular outcome is the same. With this observation we validated the concept that a single atherosclerotic plaque could be predictive for systemic adverse cardiovascular events in a second vascular territory: the femoral artery. Visualization of the atherosclerotic plaque is not yet used for risk assessment of secondary events in daily clinical practice. Furthermore, IPH might serve as surrogate marker for the detection of the so-called vulnerable patient with MRI.

INTRAPLAQUE HEMORRHAGE: ATHEROSCLEROSIS A BLEEDING DISEASE?

As early as 1936, IPH was seen as an important contributor to a violent plaque that caused clinical complications.[28,29] After these initial reports, however, studies focused on the lipid core and the inflammatory response within the plaque.[30] Research in recent years is again focusing on the repetitive occurrence of IPH.

IPH is related to the neoangiogenesis that take places from the adventitia toward the plaque. This formation of small capillaries takes place early in atheroma development and is related to lipid overload.[31] Because neovessels in plaques appear to be fragile and leaky, they could allow diffusion of blood cells, such as erythrocytes or leucocytes, and also plasma-borne molecules.[32-34] The density of these small vessels correlates with the extent of the necrotic core, IPH, and inflammatory infiltrates, suggesting that it is a determinant of plaque evolution.[35,36]

Owing to the leaky vessels and outflow of erythrocytes into the plaque, the lipid core grows expansively because the cholesterol content of erythrocytes exceeds that of all other cells in the body.[37,38] The molecules released by lysis of hemoglobin can further harm endothelial cells and neutrophiles, and mononuclear cells and leucocytes are further attracted into the plaque by repetitive IPHs.[39,40] We studied the different carotid and femoral plaques in more detail and concluded, as have some earlier studies, that different IPH types can be classified as recent, organized, and amorphous (**Chapter 6**).[41,42]

We further discovered that these different IPH types were related to different plaque phenotypes: organized IPH was related to unstable plaque characteristics, and amorphous IPH was related to stable plaque characteristics (**Chapter 6**). This is a clinically important finding, because an organized IPH would be a better surrogate marker for a vulnerable plaque and amorphous IPH more for stable plaque. Because IPH is an important marker for imaging studies,[43] our findings suggest that magnetic resonance imaging (MRI) studies could facilitate the detection of vulnerable plaques and patients by detecting the organized IPH.

It is unclear if the different IPH types (recent, organized, and amorphous) represent different stages of the same process over time, are different tissue processes after an initial hemorrhage, or have different bleeding origins. Fresh IPH evolve into organized IPH through a process of fibrovascular ingrowth of the hematoma.[44,45] However, no studies have proved that organized IPH evolve into an amorphous IPH. If this were true, a plaque would be more stable over time

because amorphous IPH is associated with stable plaque characteristics and would be in accordance with a recent study that discovered a stabilizing process occurred in the carotid plaque over time.[46]

A second possibility is that different types of IPH represent the result of different healing responses after an initial hemorrhage.[35,38,42,47] A third possibility is that the amorphous IPH is a result of insudation of the plaque matrix by large amount of plasma proteins through small defects of endothelial junctions, leading to an amorphous appearance.[32,35,38] Organized IPH could be the result of ruptured microvessels with extravasation of red blood cells that, as explained above, leads to a response with inflammatory cells and ingrowth of many types of cells.[44]

Clearly, IPH forms the basis of the development of the atherosclerotic plaque, and all subsequent mechanisms provide a snowball effect that leads eventually to a plaque rupture or thrombosis with clinical consequences. IPH is an important contributor of forming the lipid pool, it attracts all types of inflammatory cells into the plaque, and due to organizing of IPH, smooth muscle cells are among them. We may conclude that researchers at the beginning of the 20th century were already making progress focusing at IPH compared with their successors, who focused instead at macrophages and the lipid core.

FUTURE RESEARCH PERSPECTIVES

Histologic plaque characteristics associated with cardiovascular outcome provide new opportunities for imaging studies. Studies have focused on the detection of the in vivo atherosclerotic plaque with different imaging techniques, among them angiography, intravascular ultrasound, computed tomography, and MRI.[48] MRI showed that IPH and a large lipid core are independently associated with thin or ruptured plaques in patients with carotid stenosis.[49] For now, high-resolution MRI has emerged as the leading noninvasive in vivo imaging modality for atherosclerotic plaque characterization.[48] A disadvantage of MRI is a limited temporal resolution in the deeper arteries, such as the coronary arteries. However, the carotid and femoral arteries have a superficial location, with relative absence of motion, and thereby are more suitable for MRI studies.

