PREDICTORS AND NOVEL TREATMENTS FOR RESTENOSIS IN PERIPHERAL ARTERIAL DISEASE

STEVEN VAN HAELST

Predictors and novel treatments for restenosis in peripheral arterial disease

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Predictors and novel treatments for restenosis in peripheral arterial disease

Voorspellende factoren en nieuwe behandelingen
van restenose bij perifeer vaatlijden

(met een samenvatting in het Nederlands)

Proefschrift

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door

Steven Theo Willem van Haelst

geboren op 11 september 1988
te Kockengen

Promotoren: Prof. dr. G.J. de Borst
Prof. dr. F.L. Moll
Prof. dr. G. Pasterkamp

Voor Max,
de liefste en sterkste persoon die ik ken

"Een mooi verhaal is nog nooit begonnen door nee te zeggen"

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CHAPTER 1

General introduction and thesis outline

GENERAL INTRODUCTION

Atherosclerosis

Atherosclerosis is a systemic disease with a possible impact on all medium to large arteries in the human body. The process starts at a young age and its severity increases over a lifetime. The formation of atherosclerotic plaques is complicated and involves a lot of steps before the disease becomes symptomatic or leads to adverse cardiovascular events. At first, the endothelium of the arterial wall, a one cell layer lining all arteries, becomes dysfunctional. Thickening of the intimal layer of the artery occurs with accumulation of lipid filled macrophages (foam cells), but also lipid in extracellular deposits leading to fatty streaks in the arterial wall. In turn, smooth muscle cells migrate into the intimal layer where they can proliferate. Macrophages cause further inflammation and subsequently proliferation of muscle and fibrous tissue. To meet increasing oxygen demand an expanded microvasculature can evolve, which are more prone to bleeding and could cause intraplaque hemorrhage. Areas without enough oxygen supply may become necrotic. Calcium salts can deposit with cholesterol and other lipids within the plaques. In the end an irreversible atherosclerotic lesion causes narrowing of the lumen and stiffening of the arterial wall and can lead to thromboembolic events due to rupture of a plaque or due to thrombus formation at the location of a plaque due as a reaction to plaque erosion[1–3].

Peripheral Arterial Disease

Narrowing of the lumen of peripheral arteries can result in impaired blood flow to organs and tissue located distally. This causes hypo-oxygenation and often leads to symptoms, such as intermittent claudication (IC) or critical limb ischemia (CLI) in peripheral arterial disease (PAD) patients. Most PAD patients suffer from years of atherosclerotic disease development which leads to the hemodynamic problem to get a sufficient amount of blood and nutrients to the leg and foot. The disease has a severe impact on the quality of life of these patients and greatly increases morbidity and mortality risks[4,5]. Current estimated prevalence of IC increases with age and is 3% in 40-year olds and up to 6% of all 60-year olds (both sexes), or an estimated 131.000 persons in 2011 in The Netherlands[6,7]. The estimated prevalence and (global) disease burden as a result of PAD still increases, amongst others due to an increased prevalence of diabetes and an ageing population[7–9].

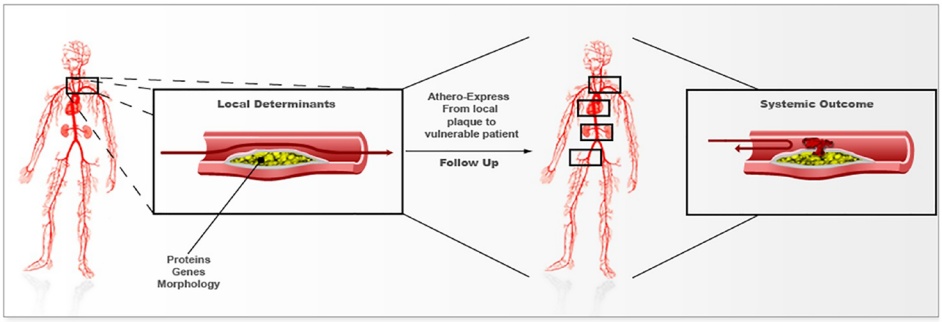
As PAD patients can have symptoms that range from no-complaints but proven atherosclerotic disease to severe tissue loss of the affected leg, two main classification systems have been developed to classify PAD patients; the Fontaine classification and the Rutherford classification[10]. These make a clear distinction between patients with impaired walking distance (IC patients) and patients with rest pain or tissue loss (CLI patients). Mortality rates and disease burden in CLI patients are higher than in IC patients, the reason for distinction within these classifications. In addition to the patient

Fontaine classification		Rutherford classification	
Stage	**Symptoms**	**Category**	**Symptoms**
I	Asymptomatic	0	Asymptomatic
II	Intermittent claudication	1	Mild claudication
		2	Moderate claudication
		3	Severe claudication
III	Ischemic rest pain	4	Ischemic rest pain
IV	Ulceration or gangrene	5	Ischemic ulceration (minor tissue loss)
		6	Ischemic gangrene (major tissue loss)

Local plaque characteristics and secondary cardiovascular events – The Athero-Express Biobank

classification, an anatomic classification was developed based on expert recommendations and available evidence, the TASC guidelines, which includes disease severity, treatment options and recommendations[6,11].

As atherosclerosis is a systemic disease affecting multiple arterial beds simultaneously; the hypothesis was raised that a local retrieved plaque, for example during femoral endarterectomy or carotid endarterectomy, might be of predictive value for adverse cardiovascular events elsewhere in the body. The Athero-Express Biobank was launched in 2002 to investigate plaque characteristics combined with blood samples of patients, patient characteristics and history, and three years of follow-up[12]. The aim was to be able to investigate which patients are prone to develop secondary cardiovascular events and whether local plaque characteristics could provide possible clues as to which patients are of increased risks for a cardiovascular event. Plaques were retrieved after surgery and in every plaque the culprit lesion was identified (e.g. the location with the largest plaque burden). The total plaque was cut into 5mm segments and the culprit lesion was processed for histological analyses. The obtained histological slides were stained for several plaque characteristics, namely: calcification, collagen, fat content, intraplaque haemorrhage, macrophages, smooth muscle cells and density of microvessels. The other segments were snap frozen and stored at -80 degrees Celsius. Currently there are over a 1000 patients with ilio-femoral retrieved plaques included in the Athero-Express Biobank.
In this thesis, mainly PAD patients and plaque characteristics retrieved from the iliac and femoral arteries were investigated.



Treatment strategies

Several guidelines have been developed to treat IC and CLI patients with ilio-femoral atherosclerosis[6,13]. At first, population-wide prevention strategies exist to inhibit the development of atherosclerosis, such as: lifestyle changes, discouraging of smoking, and healthy nutrition[14–16]. When a patient does develop risk factors or symptoms, then there are primary or secondary medical prevention therapies to inhibit further progress of atherosclerosis, such as: lipid-lowering, anti-hypertensive and anti-platelet therapy[17,18]. Patients with IC benefit from guided walking therapy for 6 months, which can dissolve their complaints[19]. Lastly, there are interventional options. These are reserved for CLI patients or IC patients who do not respond to guided walking therapy. Basically there are two main interventional treatment strategy options, both intended to preserve or restore blood flow to the distal leg. An endovascular approach is the current first choice of treatment[20,21], when this is not feasible an open approach or bypass surgery is warranted. The measure of success for these interventions is often defined as patency of the treated artery at one, two or three to five years, or as amputation free survival. One of the main culprits of failing patency of the treated artery is restenosis.

The main causes of restenosis

There are two main causes of restenosis after endovascular interventions. First, as a reaction to treatment, smooth muscle cell (SMC) and intimal hyperproliferation can develop due to endothelium injury, inflammation of the persistent atherosclerotic plaque, haemodynamic factors and mechanical stress when a permanent stent remains in situ[22,23]. Second, basically due to the same factors which cause hyperproliferation of the intimal layer, negative remodeling of the artery can occur[24,25]. This results in further narrowing of the artery and subsequent impaired flow to distal tissue and organs. A permanent stent in the artery counters the effects of negative remodeling, but may induce increased hyperproliferation of the intima due to the foreign body on the arterial wall.

Novel ways to reduce restenosis after treatment

A theorethical attractive alternative to overcome negative remodeling and intimal hyperproliferation, bioresorbable stents dissolve after the initial stenting fase, whereupon the vessel can remodel and retain some of its natural physical properties, thus reducing the risk of developing restenosis[26,27]. In a separate development, several drugs have been developed to limit hyperproliferation in cancer therapies, such as: everolimus or paclitaxel. These drugs were found to be effective against intimal hyperplasia as well[28]. New treatments with the use of these drugs on balloon angioplasty show promising results and are currently widely studied. Yet, scarce evidence on medium to long term improvement in clinical endpoints such as: amputation, Rutherford class, quality of life, and walking ability, has only recently been reported[29,30]. In addition, no long-term effects on the arterial wall have been confirmed[31]. Therefore, the search towards an effective method to restenosis after (endo)vascular treatment in PAD patients remains valuable.

In this thesis a new treatment was studied: a synthetic intimal coating (SIC) to inhibit the formation of intimal hyperplasia in an animal model.

THESIS OUTLINE

The main goals of this thesis are (1) to investigate a new treatment to counter restenosis due to intimal hyperplasia in an animal model and (2) to investigate plaque characteristics as possible predictors for secondary cardiovascular events in an existing database – the Athero-Express Biobank.

PART I

Overview of literature

Part I of this thesis starts with an overview of existing literature in the field of peripheral arterial disease. In **Chapter Two**, current literature on a novel technique to treat peripheral arterial disease is examined. Emphasis was placed on clinically important outcomes during follow-up and whether current results justify clinical use outside clinical trials. In addition, available evidence for a common clinical practice – the use of dual antiplatelet therapy after infra-inguinal endovascular procedure with stent– was critically appraised in **Chapter Three**.

Although these two topics do not directly relate to the experimental animal research conducted and described in Part II, they do relate to important clinical outcomes of endovascular interventions, namely: to prevent the occurrence of clinically important restenosis and occlusion of a treated artery. In Part III, this thesis will further investigate whether derived plaques have a predictive value for the (secondary) occurrence of cardiovascular events, and will look into the prognosis of patients with peripheral arterial disease. But first, Part II describes animal research conducted into the prevention of intimal hyperplasia, an important cause of restenosis.

PART II

Experimental animal research

To be able to use new techniques in a clinical setting (humans), these techniques have to be tested and validated in vitro and in vivo. In vascular surgery, in vivo animal experiments are often conducted in pig arteries as the anatomy, size and healing process has similarities compared with human arteries. Other animals have been used as well, as goats for venous techniques and sheep for cardiac valves. All experiments conducted for this thesis were approved by the Committee on Animal Experiments (DEC), Utrecht, The Netherlands.

Pigs do not develop atherosclerotic or stenotic lesions spontaneously and currently no golden standard pig ilio-femoral atherosclerotic or restenosis model exists. Current animal models often rely on vascular injury methods, mechanically disrupting the Internal Elastic Lamina (IEL), for the development of intimal hyperplasia (IH)[32]. The induction of IH works as a surrogate for restenosis and creates an environment to test new drugs and endovascular devices. However, creating IH in peripheral arteries is harder than in coronary arteries, often leading to sub-optimal amounts of IH[33,34]. A proposed new method in literature promised good results, but was not validated yet[35]. We decided to use this model to create IH and in the same time validate the usefulness of this model. In **Chapter Four** we describe the results of the practicality and critically examination of this model as part of a larger pilot study to the effects of a new treatment to inhibit the formation of IH.

Within a European Consortium, named "The Grail Study", a novel treatment to inhibit the formation of IH was developed, consisting of an absorbable Synthetic Intimal Coating (SIC). The SIC could be placed against a damaged arterial wall and was hypothesized to stimulate a normal healing process and inhibit the formation of IH, before being absorbed by into the arterial wall. In **Chapter Five**, the results of the pilot animal study and directives for future research into the SIC are described.

In **Chapter Six** the histological effects and outcomes of mechanochemical endovenous ablation (MOCA) and its separate components were tested in goat lateral saphenous veins to evaluate the mechanism of action of this treatment which is commonly used to treat venous insufficiency in humans, but for which the histological effects are still largely unknown.

PART III

Plaque characteristics & prognosis of Peripheral Arterial Disease patients

Part III consists of several plaque studies from the Athero-Express Biobank, currently the largest biobank for atherosclerotic plaques worldwide. **Chapter Seven** investigates in Ilio-Femoral Endarterectomy (IFE) derived plaques a finding previously observed in Carotid Endarterectomy (CEA) derived plaques: that plaque characteristics change over time within the Athero-Express Biobank[36]. To further analyse the effects of these changing plaques on secondary cardiovascular events over time, the secondary cardiovascular events of all included patients undergoing IFE or CEA stratified for year

of inclusion, were investigated in **Chapter Eight**.
The missing link in these studies was whether the investigated plaque characteristics, some of whom are considered to belong to 'vulnerable' plaques, did indeed predict the occurrence of secondary cardiovascular events in these patients. **Chapter Nine** looks into the connection between plaque characteristics and secondary cardiovascular outcomes. While **Chapter Ten** the effects of diabetes on ilio-femoral plaque characteristics were investigated and whether these patients are of increased risk of secondary cardiovascular events during follow-up.
Lastly, in **Chapter Eleven**, the Dutch peripheral arterial disease patient population was retrieved from Hospital Registries, with the use of ICD-codes. In this population based cohort study, the mortality risks of a vulnerable patient population were evaluated - stratified for sex. In addition, the cohort was split into two time-frames to assess whether the mortality rates of PAD patients improved over time.