Most studies have focused on identifying markers representative for vulnerable plaque, and showed that MRI was useful for the identification of calcification, fibrous intimal tissue, and intraplaque hemorrhage.[50,51] These studies had a cross-sectional study design and did not focus on clinical follow-up. One new study in progress is imaging patients and a control group, combined with 3-year follow-up. The goal of the study is to identify markers predictive for cardiovascular events.[52] However, we already validated in two different studies that IPH as a predictive marker for future adverse cardiovascular events. Our observations, together with other literature that proved the importance of IPH, should be enough evidence for imaging studies to focus on imaging IPH. Although images may still appear difficult to interpret for nonexperts, improvement in the technology is rapidly occurring. and is though to be the key technology.[48]

According to the literature, correlation of in vivo imaging and histology is more than acceptable in animal and human studies.[53-55] To validate our histologic observations with high-resolution

MRI, a new study could be initiated with imaging of all patients who undergo a carotid or femoral endarterectomy.

Because we studied a specific subpopulation of patients with PAD, future studies have to show that our discovered predictive histologic markers can be extrapolated to the total population of patients with PAD. An MRI of all patients suffering from PAD can be made before patients with IPH are randomized to extra intensive conservative treatment. Especially in patients with PAD who have a high morbidity and mortality a priori, one can expect an effect of optimized lifestyle and medical treatment.

High-resolution MRI can also be used for monitoring conservative treatment. Corti et al. used in vivo MRI to quantify the effects of lipid-lowering therapy by statins and observed a regression of the atherosclerotic lesions.[56] We discovered an inverse relationship between statins and IPH (**Chapter 6**); thus, MRI can also be used to monitor the effect of statins and anticoagulants on IPH.

CONCLUSION

This thesis showed that the dissected femoral atherosclerotic plaque contains a predictive value for clinical outcome after femoral endarterectomy. Plaque histology analysis should be incorporated in clinical practice to help predict the patient at risk for restenosis or secondary cardiovascular events after femoral endarterectomy.

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CHAPTER 11

Nederlandse Samenvatting

Wereldwijd sterven er jaarlijks vele mensen aan de gevolgen van atherosclerose (slagaderverkalking).
Atherosclerose is een verdikking aan de binnenkant van de slagader en dit is een progressieve ziekte. In eerste instantie zal de ziekte onopgemerkt blijven. Echter, na vele jaren kan deze verdikking uiteindelijk leiden tot een totale afsluiting van de slagader. De slagader zorgt voor zuurstofrijk bloed naar de weefsels, maar bij een plotselinge afsluiting of ernstige vernauwing, zal het weefsel achter de afsluiting zuurstofgebrek krijgen. Afhankelijk van waar dit bloedvat zich in het lichaam bevindt, kan dit lijden tot cardiovasculaire events, zoals een (fatale) hartaanval, beroerte of etalagebenen (pijn in de benen bij het lopen door zuurstofgebrek van de weefsels).

Slagaderverkalking aan de beenslagaders komt vaak voor en dit aantal neemt toe naarmate de leeftijd stijgt. Dertig procent van de mensen boven de 70 jaar heeft slagaderverkalking aan de benen. Één derde van deze mensen krijgt ook daadwerkelijk klachten bij het lopen (etalagebenen), of zelfs in rust met soms slecht genezende wonden aan de benen.

Uit onderzoek weten we dat als mensen slagaderverkalking in de benen hebben, zij dit dan ook al in andere slagaders van het lichaam hebben. Het risico op een (fatale) hartaanval bij patiënten met slagaderverkalking in de benen is 3x groter dan bij mensen zonder slagaderverkalking in de benen. Verder is 20% van de mensen met etalagebenen na 5 jaar overleden door een fataal cardiovasculair event. Bij mensen met pijn aan de benen in rust is zelfs 20% overleden na 1 jaar.