Chapter Twelve contains the summary, general discussion & future perspectives

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PART 1

OVERVIEW OF LITERATURE

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CHAPTER 2

Current status and future perspectives of bioresorbable stents in peripheral arterial disease

J Vasc Surg. 2016

ABSTRACT

Background
Endovascular treatment of peripheral arterial disease (PAD) still yields unsatisfactory patency rates, recent new developments include the use of bioresorbable stents.

Objective
To provide an overview of currently available data on the use of bioresorbable stents in lower limb PAD and to summarize the needs for future research focus.

Methods
A systematic search in the databases of Medline, Embase and the Cochrane library was performed. Studies were included using predefined in- and exclusion criteria and critically appraised by two independent reviewers. Inclusion criteria were: 1) original data on 2) bioresorbable stents in 3) lower limb arteries including the iliac tract. Primary endpoints were safety and feasibility of bioresorbable stents, including 30-day adverse events. Secondary endpoints included radial force, bioresorption process, long-term primary and secondary patency and clinical outcomes such as amputation rate, Rutherford category and ankle-brachial index (ABI) improvement.

Results
Seven published studies with a total of 316 patients were included and five conference abstracts including 272 patients were assessed. Median follow-up time was 12 months. Overall technical success rate was 99% (range; 95.0 – 100%), 30-day adverse event rates were reported in 5.0% of patients (range; 0 – 13.3%), these included one death, two major amputations and seven re-interventions. Mean primary patency rate was 61.6% in the femoral arteries (range; 32.1 – 80.0%) after six to 12 months, compared with 50.3% in below-the-knee lesions (range; 31.8 – 92.9%). Secondary patency rates were 91.5% (range; 84 – 97.1%) and 72.1% (range; 62.9 – 100%) respectively. 1-year amputation rate was 3.0% in the whole group (range; 0 – 12.4%).

Conclusion
Experience with the use of bioresorbable stents in PAD is still very limited and is investigated only in small studies. The use of bioresorbable stents in PAD appears to be feasible and safe. With current published results we are unable to fully answer all of the questions regarding the future use of bioresorbable stents in PAD, and the use should be limited to only in study-related cases in PAD.

INTRODUCTION

Current guidelines for treatment of peripheral arterial disease (PAD) advise life style adjustments and, if revascularization is necessary, an endovasular first approach with percutaneous transluminal angioplasty (PTA) and additional stenting for residual stenosis > 30%[1–3]. Unfortunately, endovascular techniques are still hampered by 1-year restenosis rates of 23 - 42.9% after PTA and 13.7 – 35% with additional stenting. Although target lesion revascularization (TLR) is often successful and improves secondary patency rates in patients, it implies additional procedures with corresponding risks[1,2,4].

The main culprit of restenosis is smooth muscle cell (SMC) and intima hyperproliferation, caused by endothelium injury, inflammation of the persistent atherosclerotic plaque, haemodynamic factors and mechanical stress from a permanent stent[5,6]. As a theorethical attractive alternative to overcome this hyperproliferation, bioresorbable stents dissolve after the initial stenting fase, whereupon the vessel can remodel and regain its natural physical properties, reducing the risk of developing restenosis[7–9].

Two main bioresorbable stent footings are used at this moment, including metal based stents (Magnesium, Mg) and polymer Poly-L-Lactic Acid (PLLA) based stents. In short, Magnesium based stents degrade gradually in the vascular wall, releasing non-toxic levels of Mg and inorganic salts and eventually dissolving completely[10]. PLLA based stents degrade by hydrolysis into lactic acid which is further converted to carbon dioxide and water[8]. Recently, experience with the use of bioresorbable stents in PAD has been gained during first clinical trials and currently ongoing studies, leading to the question whether these devices are potentially ready for large randomized controlled trials in PAD.

This systematic review aims to 1) provide an overview of currently available clinical data on the use of bioresorbable stents in lower limb PAD, 2) show safety and feasibility results, procedural adverse events and long term patency results and 3) summarize the needs for future research focus.

METHODS

Search Strategy

This systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement[11]. A systematic literature search was performed using the PubMed and Embase databases, as well as the Cochrane Library and Cochrane Trials register. The search strategy was optimized in consult with the clinical librarian available in the UMC Utrecht (shown in appendix). We manually searched the reference list of relevant articles for publications that were not identified in the initial search.

Study Endpoints
Primary endpoints were safety and feasibility of bioresorbable stents, including 30-day adverse events. Secondary endpoints included radial force, bioresorption process, long-term primary and secondary patency and clinical outcomes such as amputation rate, Rutherford category and ankle-brachial index (ABI) improvement.

Clinical Definitions
Safety and feasibility were based on the technical procedural success rate resulting in a patent vessel with < 30% residual stenosis after treatment. Reported peri-procedural complications, including complications resulting from anaesthesia and procedural failures were taken into account. Major cardiovascular events during the first 30 days after procedure were assessed, such as myocardial infarction (MI), re-intervention, amputation and death.
Radial force is defined as the absence of acute recoil of the vessel after implantation, stent fractures or change in minimal lumen area during follow-up. Bioresorption process is in the Cardiology field measured by radiological evidence of disappearing struts using intravascular ultrasound (IVUS) or Optical Coherence Tomography (OCT). Long-term patency results were analyzed based on longest available follow-up time. Primary patency was defined as radiographic <50% restenosis rate or a peak systolic velocity ratio of <2.4, without any reinterventions of the treated segment. If TLR has been performed, the secondary patency was assessed.

Study Selection
Predefined inclusion criteria for studies were: 1) original data on 2) bioresorbable stents in 3) lower limb arteries including the iliac tract. Supplements or conference abstracts were included for descriptive purposes and to circumvent possible publication bias. Studies in English or Dutch were assessed for inclusion. Exclusion criteria were: 1) non-human setting, 2) reviews and 3) case reports of less than five treated patients.
Study selection was performed by two independent researchers (S.H. and S.P.W.). Studies meeting inclusion criteria were additionally read by the two researchers independently before eventual inclusion. In case of disagreement, discussion with an independent third reviewer (G.B.) was conducted for final inclusion.

Quality Assessment
Quality assessment was also performed by the same two individual reviewers, using a pre-established format. In case of discrepancies, consensus was achieved by discussion and judgement of an independent third reviewer (G.B.).

Data Extraction and Analysis
Data extraction from the included articles has been performed by both reviewers independently by a predefined format. The possibility to pool the data was dependent on homogeneity of the articles, which was determined by observation and discussion

between the reviewers. Information from unpublished studies has been retrieved from conference presentations or conference abstracts.
For statistical analysis Statistical Package for Social Science (IBM SPSS, version 22, IBM Corp., Armonk, NY, USA) was used. A double-sided *p*-value of less than 0.05 was considered statistically significant. Non-normally distributed data were displayed as median (interquartile range, IQR) and normally distributed data were displayed as mean (standard deviation, SD), pooled data were given as a weighted (overall) mean (SD).

RESULTS

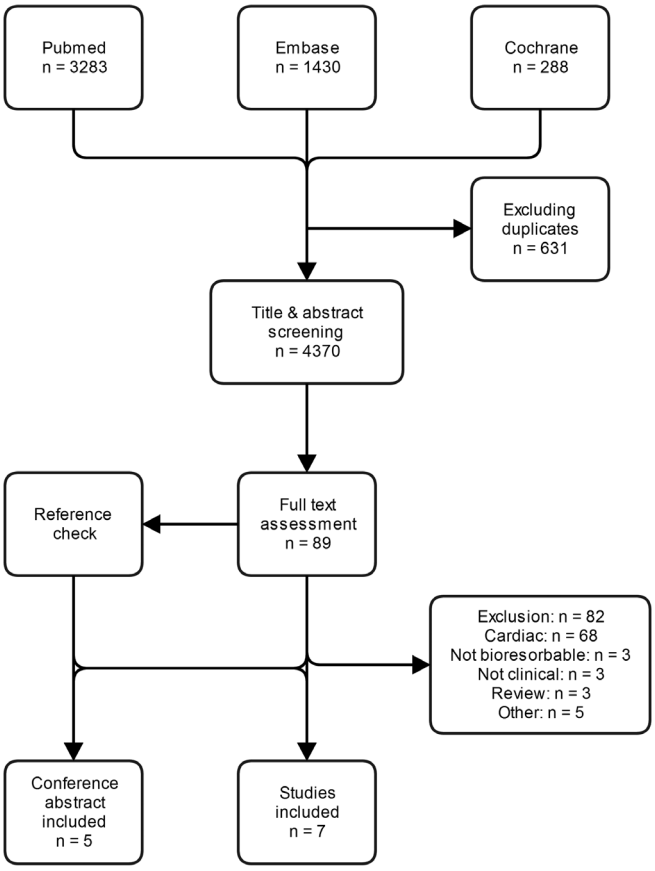
The literature search, performed in January 2016, recovered 5001 articles from the described databases. After exclusion of duplicates and subsequent title and abstract screening, 89 articles were selected for full-text evaluation. Subsequent screening and critically appraising the remaining articles, six articles and one conference abstract were included in this review. These included two randomized controlled trials[12,13], two prospective cohort studies[14,15], one retrospective cohort study[16], one pilot study[17] and one conference abstract[18]. An additional four conference abstracts[19–22] and one pilot study[23] have been included after reference checking, making a total of seven published studies and five conference abstracts. A flow chart of the search is shown in Figure 1, and a summary of included studies in Table 1.

TABLE 1 Overview of included studies

Published	Design	YI	YP	Site of lesion	Number of patients	Follow-up time (m)
Bosi*ers* pilot[23]	Pilot	2004-2005	2008	BTK	20	12
Bosiers et al[12]	RCT	2005-2007	2009	BTK	117*	6
Varcoe et al[17]	Pilot	2013-2014	2015	BTK	14	6
Linni et al[13]	RCT	2011-2013	2014	CFA	80**	12
Werner et al[14]	Prospective	na	2014	SFA	30	12
Silingardi et al[16]	Retrospective	2009-2011	2015	SFA	35	36
Werner et al[15]	Prospective	2011	2015	SFA	20	12
				Total	316	
Unpublished studies						
Biamino - PERSEUS[20]	Pilot	na	na	SFA	45	9
Messina - Absorb[19]	Retrospective	2013-2014	na	SFA/BTK	22	3
Goverde - REMEDY[18]	Prospective	2011 – est 06-2014	na	SFA	100	12
Lammer - ESPRIT[21]	Prospective	2011 – est 08-2015	na	SFA	35	12
Holden - STANZA[22]	Prospective	2013 – est 12-2015	na	SFA	70	na
				Total	272	

na = not available, RCT = randomized controlled trial, YI = year of inclusion, YP = year of publication
Prospective & retrospective cohort
* of which 60 received a resorbable stent
** of which 40 received a resorbable stent

FIGURE 1 Literature search flowchart



No discrepancies existed between both reviewers considering included studies and the quality of these studies. The quality assessment of included the studies and abstracts is shown in Table 2.

Published studies and conference abstracts

Published data consisted of seven studies with 316 patients[12–17,23], of which 125 patients received a bioresorbable stent in the iliac-femoral tract, 94 patients below-the-knee (BTK) and 97 patients were randomized in a control group (receiving either common femoral artery endarterectomy or PTA, not included in further analysis). In the five conference abstracts, which we considered unpublished studies, another 272 patients were included, all treated with bioresorbable stents for lesions in the SFA[18–22].

The majority of published studies included patients with short and de novo lesions and were focussed on lesions in the SFA or BTK. Median follow-up time was 12 months

TABLE 2 Quality assessment

Study	Design	Relevance				Validity			
Published	Design	Sample size	Domain	Determinant	Outcome	Random allocation	Concealed treatment	Complete data	Blinding
Bosiers pilot[23]	Pilot	20	Yes	Yes	Yes	No	No	Yes	No
Bosiers et al[12]	RCT	117	Yes	Yes	Yes	Yes	No	Yes	No
Varcoe et al[17]	Pilot	14	Yes	Yes	Yes	No	No	Yes	No
Linni et al[13]	RCT	80	Yes	Yes	Yes	Yes	No	Yes	No
Werner et al[14]	Prospective	30	Yes	Yes	Yes	No	No	Yes	No
Silingardi et al[16]	Retrospective	35	Yes	Yes	Yes	No	No	Yes	No
Werner et al[15]	Prospective	20	Yes	Yes	Yes	No	No	Yes	No
Unpublished studies									
Biamino - PERSEUS[20]	Pilot	45	Yes	Yes	Yes	No	No	No	No
Messina - Absorb[19]	Retrospective	22	Yes	Yes	Yes	No	No	No	No
Goverde - REMEDY[18]	Prospective	100	Yes	Yes	Yes	No	No	Yes	No
Lammer - ESPRIT[21]	Prospective	35	Yes	Yes	Yes	No	No	No	No
Holden - STANZA[22]	Prospective	70	Yes	Yes	No	No	No	No	No

Prospective & retrospective cohort

(IQR; 6 – 18) and median target lesion length was 22.2mm (IQR; 11 – 59). A full overview of patient and plaque characteristics is given in Supplemental Table 1 and Supplemental Table 2.