Om te voorkomen dat patiënten een hartaanval of beroerte krijgen, is het belangrijk om risicofactoren voor atherosclerose aan te pakken. Dit gebeurt door de leefstijl van patiënten aan te passen en ze zo optimaal mogelijk met medicatie te behandelen. Hierbij moeten mensen minimaal 30 minuten per dag actief bewegen, afvallen, een vetvrij dieet naleven en stoppen met roken. Het is belangrijk dat daarnaast de bloeddruk strikt wordt gecontroleerd en behandeld en dat het cholesterol wordt verlaagd. Verder moeten de suikerspiegels van patiënten met suikerziekte strikt worden gereguleerd. Alle patiënten met etalagebenen moeten een bloedverdunner krijgen, omdat dit de kans op een hartaanval of beroerte verkleint. Verder moeten deze mensen gesuperviseerde looptraining krijgen om de aanmaak van nieuwe slagaders (collateralen) te stimuleren en zo een betere bloedvoorziening in de benen te krijgen. Indien deze looptraining niet helpt en de etalagebenen invaliderend zijn, of patiënten pijn in rust aan de benen hebben, zal er een operatie volgen om de bloedvoorziening in het been te bevorderen. Met deze operatie wordt de slagader in het been opengemaakt en wordt de lokale slagaderverkalking (plaque) aan de binnenkant verwijderd, zodat het bloedvat weer open is. Helaas gaat de slagader, ondanks steeds vernieuwde operatie technieken, bij de helft van de geopereerde patiënten binnen 3 jaar weer dicht (restenose). Echter, goede waardes die voorspellen welke patiënten risico lopen op restenose zijn niet voorhanden.

Primaire en secundaire preventie van hart- en vaatziekten zullen verbeteren als we patiënten kunnen identificeren die een groot risico lopen op cardiovasculaire events. Echter goede

voorspellende markers die deze patiënten kunnen identificeren zijn niet bekend. De traditionele risicofactoren voor atherosclerose, zoals roken, hoge bloeddruk, suikerziekte, etc. zijn niet geschikt om het risico van de individuele patiënt (op bijvoorbeeld een hartaanval) in te schatten, aangezien vrijwel elke patiënt deze risicofactoren bezit.

Uit eerdere wetenschappelijke studies weten we dat de cellulaire en moleculaire opbouw van de atherosclerotische plaque (de plaque samenstelling), gerelateerd wordt aan de stabiliteit van de plaque. Instabielere plaques, die veel vet en ontstekingscellen bevatten, zouden eerder tot een afsluiting van de slagader zorgen.
Aangezien atherosclerose in het gehele lichaam kan ontstaan, zou het mogelijk kunnen zijn dat een lokale plaque in de hals- of beenslagader informatie bevat over de toestand van plaques elders in het lichaam, zoals in de kransslagader op het hart. De lokale plaque uit het been zou dan als "vingerafdruk" voor alle slagaderverkalking in het lichaam kunnen fungeren. Dit innovatieve concept vormt de basis voor de "Athero- Express study." Bij dit concept wordt er vanuit gegaan dat als de lokale plaque uit de hals- of beenslagader instabiele kenmerken laat zien, dat de plaque elders in het lichaam ook instabiel is en dus mogelijk een toekomstige hartaanval of beroerte kan voorspellen. In de Athero- Express studie worden patiënten bestudeerd die aan de hals- of beenslagader worden geopereerd. Hierbij worden hun medische gegevens vóór de operatie, de atherosclerotische plaque en medische gebeurtenissen in de 3 jaar ná de operatie in de biobank- studie opgeslagen. Het doel van dit proefschrift is om plaque- kenmerken te vinden uit de beenslagader die voorspellend zijn voor cardiovasculaire events, zowel lokaal: terugkerende slagaderverkalking op de plek van de plaque (restenose), als systemisch: een hartaanval, beroerte, etc. elders in het lichaam.

Deel 1 van dit proefschrift bevat de hoofdstukken 1 tot en met 3.
Hoofdstuk 1 bevat de algemene inleiding van het proefschrift. **Hoofdstuk 2** beschrijft de operatietechniek om de plaque uit de beenslagader te halen. Een groot probleem van deze techniek is dat er relatief vaak een restenose optreedt, met name gedurende het eerste jaar na de operatie. Welke patiënten er veel risico lopen op een restenose weten we niet. Als we deze patiënten op voorhand wél kunnen identificeren, kunnen we hen intensiever controleren en behandelen om zo de slagader open te houden.
In **hoofdstuk 3** worden de voordelen van een biobank beschreven met als goed voorbeeld de Athero- Express biobank. Een biobank met klinische follow-up is noodzakelijk om onderzoek te kunnen verrichten waarbij patiëntgegevens worden gekoppeld aan menselijk weefsel (zoals het onderzoek in dit proefschrift) .