Stent materials

In all studies a variety of bioresorbable stents have been used, in BTK lesions mostly Magnesium (Mg) based stents were used, in the femoral arteries most stents are based on the polymer Poly-L-Lactic Acid (PLLA), some with eluting drugs. Stent bioresorption properties of these two platforms significantly differ, both in pathway and length of the process as briefly described in the introduction and Table 3. The complete list of used stents and stent properties is shown in Table 3.

Pooling and analyzing published study data

Included published studies differed in study design, applied type of stents and stent materials, patient population and used a variety of end-points such as safety and feasibility, primary patency or the need for re-intervention, major adverse events and ABI. Because of these differences we considered these studies to be inhomogeneous and were therefore mostly not able to pool the study data, although we did consider technical success rate and 30-day complication rate feasible to pool. We attempted to pool long-term patency rates in lesions in the SFA and BTK separately but this evidence is marginal.

TABLE 3 Stent composition and properties

Published	Scaffold	Material	Coating	Eluting drugs	Strut thickness	Radial support	Bioresportion	Current status
Bosiers pilot[23]	AMS Insight	Magnesium	No	No	na	na	2-3 months	Finished
Bosiers et al[12]	AMS Insight	Magnesium	No	No	na	na	4 months	Ongoing
Varcoe et al[17]	Absorb BVS	PLLA	PDLLA	Everolimus	0.15mm	na	<3 years	Ongoing
Linni et al[13]	Remedy	PLLA	No	No	0.24mm	na	8 months*	Stopped*
Werner et al[14]	Igaki-Tamai	PLLA	No	No	0.24mm	6 months	<3 years	Finished
Silingardi et al[16]	Remedy	PLLA	No	No	0.24mm	6 months	12-18 months	Finished
Werner et al[15]	Igaki-Tamai	PLLA	No	No	0.24mm	6 months	<3 years	Finished
Unpublished studies								
Biamino[20]	Igaki-Tamai	PLLA	No	No				
Messina[19]	Absorb BVS	PLLA	PDLLA	No				
Goverde[18]	Remedy	PLLA	No	No				
Lammer[21]	Esprit	PLLA	PDLLA	Everolimus				
Holden[22]	STANZA	PLGA	Elastomer	No/paclitaxel**				

AMS = absorbable metal stent, BVS = bioresorbable vascular scaffold, na = not available
PDLLA: poly DL-lactide, PLLA: poly-L-Lactic Acid, PLGA: poly(d,l-lactic-co-glycolic acid)
* study ended because of results
** bare stent & drug eluting stent available
+ drug eluting (paclitaxel) balloon dilatation of stenosis before stent placement

Safety and feasibility

In the published studies, overall technical success rate during the procedures was 99% (range; 95.0 – 100%). Reported technical failures consisted of one intra-operative stent occlusion[13], which required the patient to cross-over to the other study-arm receiving surgical endarterectomy. Furthermore, one non-study stent was placed due to severe tortuosity of the iliac artery[12], one stent deployed distally from the target side due to a technical error[15], and one device failure occurred for which a non-study stent was placed[14].

All studies reported safe stent use with no unforeseen side effects. 30-day adverse events occurred in a minority of cases, pooled analysis showed an overall 30-day adverse event rate of 5.0% (range; 0 – 13.3%) (Table 4). Serious adverse events included 2 major amputations[12], one death[12] one patient with acute thrombotic events in two stents[17], one patient with femoral pseudo-aneurysm, both requiring reintervention[17] and 5 cases of <30 day binary restenosis requiring reintervention[13,14].

Radial force

One study found a significant decrease in minimal lumen diameter on angiography six months after implantation of a bioresorbable stent compared with PTA BTK, and attributed this to too swift bioresorption and subsequent inadequate radial force of the Mg-based stent[12]. Two studies reported on stent fractures during follow-up using

grayscale ultrasound or clinically indicated angiography; both found no fractures[13,16]. The Igaki-Tamai stent has been investigated in the coronary arteries and was found to keep radial strength and flexibility for up to six months, in the SFA the mean diameter stenosis directly post-stenting was reduced from 89.9% to 6.2%[14].

Bioresorption process

One study performed echo duplex analysis of the stent resorption process during follow-up and found total bioresorption within the follow-up time of 30 months in all patients[16]. Another study performed histopathological examination of by atherectomy retrieved tissue in 8 patients and found mostly inflammatory cells (50%) and stent struts (37.5%) in the restenotic tissue[14]. The remaining included studies did not report on the bioresorption process in peripheral arteries.

Clinical results

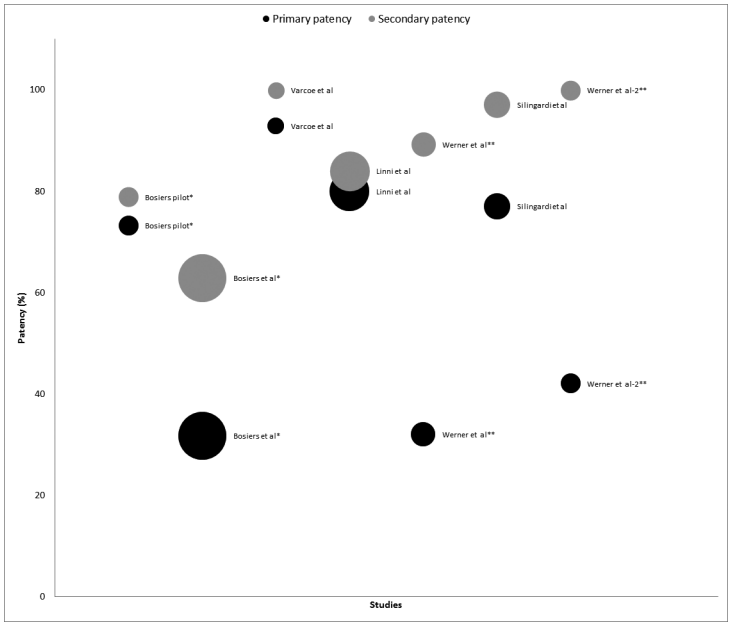
Patients treated for lesions in the femoral artery had a mean one-year primary patency rate of 61.6% (range; 32.1 – 80%). TLR was performed in 30.8% (range; 12.5 – 57.1%) of patients, leading to an overall secondary patency rate of 91.5% (range; 84 – 97.1%)[13–16]. Similarly, the subgroup of patients treated for lesions BTK had an overall six-month primary patency rate of 50.3% (range; 31.8 – 92.9%), with TLR in 21.8% of patients (range; 5.6 – 31.1%) and a secondary patency rate at six months of 72.1% (range; 62.9 – 100%)[12,17,23]. An overview of primary and secondary patency rates is shown in Figure 2. Clinical improvement was reported in most studies using either the ABI[12,14–16,23] or Rutherford category[12–14,17,23] as parameter. A notable decrease in mean Rutherford category and ABI improvement was seen in all studies (Table 4). One study found an amputation rate of 12.4% in patients treated for lesions BTK12, which was not reproduced in other studies (0-5%). Overall mortality ranged from 0-15%, the highest rates being in studies treating BTK lesions.

Results from unpublished studies

Current ongoing and/or unpublished studies all focused on lesions in the SFA, with the REMEDY and STANZA trial being large prospective cohort studies[18,22]. The REMEDY trial reported a 98% technical success rate, three studies all had 100% success rates, and STANZA has not yet reported on technical success.

Preliminary results of the REMEDY and ESPRIT trial showed one-year primary patency rates of 65% and 91.2% respectively, after TLR resulting in 85.9% and 100% secondary patency rates. Clinical outcomes in the REMEDY and ESPRIT trial indicated a Rutherford improvement in 77.4% of patients and a less than 3% amputation rate18,21. A small retrospective study reported no peri-procedural complications and a 100% primary patency with the Absorb bioresorbable stent in 22 patients with SFA/BTK lesions with three months follow-up thus far[19] (Table 5).

FIGURE 2 Patency rates published studies+



+Size of the circle compares to the relative size of included study
*Same first author, Bosiers pilot is the earlier conducted and smaller pilot study
**Same first author, Werner et al-2 is the latest study including a DEB balloon inflation before stent placement

TABLE 4 1-Year clinical results published studies

Study	**Bosiers pilot[23]**	**Bosiers *et al*[12]**	**Varcoe *et al*[17]**	**Linni *et al*[13]**	**Werner *et al*[14]**	**Silingardi *et al*[16]**	**Werner *et al*[15]**
Artery Operated	**BTK**	**BTK**	**BTK**	**CFA**	**SFA**	**SFA**	**SFA**
Technical success rate (%)	100	100 vs 96.4	100	97.5 vs 100	96.7	100	95
30-day adverse events*	0	5 vs 5.3	13.3	7.5 vs 18	6.9	0	0
Primary Patency (%)	73.3	31.8 vs 58**	92.9**	80 vs 100	32.1	77.1^	42.1
TLR (%)	5.6	31.1 vs 16	7.1	12.5 vs 0	57.1	22.8^	42.1
Secondary patency (%)	78.9	62.9 vs 74**	100**	84 vs 100	89.3	97.1^	100
Bypass (%)	5	na	0	0 vs 5	0	0	0
Amputation (%)	5	12.4 vs 7.6	0	2.5 vs 0	0	0	0
Death (%)	15	8.7 vs 7.5	0	12 vs 10	3.3	0	5
Rutherford improvement (%)	na	69.2 vs 65.9	80	80 vs 85	85.7	na	94.7
ABI improvement	na	>0.1 vs > 0.2	na	na	>0.15	na	>0.15

TLR = target lesion revascularization
Bosiers & Linni first number is resorbable stent group vs alternative treatment group
* all reported adverse event including binary restenosis within 30 days
** after six months follow-up
^ 36 months of follow-up

TABLE 5 Post-operative and 1-year patency results unpublished studies

Study	Artery treated	Technical success rate %	Primary patency (1 year) %	TLR (1 year) %	Secondary patency (1 year) %
Unpublished studies					
Biamino[20]	SFA	100	91*	30	91*
Messina[19]	SFA/BTK	100	100**	0	100**
Goverde[18]	SFA	98	65	29.5	85.9
Lammer[21]	SFA	100	91.2	8.8	100
Holden[22]	SFA	na	na	na	na

na = not available, TLR = target lesion revascularization
*9 months after surgery + ´primary assisted primary patency´
** after 3 months

DISCUSSION

This systematic review provides an overview of seven published and five unpublished studies on bioresorbable stents in PAD. The studies were relatively small and heterogeneous, using various bioresorbable stents in either the iliac-femoral tract or BTK. Included studies and conference abstracts showed satisfactory safety and feasibility results with a high technical success rate and low 30-day adverse events for both Mg-stents and PLLA based stents.

Current evidence is not strong enough to reach any well-founded conclusions concerning long-term follow-up, with most evidence coming from small, single arm prospective or retrospective studies. The two main stent structures were mainly used in lesions in different locations, PLLA stents in the SFA and Magnesium stents in BTK arteries, making direct comparisons difficult. One RCT comparing bioresorbable PLLA stent placement with open surgery, was terminated prematurely because of disappointing patency rates in the stent group at interim analysis[13]. The other well conducted RCT, comparing Mg-stents with PTA only in BTK lesions, proved safety and efficacy of the stent, but found a significantly lower patency rate, possibly due to early bioresorption and subsequent recoil of the vessel[12]. Most studies reported low amputation rates and improved clinical parameters such as Rutherford category and ABI[13–17,23].

In BTK lesions recoil of the stent was suspected with the use of the AMS stent, presumably as a result of too swift bioresorption and loss of radial strength of the Mg-stent[12]. Animal studies proved Magnesium to be safe to use and to decrease intimal hyperplasia. Conversely, they also revealed to induce negative remodelling, resulting in a smaller lumen size after 3 months, which might contribute to the relatively high restenosis rates in the RCT[10,24].

Gradual PLLA stent bioresorption has been reported in only one study in PAD, using grayscale ultrasound as imaging modality to examine this process[16]. Cardiac studies showed good and safe gradual bioresorption of PLLA stents over time using IVUS or OCT[8,16,25]. Two studies reported in respect to stent fractures, a known risk factor after

stent placement[26,27], and both studies found none[13,16]. Also, 30-day restenosis did not often occur in included studies, implying that the radial force is adequate in PLLA stents[13–17]. In future studies more emphasis should be put in the actual bioresorption process during follow-up of these newly developed devices, best measured with either IVUS or OCT.

Currently, most data is retrieved from the Cardiology field, and it remains questionable whether this data can be extrapolated to PAD patients[28]. Coronary stenting presents other challenges and more severe risks and effects of complications when compared to peripheral stenting. Focus of Cardiac studies has been on technical properties of stents, imaging techniques and follow-up endpoints. Whilst most cardiac stents have different properties, some sort of consensus seems to form on radial force and duration, drug eluting properties, and placement of the stent. For comparison, technical stent properties needed to ensure clinical benefit in peripheral arteries are currently unclear. In addition, imaging techniques in most coronary studies are corresponding and more often invasive during follow-up. In peripheral arteries it should be possible to evaluate stent patency with color flow Doppler ultrasound (CFDU), although in clinical studies stent bioresorption should be measured, which is best measured with either IVUS or OCT. We tried to give an overview of Cardiac lessons applicable for peripheral arterial use of bioresorbable stents in Table 6[8,29–34].

Present PAD studies remain relatively small and will probably never achieve the large numbers of patients that are common in cardiac trials. Yet, with a multicenter, and possibly even multinational, approach reaching sufficient power should be a possibility. In- and exclusion criteria should adequately reflect the average vascular patient population, since some of these new devices offer very strict instructions for use (IFU) and vague contra-indications for use, which could hamper inclusion rates and practicability of these devices.

A recently published review reported on bioresorbable stents in PAD, but this study has some shortcomings. No specification of the search strategy or flow chart was included, making reproduction of their outcome impossible. Furthermore, no quality appraisal of the included studies was given, making it difficult for the reader to draw firm conclusions. The current review included essential data on unpublished trials which were not included in the previous mentioned review, causing some differences in conclusions[35].

Study limitations

This study has some limitations. As a result of the small number of studies and included patients and large heterogeneity of the studies, we were unable to pool most of the study data. The various studies used different types of stents to treat different lesion sites and lengths, which makes it impossible to directly compare PLLA and Magnesium stents. Similarly, we cannot compare SFA and BTK lesions, and a patient with critical ischemia has a different clinical approach compared with a claudicant patient. Moreover, there were varying in- and exclusion criteria, as well as different follow-up times.

TABLE 6 Cardiac lessons applicable for peripheral arterial use

	Coronary[8,29–34]	**SFA**
Technical properties		
Stent radial force duration	3-6 months?	?
Strut thickness	As small as possible	?
Drug eluting properties	Yes	√
Minimal duration drug eluting properties	6 months?	?[15]
Lesion preparation: predilatation	Yes	√
Overdilatation stent	No	√
Postdilatation 1:1	Yes	√
Restart research process with new stent design	Yes	√
Off-label use	No	√
Imaging		
Non-invasive imaging	Yes: Radiopaque marker	√: Radiopaque marker
Angiography/IVUS/OCT: Sizing	Yes	√
Angiography/IVUS/OCT: Evaluation stent positioning	Yes	√
Angiography/IVUS/OCT: Direct evaluation strut fracture	Yes	√
Follow-up		
Angiography/IVUS/OCT: Follow-up restenosis/lumen area/recoil	Yes	√: In clinical studies
CFDU: Follow-up restenosis/lumen area/recoil	na	Applicable
Evaluation bioresorption progress and duration	Yes in clinical studies	√
Vasomotion function during follow-up	Yes	X
Antiplatelet regimen	DAPT 6 - 12 months?	?

IVUS: Intravascular ultrasound, OCT: Optical Coherence Tomography, CFDU: Color flow doppler ultrasound, DAPT: dual antiplatelet therapy, na: not applicable
√ = coronary findings applicable in peripheral arteries
? = currently unknown in peripheral arteries
X = coronary findings not applicable

After performing a quality assessment of included studies we concluded that most studies were of low quality. As a result of abovementioned shortcomings we have chosen to describe the separate results of the studies per clinical important stent property. As a result, with this evidence, we are unable to fully answer all questions regarding the future use of bioresorbable stents in PAD, which necessitates large randomized controlled trials. Currently, most data is retrieved from the Cardiology field, and it remains questionable whether this data can be extrapolated to PAD patients.

Future needs

In our opinion, abovementioned PLLA stent properties imply that current bioresorbable PLLA based stents are ready for a set-up in a large 1:1 randomized trial concentrated on the SFA.

- Future studies should focus on investigating abovementioned stent characteristics, such as bioresorption process, radial force and patency.
- External monitoring should use standardized radiological endpoints in both study arms, preferably with the same modalities as currently used in cardiologic studies such as CFDU, IVUS or OCT.
- Control group should receive either PTA or non-resorbable stenting.
- Clinical outcomes such as amputation, Rutherford category and ABI should be taken into account with a sufficient follow-up time (> 12 months).
- A multicenter approach is advisable to create sufficient statistical power.

In conclusion, experience with the use of bioresorbable stents in PAD is still very limited and investigated in small studies only, with short term follow-up. The use of bioresorbable stents seems safe and feasible. With current published results we are unable to fully answer all of the questions regarding the future use of bioresorbable stents in PAD, and the use should be limited to only in study-related cases in PAD. In order to address these questions, larger and randomized trials are warranted longer follow-up time. Diagnostic tools should be implemented to examine the stent bioresorption process in better detail.

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SUPPLEMENTAL

SUPPLEMENTAL TABLE 1 Baseline Characteristics

Study	Patients		Lesion classification	
	Age (y)	Male sex (%)	Ruther ford 2-4	Ruther ford 5-6
Bosiers pilot[23]	76	50	45	55
Bosiers et al[12]	74.7	51.7	26.7	73.3
Varcoe et al[17]	82	60	60	40
Linni et al[13]	71.6	60	72.5	27.5
Werner et al[14]	67.7	76.7	-	-
Silingardi et al[16]	71	80	94.3	5.7
Werner et al[15]	66.7	70	100	-

SUPPLEMENTAL TABLE 2 Plaque Characteristics

Study	TASC A/B	TASC C/D	Lesion site				Total occlusion %	Target lesion length (mm)	Stent length <36mm	Stent length >36mm	Multiple stents
			Iliac	CFA	SFA	BTK					
Bosiers pilot[23]	-	-	0	0	0	20	0	11	23	0	3
Bosiers et al[12]	-	-	0	0	0	60	0	10.6	74	0	14
Varcoe et al[17]	-	-	0	0	0	15	26.7	22.2	21	0	6
Linni et al[13]	na	na	0	40	0	0	30	na	40	2	1
Werner et al[14]	100%	0%	0	0	30	0	10	59	11	28	9
Silingardi et al[16]	100%	0%	0	0	35	0	57.1	68	22	21	8
Werner et al[15]	na	na	0	0	20	0	10	43.6	18	-	2

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CHAPTER 3

Lack of Evidence for Dual Antiplatelet Therapy after Endovascular Arterial Procedures: A Meta-analysis

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ABSTRACT

Introduction

Dual antiplatelet therapy (DAPT) has mainly replaced mono antiplatelet therapy (MAPT) and is recommended after arterial endovascular revascularization. Aim of this meta-analysis was to summarize the available evidence for DAPT after endovascular revascularization throughout the arterial system.

Methods

A systematic search was performed in Medline, Embase and the Cochrane Register. Two reviewers independently performed data extraction and quality assessment using the risk of bias assessment tool of the Cochrane Collaboration. Included were randomized controlled trials (RCTs) comparing DAPT with MAPT after endovascular procedures for the treatment of coronary, carotid or peripheral artery disease, reporting at least one clinical outcome. Articles were excluded if patients received anticoagulation in addition to antiplatelet therapy in the post-procedural phase. Primary outcome was restenosis or stent thrombosis and secondary outcomes were major adverse cardiac events (MACE), target lesion revascularization, cerebrovascular accident or transient ischemic attack, bleeding and death. Meta-analyses of binary outcomes were performed using the random effects model and described as risk ratios (RR) and 95% confidence intervals (95% CI). Chi-square tests were used to test for heterogeneity.

Results

Nine articles were included in this study, involving lower limb peripheral arteries (1), carotid arteries (2) and coronary arteries (6). A meta-analysis of data from all included trials showed a RR for restenosis of 0.64 (95% CI 0.36-1.13),for MACE of 0.70 (95% CI 0.19-2.50) and for any bleeding 1.06 (95% CI 0.32-3.52) with DAPT. Pooled results of coronary trials only showed a RR for restenosis with DAPT of 0.60 (95% Ci 0.28-1.31) and for myocardial infarction 0.49 (95% CI 0.12-2.03). In the carotid artery trials the RR for restenosis was 0.22 (95% CI 0.04-1.20) and for peripheral arteries 1.02 (95% CI 0.56-1.82).

Conclusion

The available evidence comparing DAPT with MAPT after endovascular arterial revascularization is limited and the majority of trials were conducted in the cardiology field. No significant evidence for superiority of DAPT compared to MAPT was found, but there was also no evidence of an increased bleeding risk with DAPT over MAPT.

What this paper adds

Dual antiplatelet therapy (DAPT) is widely prescribed after endovascular arterial revascularizations, but recommendations vary. We summarized the evidence for the use of DAPT by performing a meta-analysis of randomized controlled trials comparing DAPT with mono antiplatelet therapy (MAPT) after coronary, carotid and peripheral arterial revascularization. No clear benefit of DAPT over MAPT could be demonstrated in this meta-analysis.

INTRODUCTION

Patients with peripheral arterial disease (PAD) requiring arterial revascularization can be treated by percutaneous transluminal angioplasty (PTA) with or without additional stent placement, or surgery[1]. Endovascular intervention has largely taken over the role as primary choice of intervention as it offers a minimally invasive treatment option with lower morbidity rates. Unfortunately, the patency rates remain rather low, patency rates as low as 49% at five years have been reported[2–7].

In these processes of restenosis and stent thrombosis, several mechanisms are involved. PTA induces atherosclerotic plaque disruption, causing aggregation of platelets at the site of the damaged arterial wall, followed by organization of the thrombus and attraction of inflammatory cells. During the later stages of healing, neointima formation is caused by smooth muscle cells that organize over the luminal surface of the lesion, growing thicker over time and reducing the lumen of the artery[8,9]. Because of the important role of platelets in this process of restenosis, there is strong rationale to expect less restenosis when adequate antiplatelet therapy is applied after endovascular procedures. Therefore it should be of no surprise that parallel with the increase in endovascular interventions, the prescription of dual antiplatelet therapy (DAPT) after peripheral endovascular revascularization to prevent restenosis as well as other cardiovascular events has become favorable over mono antiplatelet therapy (MAPT)[10–12]. A survey held in the Netherlands showed that the use of DAPT after infrainguinal PTA without stenting increased from 4% (95% CI 1-14) in 2004 to 14% (95% CI 9-21) in 2011 and after PTA with stent placement DAPT use increased from 17% (95% CI 8-30) to 38% (95% CI 30-47) in 2004 and 2011. It is expected that these percentages continued to rise[13].

Indication, duration and type of DAPT vary across the world, and even among specialists in the same medical center[12]. Guidelines provide different recommendations regarding DAPT after peripheral endovascular lower limb arterial revascularization, mainly because of low grade of evidence[2,14,15]. Not only after peripheral arterial revascularization dual antiplatelet therapy is given. After endovascular carotid artery stenting the use of DAPT is recommended for a minimum of 30 days[16,17] and following endovascular coronary artery revascularization DAPT is recommended for at least 12 months[18]. In the coronary field the question is no longer whether to give mono or dual antiplatelet therapy, but whether to give dual or triple therapy and for which duration.

The aim of this systematic review and meta-analysis was to summarize the currently available evidence for the benefits of use of DAPT compared to MAPT after endovascular procedures in lower limb peripheral, carotid and coronary artery disease.

METHODS

This systematic review and meta-analysis was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines[19].

Search methods

In October 2015, the Embase, Medline and Cochrane clinical trial databases were used to systematically search for articles. Used search terms were: dual antiplatelet therapy, antiplatelet therapy, thienopyridine, clopidogrel, ticagrelor, prasugrel, cangrelor, abciximab, dipyridamole, cilostazol, tirofiban, eptifibatide, indobufen, aspirin, acetylsalicylic acid, platelet inhibitors, platelet inhibition, angioplasty, intervention, percutaneous transluminal angioplasty, stent, stenting, angiography, intravascular and endovascular. The search was limited to articles in English or Dutch, there were no limitations in publication year or publication status (see appendix 1).

Article selection

Articles were included if they were randomized controlled trials (RCTs) in humans, comparing MAPT with DAPT for a minimum of seven days after endovascular arterial revascularization, without the concomitant use of anticoagulants. A minimum period of seven days post procedural DAPT treatment was chosen, because we didn't want to include articles investigating periprocedural short-term DAPT. Periprocedural anticoagulant therapy was allowed up to 48 hours after the procedure. The inclusion and exclusion criteria were defined before the search and are displayed in Table 1. Two reviewers independently screened the articles for eligibility = (S.P.W. and S.H.) and disagreements were resolved by an independent third reviewer (G.B.).

Quality assessment

After inclusion, data extraction and quality assessment were performed by the same two reviewers, using the risk of bias assessment tool of the Cochrane Collaboration[20].

Data collection

Two authors (S.P.W., S.H.) independently recorded information from publications and discrepancies were resolved through discussion with a third reviewer (G.J.B.). Only published data was recorded and data from the latest follow-up available was used. The primary outcome was restenosis or stent thrombosis, as defined by angiography or duplex ultrasound. Secondary end points included: major adverse cardiovascular events (MACE), any bleeding and major bleeding, target lesion revascularization (TLR), cerebrovascular accident (CVA) or transient ischemic attack (TIA) and death. MACE was defined as myocardial infarction, cardiovascular death or target lesion revascularization. Restenosis was defined as >50% lumen loss of the previously treated lesion, defined by duplex ultrasound or angiography and major bleeding was defined as any episode of bleeding requiring transfusion or intervention.