Deel 2 van dit proefschrift bevat de hoofdstukken 4 tot en met 6. In **deel 2** van dit proefschrift wordt gekeken welke patiëntkarakteristieken geassocieerd zijn met plaque samenstellingen.

Hoofdstuk 4 beschrijft de relatie tussen alcoholgebruik en het optreden van cardiovasculaire events na de initiële operatie. Patiënten die regelmatig alcohol gebruikten en geopereerd werden aan de beenslagaders, hadden significant minder cardiovasculaire events in de jaren na de operatie, dan patiënten die nooit alcohol gebruikten. Dit kan mogelijk verklaard worden doordat de patiënten die alcohol dronken stabielere plaques (minder ontstekingscellen en

vet) hadden dan patiënten die geen alcohol dronken. Alcohol zou dus, zoals ook al vaker in de literatuur is beschreven, een beschermend effect kunnen hebben tegen cardiovasculaire events. Echter, dit kon alleen worden aangetoond voor patiënten die aan de beenslagaders waren geopereerd en niet voor patiënten die aan de halsslagader werden geopereerd. Bij deze laatste patiëntengroep hadden de alcoholdrinkers en niet- alcoholdrinkers evenveel cardiovasculaire events in de jaren na de operatie en was er ook geen verschil in de plaque samenstelling. Een verklaring kunnen we niet geven voor dit opmerkelijke verschil tussen deze twee patiëntengroepen. Patiënten met slagaderverkalking aan de beenslagaders worden vaak pas jaren na de eerste symptomen geopereerd, terwijl patiënten met slagaderverkalking in de halsslagader al enkele weken na de eerste symptomen worden geopereerd. Mogelijk heeft alcohol een lange termijn effect op de plaque samenstelling.

Een zeer belangrijke factor bij het ontstaan en ontwikkelen van de atherosclerostische plaque is de bloeding in de plaque. Door deze bloedingen wordt de plaque steeds groter en worden er veel ontstekingscellen aangetrokken. Daarbij wordt de plaque door repeterende bloedingen steeds "vetter." Het aanwezig zijn van een plaquebloeding is dus een marker voor een instabiele plaque. Echter patiëntkarakteristieken die geassocieerd zijn met plaquebloedingen waren nog niet bekend. **Hoofdstuk 5** beschrijft dat patiënten die gemiddeld ouder waren, of patiënten die een bepaalde bloedverdunner (coumarines) gebruikten, significant vaker een plaquebloeding hadden. Patiënten met een cholesterol verlagend medicijn (statines) hadden significant minder vaak een plaquebloeding. Echter, dit gold alleen voor patiënten die geopereerd werden aan hun halsslagader. Er konden geen patiëntkarakteristieken worden gevonden, die geassocieerd waren met plaquebloedingen binnen de patiëntengroep geopereerd aan de beenslagader. Onze resultaten tonen aan dat oudere mensen instabielere plaques hebben (overeenkomstig met bekende literatuur). Verder moeten coumarines worden vermeden vanwege het ongunstige effect op de plaque samenstelling en hebben statines een gunstig effect op de plaque samenstelling.

Aangezien de plaquebloeding zo'n belangrijke factor speelt binnen atherosclerose hebben we van alle plaques, van meer dan 1000 patiënten, de plaquebloedingen in detail bestudeerd (**hoofdstuk 6**). Hierbij viel op dat we de bloedingen in de plaque van de hals- en beenslagader in 3 verschillende categorieën konden verdelen: verse bloeding, georganiseerde bloeding en amorfe bloeding. Als we deze bloedingen correleerden met de plaque samenstelling bleek dat de georganiseerde bloedingen significant vaker voorkwamen met andere instabiele plaque kenmerken (bijvoorbeeld vet en ontstekingscellen) en dat de amorfe bloeding significant vaker voorkwam met stabielere plaque kenmerken (bijvoorbeeld kalk en gladde spiercellen). Dit betekent dat de georganiseerde bloeding eigenlijk beter de instabiele plaque kenmerkt dan de amorfe bloeding. Deze resultaten zijn van belang aangezien plaquebloedingen worden gebruikt als marker om met een scan instabiele plaques aan te tonen. Onze resultaten suggereren dat de focus beter kan worden gelegd op de georganiseerde plaque bloeding.