TABLE 1 In and exclusion criteria

Inclusion criteria	Exclusion criteria
Randomized controlled trial	Concomitant oral anticoagulants
Dual vs mono antiplatelet therapy	Concomitant heparin/warfarin >48h
≥7 days DAPT vs MAPT	<10 patients included
Arterial endovascular revascularization	Follow-up <14 days
At least one clinical endpoint	Non-human studies
	Start DAPT >48h after revascularization
	Initial DAPT >48h after procedure in both arms

Only patients receiving the allocated medication within 48 hours after the intervention were included in the analyses.

Statistical analysis

A meta-analysis of available results was performed. Meta-analyses were performed using Review Manager 5.1 software (The Cochrane Collaboration, Copenhagen, Denmark). A random effects model was applied to calculate treatment effects, because a large heterogeneity between studies was assumed. The relative risks (RR) and 95% confidence intervals (CI) were calculated to express treatment effects. Heterogeneity between included studies was determined using the Chi^2 test. Inconsistency was quantified with the I^2 statistic, where I^2 values <25% represent mild inconsistency, values between 25% and 50% represent moderate inconsistency and values >50% suggest severe heterogeneity between the studies. Subgroup analyses were performed for trials on coronary, carotid and peripheral arteries.

Statistical significance was assumed at P <0.05. The presence of publication bias was assessed by visual inspection of the funnel plots. A Begg funnel plot was created to compare the RR with the standard error of the log RR of the included studies.

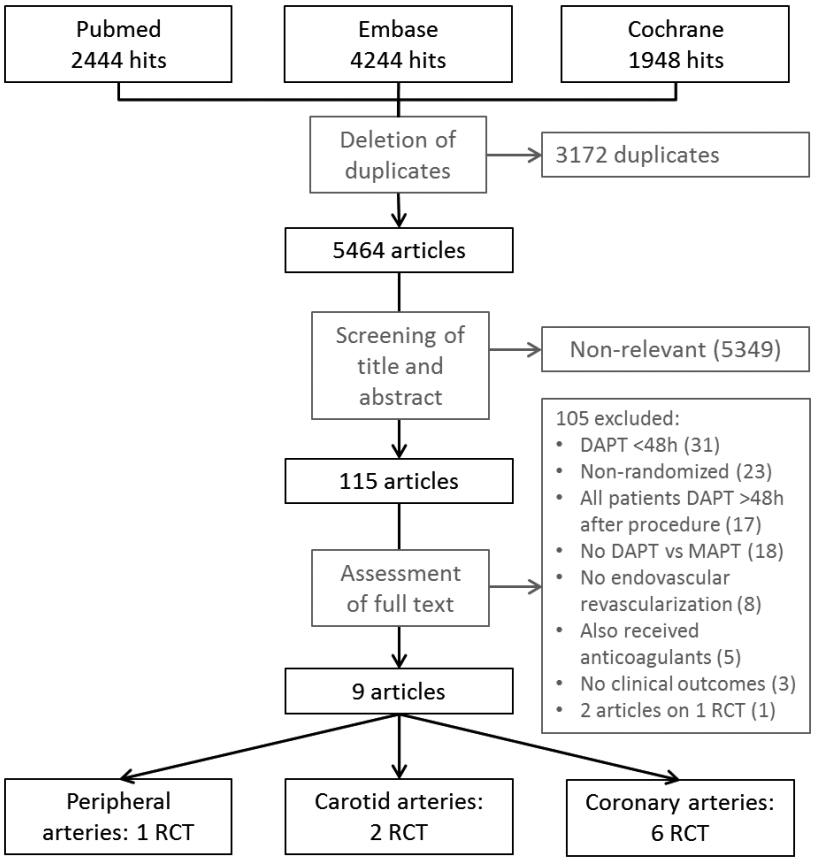
RESULTS

The search in the Medline database resulted in 2444 hits, in the Embase database in 4244 hits and in the Cochrane register in 1948 hits. After duplicate removal, 5464 titles and abstracts were screened. Ultimately, 115 articles were assessed based on full text and 10 articles seemed eligible for inclusion in this review. Two articles regarded the same clinical trial[21,22], which ultimately resulted in a total of nine included trials. A flow chart of this search is displayed in Figure 1.

Of nine included articles, one regarded peripheral lower limb arteries[22], two regarded carotid arteries[23,24] and six regarded coronary arteries[25–30]. No consultation with the third independent reviewer was necessary to reach consensus about included studies. Characteristics of all included studies are displayed in Table 2. A risk of bias summary

of included studies is displayed in Figure 2. Funnel plots were suggestive for publication bias or small study bias for the pooled primary outcome of restenosis (Figure 3). Larger, more precise studies with smaller standard errors tended to find larger relative risks for restenosis or stent thrombosis, than did smaller studies with larger standard errors.

FIGURE 1 Flow chart of search strategy



Endovascular lower limb artery revascularization

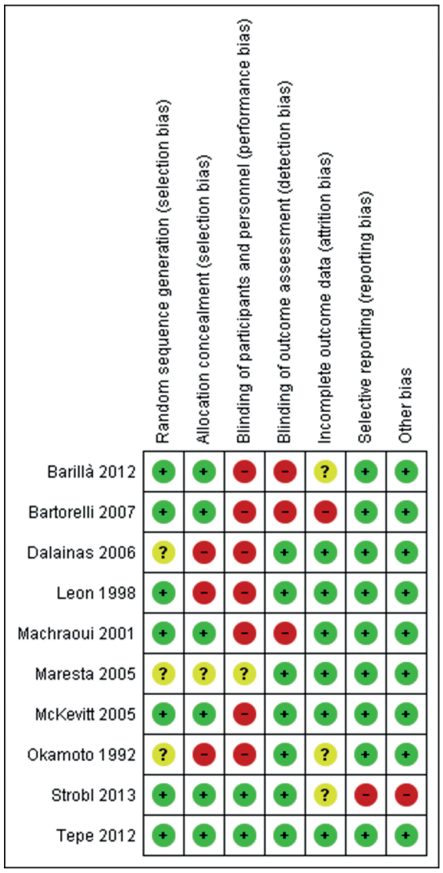
One RCT was available on lower limb arteries. This study compared aspirin plus clopidogrel with aspirin mono therapy after PTA with or without stenting in the femoropopliteal segment; the MIRROR trial[22]. The MIRROR trial was a randomized, double-blinded, placebo-controlled trial including 80 patients. Patients received either a loading dose of 500 mg aspirin and 300 mg clopidogrel before the intervention and a daily dose of 100 mg aspirin and 75 mg clopidogrel for six months after the intervention, or the same dose of aspirin plus placebo instead of clopidogrel.

TABLE 2 Characteristics of included articles

Author	Year	N (DAPT/ MAPT)	Technique used	DAPT	MAPT	Duration DAPT	Follow-up	Placebo-controlled
Peripheral arterial revascularization								
Tepe[21] (MIRROR trial)	2012	40/40	PTA (37.5%) / PTA + BMS (62.5%)	Aspirin 100mg Clopidogrel 75mg	Aspirin 100mg	6 months	6 months	Yes
Strobl[22] (MIRROR trial)	2013	36/37	PTA (37.5%) / PTA + BMS (62.5%)	Aspirin 100mg Clopidogrel 75mg	Aspirin 100mg	6 months	12 months	Yes
Carotid artery revascularization								
McKevitt[23]	2005	23/24	Carotid artery stenting (BMS)	Aspirin 75mg Clopidogrel 75mg	Aspirin 75mg	28 days	1 month	No
Dalainas[24]	2006	50/50	Carotid artery stenting (BMS)	Aspirin 325mg Ticlopidine 250mg BID	Aspirin 325mg	30 days	1 month	No
Coronary artery revascularization								
Okamoto[30]	1992	36/36	PCI	Aspirin 300mg Dipyridamole 150mg	Trapidil 600mg	4-6 months	6 months	No
Leon[27]	1998	546/557	PCI + BMS	Aspirin 325mg Ticlopidine 250mg BID	Aspirin 325mg	4 weeks	1 month	No
Machraoui[28]	2001	122/121	PCI + BMS	Ticlopidine 250mg BID Aspirin 100mg	Ticlopidine 250mg BID	4 weeks	3 months	No
Maresta[29]	2005	35/40	PCI (42.5%) / PCI + BMS (57.5%)	Aspirin 100-325mg Trapidil 200mg TID	Aspirin 100-325mg	6 months	12 months	Yes
Bartorelli[26] (SAFE trial)	2007	244/235	PCI + BMS	Aspirin 325mg Thienopyridine	Aspirin 325mg	1 month	6 months	No
Barillà[25] (STARC II)	2012	20/21	PCI + stent (57.1% BMS + 42.9% sirolimus-eluting stent)	Clopidogrel 75mg Indobufen 100mg BID	Clopidogrel 75mg	N/A	12 months	No

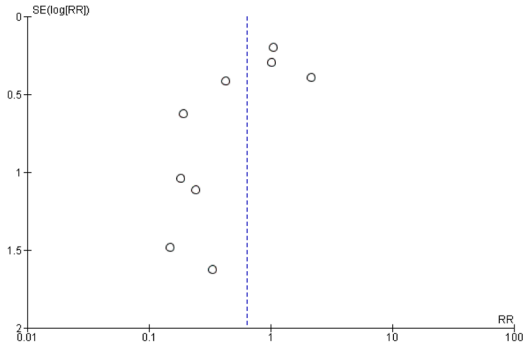
The six-months results showed restenosis rates of 40% in the DAPT group vs 39.4% in the MAPT group (RR 1.02, 95% CI 0.56-1.82). TLR rates were significantly lower in the DAPT group than in the MAPT group (5% vs 20%, P=0.040). Notably, the only two patients who needed TLR in the DAPT group proved to be resistant to clopidogrel, measured using the Chandler-Loop vessel model[21]. MACE was not significantly different (30% in the DAPT group and 37.5% in MAPT group (P=0.508)), nor were bleeding complications (any bleeding 2.5% vs 5% and major bleeding 2.5% vs 0%, respectively). At twelve months, long-term restenosis rates, MACE and bleeding were not reported, but the difference in TLR seen after six months no longer existed (25% in DAPT vs 32.5% in MAPT group, RR 0.73, 95% CI 0.35-1.52). All-cause mortality was zero in the DAPT group versus three in the MAPT group at 12 months follow-up (p=0.08). Causes of death were sepsis, myocardial infarction and heart failure[22].

FIGURE 2 Risk of bias summary



Review authors' judgements about each risk of bias item for each included study

FIGURE 3 Funnel plot for pooled restenosis or stent thrombosis



Endovascular carotid artery revascularization

Two eligible studies were found that compared DAPT with MAPT after endovascular carotid artery revascularization in a randomized matter, together including only 147 patients[23,24]. One of the trials investigated the safety and efficacy of clopidogrel added to aspirin in 47 patients[23] and the other of ticlopidine added to aspirin in 100 patients[24], both after carotid artery stenting (CAS). The first study compared aspirin plus clopidogrel for 28 days with aspirin indefinitely plus heparin for 24 hours. The latter had the same regime, but with ticlopidine instead of clopidogrel and continued DAPT for 30 days. Important to note is that both trials were ended prematurely because of large differences in outcome between the two arms of the trials (eight neurological complications in MAPT vs one in DAPT group[24] and six neurological complications in MAPT vs zero in DAPT group[23]).

Meta-analysis of trials on DAPT after carotid artery interventions

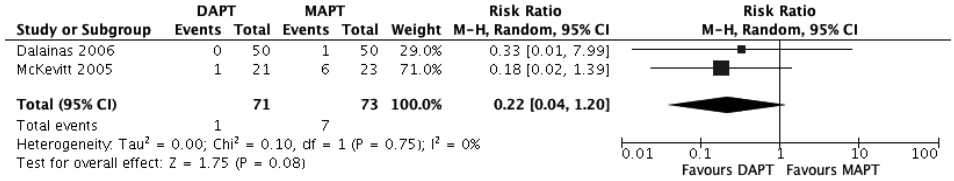
A meta-analysis of the two included trials, analyzing 73 patients in the DAPT group and 74 patients in the MAPT group, showed neither significant differences in restenosis rates between DAPT and MAPT groups (RR 0.22, 95% CI 0.04-1.20, Figure 4A), nor in bleeding rates (RR 0.51, 95% CI 0.14-1.94). The risk of CVA was significantly lower with DAPT compared to MAPT (RR 0.11, 95% CI 0.02-0.56). However, the follow-up of the two included trials was only 30 days, so only short-term results are displayed. One death was reported in the DAPT group, vs zero in the MAPT group.

Coronary endovascular procedures

Six RCTs were identified that compared DAPT with MAPT after coronary endovascular revascularization[25–30]. These trials jointly included 2013 patients, of whom 1003 received DAPT and 1010 received MAPT. One trial compared aspirin plus ticlopidine with aspirin monotherapy[27], one aspirin plus ticlopidine with ticlopidine monotherapy[28], one aspirin plus ticlopidine or clopidogrel with aspirin monotherapy[26], one aspirin plus trapidil with aspirin monotherapy[29], one aspirin plus dipyridamole with trapidil monotherapy[30] and

one trial only included patients with hypersensitivity to aspirin and compared clopidogrel plus indobufen with clopidogrel only[25] (see Table 2). The duration of the DAPT varied from one month to 12 months and follow-up duration also varied between one and 12 months. Three studies investigated superiority of DAPT versus MAPT and all found reduced restenosis or thrombosis with DAPT[25,27,29]. Three other trials investigated non-inferiority of MAPT compared to DAPT and all these concluded that MAPT was as safe and effective as DAPT[26,28,30].

FIGURE 4A

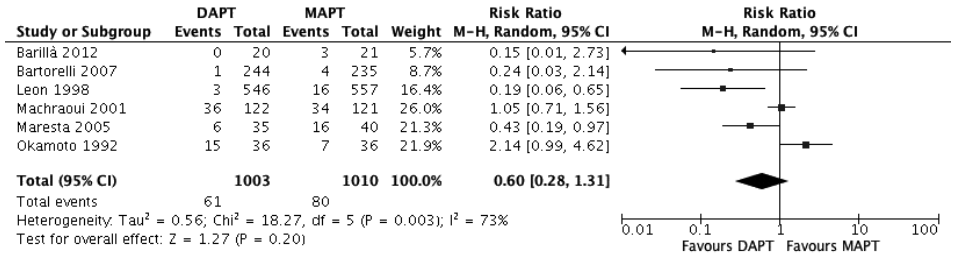


Meta-analysis of the primary endpoint of restenosis or stent thrombosis after carotid revascularization

Meta-analysis of trials on DAPT after coronary interventions

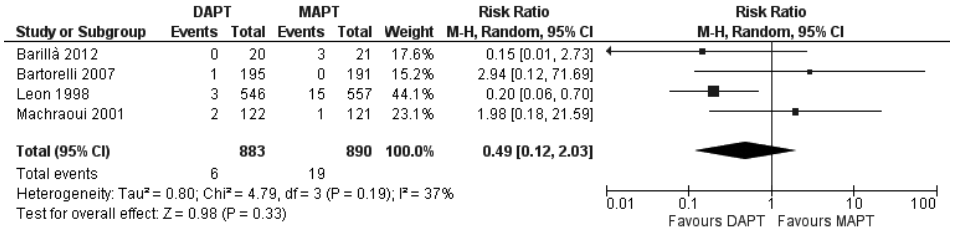
The RR for restenosis or stent thrombosis after endovascular coronary revascularization with DAPT compared to MAPT was 0.60 (95% CI 0.28-1.31, Figure 4B), with significant heterogeneity between the included trials. The RR for myocardial infarction with DAPT after coronary revascularization was 0.49 (95% CI 0.12-2.03, Figure 5). The RR for MACE after coronary interventions was 0.61 (95% CI 0.04-8.81) with DAPT compared to MAPT. The RR for major bleeding with DAPT was based on results of only one trial[28] and was 0.98 (95% CI 0.18-21.59) and RR for any bleeding was also retrievable in only one trial and was 3.06 (95% CI 1.51-6.20). In total, one death was reported in the MAPT group, compared to zero in the DAPT group.

FIGURE 4B



Meta-analysis of the primary endpoint of restenosis or stent thrombosis after coronary revascularization

FIGURE 5

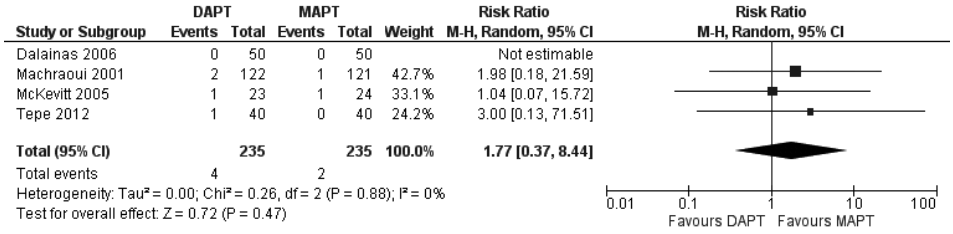


Meta-analysis of myocardial infarction after coronary revascularization

Risk of bleeding in all included trials

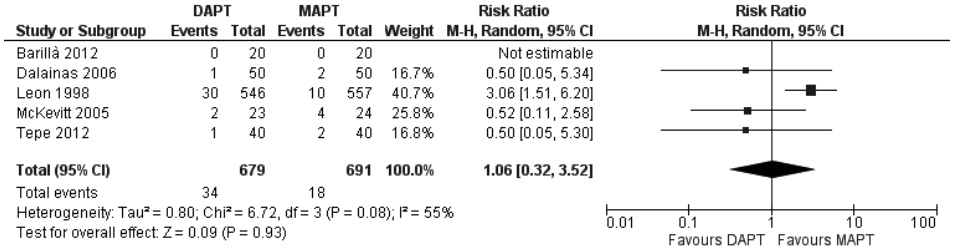
A meta-analysis of all trials reporting on major bleeding showed a RR of 1.77 (95% CI 0.37-8.44) and the RR for any bleeding was 1.06 (95% CI 0.32-3.52) with DAPT versus MAPT, as shown in Figure 6A and 6B.

FIGURE 6A



Meta-analysis of major bleeding in trials on DAPT after peripheral, carotid and coronary artery revascularization

FIGURE 6B



Meta-analysis of any bleeding in trials on DAPT after peripheral, carotid and coronary artery revascularization

DISCUSSION

This systematic review and meta-analysis summarizes all RCTs on DAPT versus MAPT after endovascular revascularization. For lower limb peripheral and carotid artery revascularization very few RCTs exist on the use of DAPT. The trials on carotid artery revascularization showed a significant reduction in CVA rates with DAPT, but jointly included only 147 patients. A larger number of available RCTs compared DAPT with MAPT after endovascular coronary artery revascularization. Meta-analysis of these coronary trials showed a non-significant risk reduction for restenosis or stent thrombosis with DAPT versus MAPT, and also the risk of myocardial infarction was not significantly reduced with DAPT in these patients.

Since the aim of DAPT after peripheral endovascular revascularization is not only to prevent restenosis, but also other cardiovascular events[11], we pooled the data on peripheral, carotid and coronary revascularization to analyze the risk of restenosis or stent thrombosis, MACE and bleeding. The RR for bleeding was not significantly different for DAPT or MAPT. Interestingly, two trials that showed results favoring MAPT for the prevention of restenosis, happened to be trials that were aimed at showing that MAPT is as safe and effective as DAPT[28,30], while the vast majority of trials aimed at showing superiority of DAPT. Since seven out of nine trials were non-blinded, there might have been some bias in the results.

Both trials investigating DAPT after carotid endovascular revascularization were ended prematurely because of significant benefit in the prevention of CVA in the DAPT treated groups. As a result, both studies randomized only small numbers of patients. In contradiction to the results of these 2 small RCTs, a large retrospective study with 1083 patients couldn't find any advantage of the use of DAPT compared with MAPT and stated that the suggested benefit of clopidogrel in decreasing the incidence of complications in patients undergoing CAS may be overestimated due to the overlapping effect of other more relevant factors (such as plaque stabilization from statins)[31]. The present results also contradict the results of the MATCH trial, which was a double blind placebo-controlled trial investigating the addition of aspirin to clopidogrel after recent stroke and found a significant increase in both life-threatening and major bleeding with aspirin plus clopidogrel[32].

In PAD an attempt was made to compare DAPT with MAPT after femoropopliteal endovascular revascularization in the CAMPER trial. Unfortunately, this trial was discontinued because of lack of enrollment as physicians were uncomfortable with patients receiving aspirin monotherapy after endovascular revascularization[33]. Therefore the lack of evidence for DAPT after lower limb endovascular revascularization can partly be explained by the fact that physicians had already adopted the DAPT regime used in the cardiology field after coronary endovascular revascularization. This makes it very difficult, if not impossible, to gather new evidence for DAPT after peripheral endovascular revascularization in well conducted, large randomized controlled trials.

As stated before, the DAPT regime after endovascular carotid and lower limb peripheral artery revascularization is mainly based on evidence from coronary artery studies. Nonetheless, evidence exists that coronary artery disease and peripheral artery disease have different risk factors and prognosis, which makes it questionable to simply copy this treatment[34]. The possible (post)procedural complications and the indication for DAPT are different as well, since in carotid artery disease the main goal is to prevent stroke and restenosis seems less relevant in these arteries, in contrast to peripheral arteries. In coronary arteries stent thrombosis is the most devastating event.

More recent trials accept this lack of evidence and are focusing on the duration of DAPT and the addition of a third antiplatelet drug to DAPT treatment[35–40]. Besides these new strategies, personalized antiplatelet therapy is gaining territory, since it appears that up to 40% of patients is a non-responder to clopidogrel, currently one of the most used antiplatelet agents. Response to clopidogrel and other antiplatelet drugs can be investigated by different tests and therapy could be adjusted based on these tests[41]. However, up until now, trials investigating the benefit of personalized APT based on platelet function tests show diverging results[42].

Limitations of this meta-analysis

Only RCTs were included in this review, meaning that non-randomized or retrospective studies were excluded. This means on one hand that only high quality evidence is included in this review, but on the other hand that we could have missed some non-randomized evidence supporting the use of DAPT after endovascular procedures. Moreover, we excluded trials comparing DAPT with anti-coagulants and therefore excluded a group of trials in which DAPT was compared with aspirin plus warfarin. In particular in cardiology trials the first change in treatment approach was the administration of aspirin plus anti-coagulants. Subsequently, the majority of trials compared DAPT with aspirin plus anti-coagulants[43–46]. This could explain the lack of evidence we found, comparing DAPT with MAPT after endovascular (coronary) artery revascularization and also why the included studies in the coronary field are dated and do not represent current practice.

The trials included in this review were conducted during a relatively large period of time. During this period secondary prevention of cardiovascular patients has changed, which could have led to differences in prognosis and outcome between the early and late trials. Moreover, ticlopidine (which is studied in three of the included trials) is no longer used in most European countries due to its side effect of bone marrow depression[47]. A significant heterogeneity existed between the included trials and between the definitions of end points such as stent thrombosis, restenosis, and major bleeding. The large heterogeneity may have been caused by the inclusion of patients undergoing different procedures, the use of different platelet inhibitors and different definitions of restenosis or stent thrombosis.

We cannot eliminate the potential for publication bias, which might have affected the results of the present meta-analysis. In addition, language restriction could have provided another source of publication bias.
Currently, in the cardiology field an important question raised about the use of DAPT after implantation of drug-eluting stents. However, this discussion is beyond the scope of this meta-analysis.

CONCLUSION

The current available evidence comparing DAPT with MAPT after endovascular arterial revascularization is limited and mainly comes from dated RCTs investigating coronary artery intervention, making them unsuitable to draw conclusions for the peripheral and carotid field. Overall, no significant evidence for superiority of DAPT compared to MAPT was found. However, there is also no evidence of an increased bleeding risk with DAPT over MAPT.
At this point, although a large RCT to compare DAPT with MAPT after peripheral revascularization is highly desirable, it seems to be an unachievable goal.

Conflict of interest
None.

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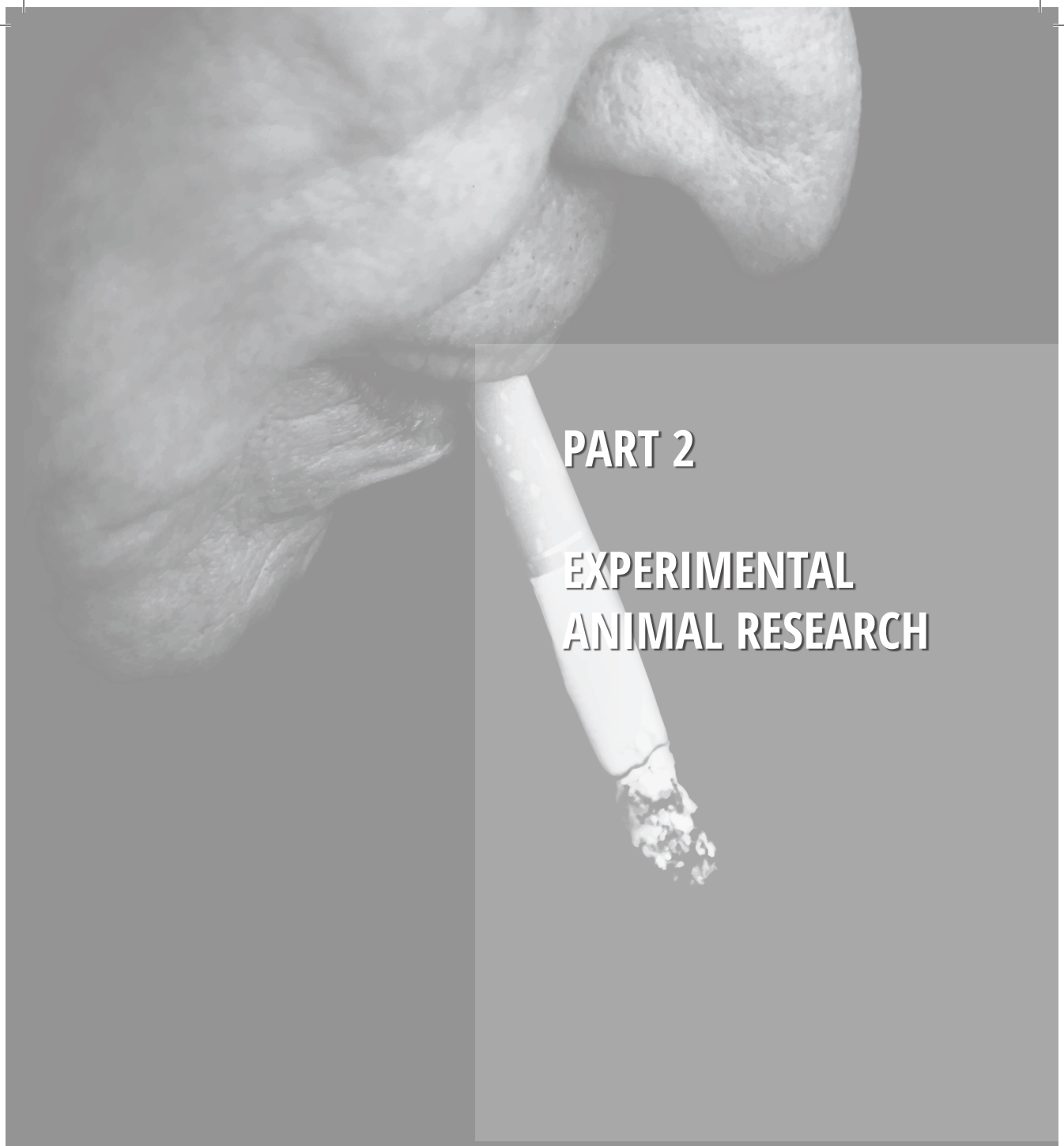
APPENDIX