Deel 3 bevat de hoofdstukken 7 tot en met 10. In **deel 3** van dit proefschrift beschrijven we de voorspellende waarde van de beenslagader plaque.
In **hoofdstuk 7** worden voorspellende waardes omschreven voor restenose. Oudere patiënten, patiënten met een lange periode van pijnklachten voordat de operatie wordt ondergaan en patiënten met een kleine diameter van de slagader, ontwikkelen significant eerder restenose in het eerste jaar na de operatie. Deze patiënten zullen een strengere controle na de operatie moeten ondergaan met optimale medicamenteuze behandeling. Eventueel beginnende restenose kan dan in een vroeg stadium worden aangepakt.
In **hoofdstuk 8** wordt beschreven dat plaque samenstelling voorspellend is voor restenose. Een "verkalkte" plaque (collageen) is een onafhankelijke voorspeller voor restenose in de eerste 3 jaar na de operatie. Opvallend is dat een uitgenomen plaque toch nog voorspellend kan zijn voor restenose op de plek waar de plaque heeft gezeten. Waarschijnlijk zijn vaten met veel collageen verlittekend en versmald, waarna er na het openen van het vat nog maar een kleinere diameter overblijft. Een smallere slagader gaat weer eerder dichtzitten (wat in **hoofdstuk 7** ook wordt beschreven).
Hoofdstuk 9 beschrijft dat de plaquebloeding een voorspellende marker is voor toekomstige cardiovasculaire events zoals een (fatale) hartaanval of beroerte.

De resultaten uit **hoofdstuk 8** en **9** komen overeen met resultaten die onze onderzoeksgroep heeft gevonden in de plaque van de halsslagader. Dit is belangrijk aangezien het concept van de Athero- Express, waarbij een lokale plaque een "vingerafdruk" is van alle slagaders, lijkt te kloppen.
De analyse van de plaque samenstelling moet nu worden ingevoerd in de dagelijkse kliniek, om secundaire cardiovasculaire events beter te kunnen bestrijden. Patiënten met een hoog risico op restenose of een cardiovasculair event moeten een striktere controle en behandeling ondergaan.
Met beeldvormende technieken, zoals een CT-scan (Computed Tomography scan) of MRI-scan (Magnetic Resonance Imaging scan) is het in de nabije toekomst waarschijnlijk mogelijk onze gevonden markers voor cardiovasculaire events af te beelden in plaques die nog in het lichaam zitten. Hiermee zou je mensen, voordat er klachten van de atherosclerotische plaque zijn opgetreden,
op kunnen sporen en vroegtijdig kunnen behandelen.
In **hoofdstuk 10** worden de voorgaande hoofdstukken samengevat en bediscussieerd

CHAPTER 12

Review Committee
Dankwoord - Acknowledgments
Publications
Curriculum Vitae

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CURRICULUM VITAE

Wouter J.M. Derksen was born on October 10, 1979 in Utrecht, The Netherlands. After graduating from High School (St. Bonifatius College, Utrecht), he started to study Business Administration at the University of Groningen. Due to the Dutch drawing system for Medicine study, he finaly could start his study in Medicine in 2000. In 2004 he followed a research internship for a half year in The Hospital for Special Surgery in New York, USA, supervised by Professor Dr. M.D. Lockshin. This internship resulted in two peer reviewed published articles.

In August 2006 he obtained his medical degree from the University of Groningen. The same year he started as a resident at the Department of Surgery in St. Antonius Hospital Nieuwegein. While working as a resident, he first started his scientific research for this thesis. In October 2007 he worked full-time one year at the Experimental Cardiology Laboratory in collaboration with the department of Vascular Surgery at the University Medical Center Utrecht. In this year he made the extensive foundation for this thesis. In January 2009 he started his surgical training at the Department of Surgery at the University Medical Center Utrecht (supervised by Professor Dr. I.H.M. Borel Rinkes). During his surgical training he finished his scientific research published in this thesis. In January 2012 he will start his last three years of surgical training in the St. Antonius hospital Nieuwgein (supervised by Dr. P.M.N.Y.H. Go).