Search syntax Embase

'dual antiplatelet therapy':ab OR **'antiplatelet therapy'** OR **'thienopyridine'**/exp OR **'thienopyridine'** OR **'clopidogrel'**/exp OR **'clopidogrel'** OR **'ticagrelor'**/exp OR **'ticagrelor'** OR **'prasugrel'**/exp OR **'prasugrel'** OR **'cangrelor'**/exp OR **'cangrelor'** OR **'abciximab'**/exp OR **'abciximab'** OR **'dipyridamole'**/exp OR **'dipyridamole'** OR **'cilostazol'**/exp OR **'cilostazol'** OR **'tirofiban'**/exp OR **'tirofiban'** OR **'eptifibatide'**/exp OR **'eptifibatide'** OR **'indobufen'**/exp OR **'indobufen'** OR **'aspirin'**/exp OR **'aspirin'** OR **'acetylsalicylic acid'**/exp OR **'acetylsalicylic acid'** OR **'platelet inhibitors'**/exp OR **'platelet inhibitors'** OR **'platelet inhibition'** AND (**'angioplasty'**/exp OR **'angioplasty'** OR **'intervention'** OR **'percutaneous transluminal angioplasty'**/exp OR **'percutaneous transluminal angioplasty'** OR **'stent'**/exp OR **'stent'** OR **'stenting'**/exp OR **'stenting'** OR **'angiography'**/exp OR **'angiography'** OR **'intravascular'** OR **'endovascular'**) AND ([controlled clinical trial]/lim OR [randomized controlled trial]/lim) AND ([article]/lim OR [article in press]/lim) AND [humans]/lim AND [english]/lim AND [embase]/lim



PART 2

EXPERIMENTAL ANIMAL RESEARCH

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CHAPTER 4

Validation of a vascular injury model using a cutting and thrombectomy balloon to induce intimal hyperplasia in pig iliac arteries

Submitted for publication

ABSTRACT

Background
Intimal hyperplasia (IH) limits the patency after (endo)vascular interventions. To effectively study new (endo)vascular techniques to prevent or treat IH, a reliable animal model is crucial. The pig has been the preferred model in vascular surgery. However, a model to induce IH in peripheral arteries in pigs using a Cutting Balloon (CB) combined with Thrombectomy Balloon (TB) has not been validated yet.

Aim
To validate the use of the CB and TB in pig iliac arteries as an effective model to induce IH.

Method
In 12 pigs, the common iliac artery (CIA) was damaged endovascular with the combined use of CB and TB. After follow-up of respectively six (n=6) and twelve weeks (n=6), all arteries were explanted for macroscopic and blinded histological examination to the extent of vascular damage, amount of IH and intima/media (I/M) ratio.

Results
One pig died due to bleeding after perforation due to unavoidable oversizing of the CB, and was replaced according to protocol. The extent of vascular damage was associated with an increased IH area (p=0.042) and I/M ratio (p=0.042). In arteries where the media was totally cut, it was difficult to make a correct distinction between repair reaction of the media and IH. After six weeks complete endothelialisation of all lesions had occurred. No difference in IH areas was observed between six and twelve weeks (p=0.485).

Conclusion
Current endovascular technique is an effective model to induce IH in pigs within six weeks. However, due to limited available sizes of the CB and no standardisation to inflate the TB, precise dosing of the extent of vascular wall damage is difficult.

INTRODUCTION

Treatment for peripheral arterial disease (PAD) yield unsatisfactory long term results with 1-year restenosis rates of up to 30%, often resulting from intimal hyperplasia or constrictive remodelling[1–5]. New endovascular treatment modalities and devices are first being tested in animal models. The pig model is the model of choice because of the similarities in anatomy and arterial healing process as compared to the human arterial system[6–9].

Unfortunately, inducing atherosclerosis in peripheral arteries in pigs is a difficult process which yields unsatisfactory results, especially in the peripheral arteries. Current animal models often rely on vascular injury methods, mechanically disrupting the Internal Elastic Lamina (IEL) for the development of intimal hyperplasia (IH)[10–14]. The induction of IH works as a surrogate for restenosis and creates an environment to test new drugs and endovascular devices in pigs. However, creating IH in peripheral arteries is harder than in coronary arteries, often leading to sub-optimal amounts of IH[13]. Recently a novel method to induce IH in peripheral arteries in pigs was presented by the use of the Cutting Balloon (CB) (Boston Scientific, Marlborough, United States) and a Thrombectomy Balloon (TB) (Fogarty Balloon, Edwards Lifesciences, Irvine, US). This method showed promising results, but has not been validated yet[15].

In the current study, we sought to validate the induction of IH in iliac arteries in pigs, using the same method as described by houbballah et al[15]. In addition, the follow-up period was extended to evaluate the longer term effects of the vascular damage model.

METHODS

Ethical Statement

The study protocol was approved by the local ethical commission on animal testing (DEC), which consists of an expert panel on animal research and welfare, under protocol number: 2014.II.12.103.

Study Design

In total, 12 female land/Yorkshire pigs were obtained from a local qualified supplier (van Beek, Lelystad), which breeds pigs according to local and international standards. The pigs were delivered at a weight of approximately 60 kg, which correspondents with an approximate age of around 6 months. This weight was chosen to optimize the chance of diameter of the common iliac artery (CIA) to be around 7 mm[16]. All pigs received normal chow without supplements and water was freely available. The pigs were housed in pairs, except for pre- and post-operative days when they were housed solitary for procedural work-up and post-operatively for their own protection. A minimal of once daily visits from animal caretakers was protocolled to assess animal welfare. All animals received pre- and post-procedural pain killers as per protocol. Dual anti-

platelet aggregation therapy was given starting 10 days before the initial procedure with a daily dose of Aspirin (Carbasalate Calcium 80mg) and Clopidogrel (75mg).

Arterial Damage

The CB and TB were used to inflict damage to the arterial wall. The carotid artery was exposed for cut-down access, after which a guiding catheter was placed in the abdominal aorta. The bilateral CIA's were visualized on angiography and the diameters were measured from outer range to outer range of contrast. The arteries on both the right and the left side were damaged using a CB (at 1:1 sizing compared with the artery diameter, the available diameter of the CB closest to the arterial diameter was used). The CB was slowly pulled back over the trajectory between the iliac bifurcation and the parting of the circumflex artery, at approximately one third distally of the aortic bifurcation in the CIA (Figure 1). After this, an inflated TB was slowly pulled-back once over the same trajectory on both sides. It was not possible to standardise the inflation of the TB, thus we used angiographic control to observe a clear flattening of the balloon before pulling back. During pull-back the balloon was kept inflated under pressure to assure a feeling of resistance over the trajectory. Angiographic control for perforation or dissection was performed after each step.

Experimental Groups

A small feasibility study was conducted (n=4 animals) to assess the study protocol and custom made catheter for The Grail study (see below). The 12 follow-up pigs were divided into 2 different groups with different follow-up: Group 1 consisted of 6 pigs with six weeks follow-up and group 2 consisted of 6 pigs with twelve weeks follow-up. Both group 1 and group 2 received arterial damage in both CIA, after which a bioresorbable gel was placed in the CIA on one side with the use of an endovascular balloon. The objective of that study was to assess the effect of a bioresorbable gel on the prevention of IH after vascular damage (Grail study, European Union funded: HEALTH.2011.1.4-2-278557). The untreated arteries (controls) were included in the present study (12 animals, n=12 arteries).

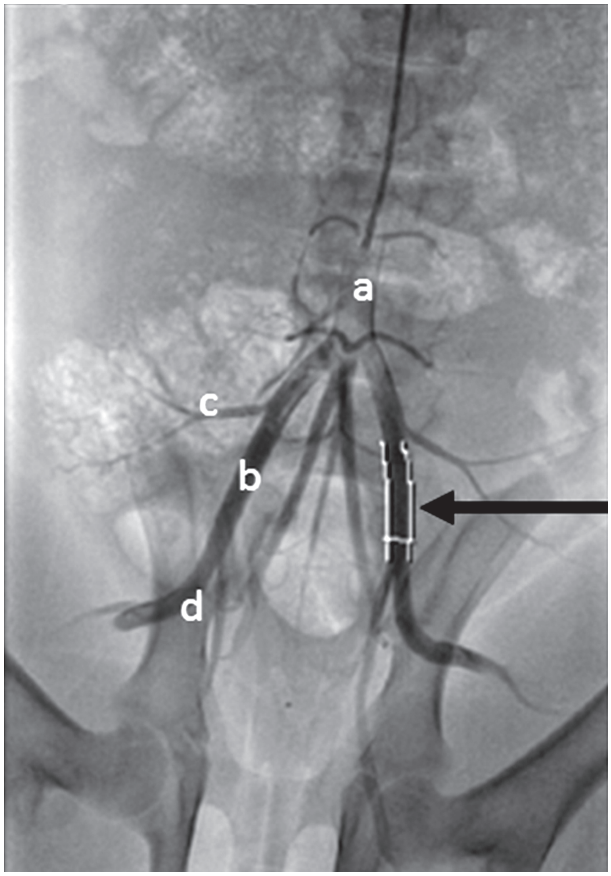
Imaging Assessment

At termination, before explantation of the arteries, a follow-up angiography was performed as well as a rollscan with the use of a fixed C-arm available in the operating theatre (Philips). These scans and images were analysed with the use of XCelera software (Philips).

Histological Assessment

All vascular explanted tissue was fixed in Formalin 4% for at least 48 hours (but no longer than 5 days). The arteries were cut into 5mm segments which were embedded in paraffin. Cross sections were made from these segments at 4 micrometre per slide. The slides were initially stained with Hematoxylin & Eosin (H&E) for general inspection

FIGURE 1 Anatomy of the pig iliac arteries



Anatomy of the pic iliac artery system. a: aorta, b: common iliac artery (CIA), c: circumflex artery branch, d: bifurcation of external and internal iliac artery. Red arrow and lined segment: treated segment of CIA.

of the arterial wall. Elastic van Gieson (EvG) staining was used to visualize the IEL and External Elastic Lamina (EEL), and to calculate intima area, media area and the intima/media (I/M) ratio. The boundary between media repair and IH was assessed on EvG slides, in which media repair was defined as continuing of lengthwise fibres of the media layer surrounding the newly formed lesion. IH was defined as hyperproliferation of myofibroblasts in the newly formed lesion towards the lumen of the artery. An alpha Smooth Muscle Actin (αSMA) immuno-stain was used to visualize smooth muscle cells and myofibroblasts in the media and neo-intima. An ETS related gene (ERG) immuno-stain was used to visualize endothelial cells. The slides were scanned with the use of the Aperio coupes scanner, at 20x magnification. Histological analysis and measurements were performed with the use of ImageScope (Aperio, Leica Biosystems, Germany).

Artery Injury Score

The used Artery Injury Score was based on a previously published scoring list[17], used to score the damage to the arterial wall after Cutting balloon and Fogarty balloon application:

0: IEL, media and EEL intact
1: IEL ruptured, media and EEL intact
2: IEL ruptured, profound media damage, EEL intact
3: Both IEL, media and EEL ruptured

Statistical Analysis

The obtained data is described as the median and range or mean and standard deviation of the mean. Final analysis was performed on the control arteries in 6 pigs with six weeks follow-up and the 6 pigs with twelve weeks of follow-up (total n=12). The 4 acute animals in the feasibility study were not further analysed in this study, apart from histological assessment in the acute setting. The artery segment with the highest Artery Injury Score and the greatest IH extent was used for further analyses. Student's t-tests were used for normally distributed continuous variables, for non-normally distributed continuous variables Mann-Whitney U tests were performed. Categorical variables were compared by chi-square tests. Values with a p value of < 0.05 were considered statistically significant. All statistical analysis were performed with the use of SPSS version 22.0.

RESULTS

Baseline Characteristics

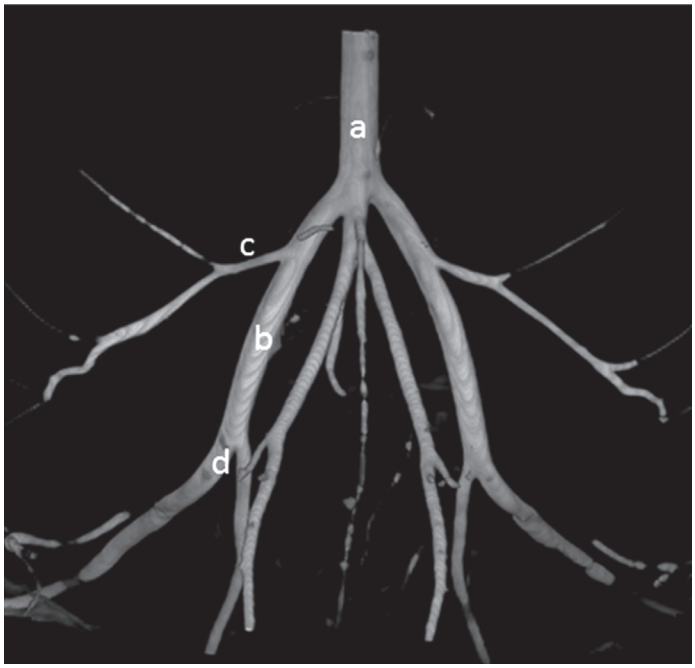
In total, 12 pigs were included in this study; 6 pigs were followed for six weeks (mean 41 days, range 38-43 days) and the other 6 for twelve weeks (mean 84 days, range 80-87 days). Average weight was 59.5 kg at inclusion (range: 52-68 kg). Mean diameter of the CIA was 7.1mm (range: 6.4-7.8). In the six week follow-up group one pig died at the first postoperative day due to blood loss as a result of a perforation in the iliac arteries (both sides) as a complication of the procedure, which was caused by a relative oversizing (0.2mm) of the CB in relation to the arterial diameter. The complication was reported and, after approval, the animal was replaced according to protocol. Limited over- or under-sizing of the CB was unavoidable due to the available sizes of the CB, which comes at intervals of 1.0mm (e.g. 5.0 – 6.0 – 7.0 – 8.0mm), and which does not cover all vessel diameters (e.g. a balloon size 6.0 can be inflated to a diameter of 5.88 to 6.05).

Radiographic Evaluation

After six and twelve weeks follow-up, angiography showed no significant (>50%) stenosis of the iliac arteries. Mean diameter of the CIA's was 7.3 (range: 6.5-8.6). No thrombosis occurred in any of the arteries. Rollscan of the CIA's revealed no significant

stenosic lesions (Figure 2). Two arteries (16.7%) showed an increase in diameter of the artery on angiography greater than 0.5mm (0.7mm and 0.8mm), which could indicate expansive remodelling. All other post-treatment diameters were within 0.5mm range as compared to pre-treatment diameters.

FIGURE 2 Rollscan of the pig iliac arteries

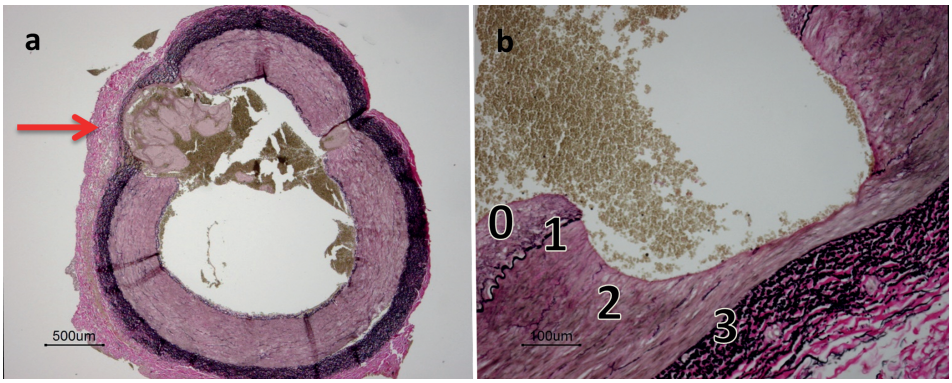


Anatomy of the pic iliac artery system on rollscan. a: aorta, b: common iliac artery (CIA), c: circumflex artery branch, d: bifurcation of external and internal iliac artery.

Histological Evaluation

Profound histological vascular damage was observed in most treated arteries. In total, 75% of the arteries reached the maximum score which includes damage to the EEL, 17% had profound media damage and 8% only IEL breaks (Table 1). None of the arteries was without damage. As the Cutting Balloon has 4 knifes on the balloon, a maximum amount of 4 damaged regions within an artery were possible (Figure 3). The maximum extent of damage to the vascular wall resulted in an increase in IH area (p=0.042) and I/M ratio (p=0.042) (Table 2 and Figure 4). The number of locations of vascular damage was closely correlated with the maximal extent of damage to the vascular wall and was associated with increased intimal area (p=0.046). No differences between six and twelve weeks follow-up in the extent of arterial wall damage (p=0.375) and IH (p=0.485) were observed.

FIGURE 3 Acute arterial damage



Assessment of common iliac artery directly post-injury. (a) Red arrow: damaged segment of CIA, initially filled with thrombus. (b) Scoring system of a CIA lesion. 0: intact segment of intimal layer, 1: broken IEL, 2: profound media damage, 3: intact EEL.

TABLE 1 Baseline characteristics Common Iliac arteries

Presented as mean ± SD	**Common Iliac arteries n = 12**
Diameter (mm)	3.6 (0.9)
Thrombosis, yes	0%
Injury score	2.7 (0.7)
Max Injury score per artery 0 1 2 3	n = 12 (%) 0 1 (8) 2 (17) 9 (75)
Lumen area (mm^2)	6.3 (4.0)
Intimal area (mm^2)	2.3 (1.9)
EEL: external elastic lamina, I/M ratio: intima/media ratio.	
Media area (mm^2)	5.0 (0.8)
I/M ratio	0.42 (0.3)
EEL area (mm^2)	13.7 (6.4)

EEL: external elastic lamina, I/M ratio: intima/media ratio.

Further histological assessment showed that the cut of the blades of the CB were initially filled up with thrombus (Figure 3). In time, these thrombi evolved to newly formed intima and media mostly consisting of a proliferation of smooth muscle actin positive myofibroblasts. In lesions with the maximum extent of damage, fibrotic thickening of the adjacent adventitial layer was observed (Figure 5). Due to the profound damage, it was difficult to make a correct distinction between neo-intima and media repair reaction in arteries where the EEL was totally cut. The lesion in the media was

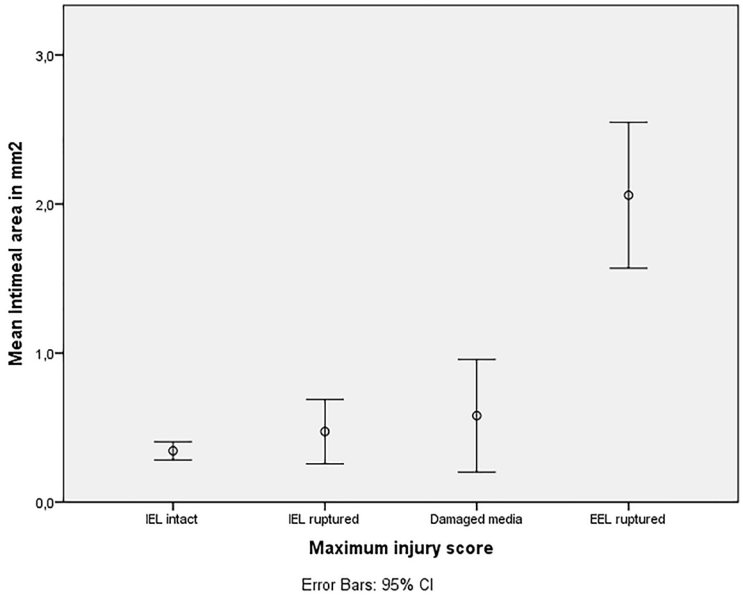
filled up with the proliferation of myofibroblasts, which is a repair reaction of the media, more than a new neointimal layer at the luminal side of the media. This phenomenon limits the possibility to carefully assess the intima-area and I/M ratio. ERG staining revealed complete endothelialisation of all lesions after six weeks of follow-up.

TABLE 2 Depth of arterial wall damage compared to wall layer areas

Mean ± SD	IEL ruptured	Damaged media	EEL ruptured
Lumen area (mm^2)	4.7	3.1 (3.5)	7.2 (4.1)
Intimal area (mm^2)	0.8	0.4 (0.1)	2.9 (1.8)
Media area (mm^2)	5.4	3.8 (0.7)	5.3 (0.6)
I/M ratio	0.16	0.10 (0.01)	0.53 (0.30)
EEL area (mm^2)	10.9	7.3 (4.3)	15.4 (6.3)

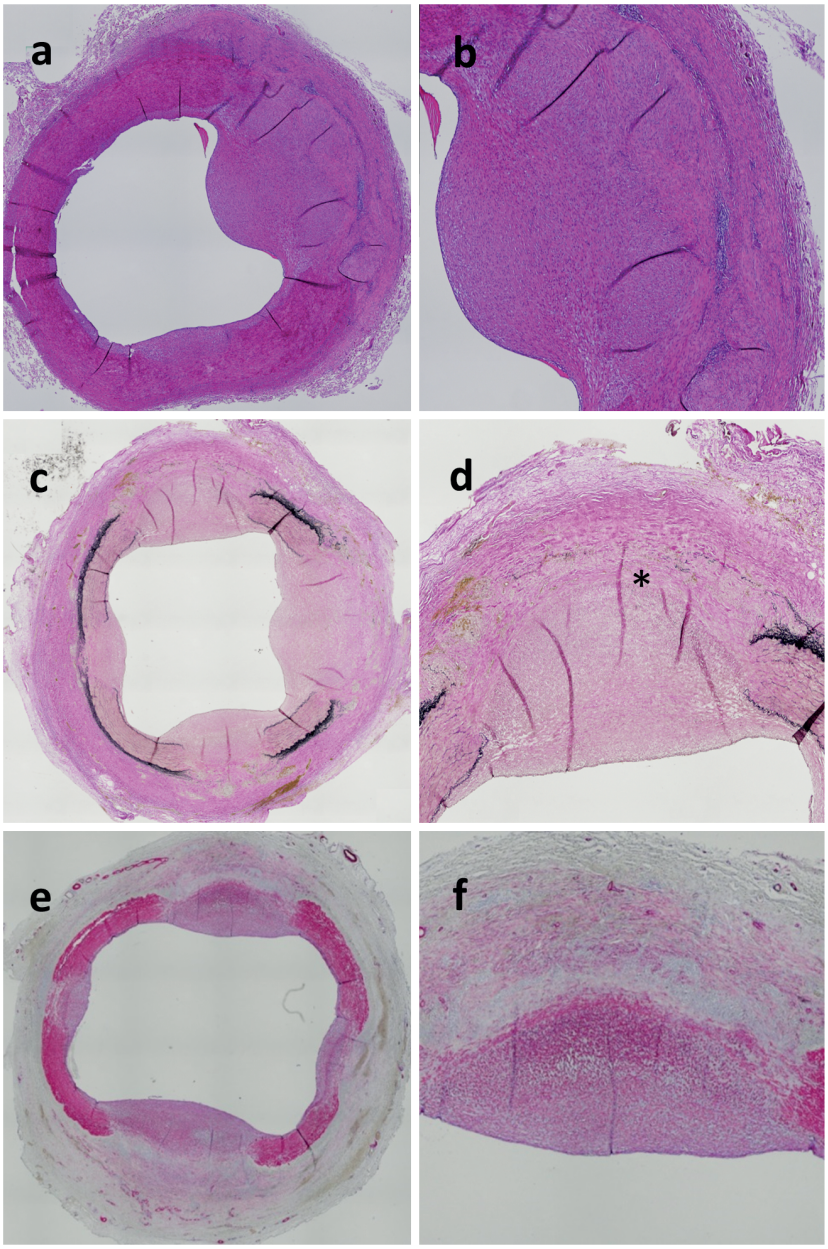
EEL: external elastic lamina, IEL: internal elastic lamina, I/M ratio: intima/media ratio.

FIGURE 4 Arterial damage vs intimal hyperplasia



Increased intimal area in mm^2 in artery segments with a higher vascular wall damage score.

FIGURE 5 Histological assessment



Histology of damaged common iliac arteries (CIA). (a,b) Hematoxylin and eosin stain showing a damaged artery with intimal hyperplastic lesion (right side). Close-up shows a cellular rich lesion with reactive surrounding adventitial tissue. (c,d) Elastic van Gieson stain showing a CIA damaged in 4 regions with complete external elastic layer laceration in 3 regions. Close up shows a defect in the media that is filled up leading to widening of the artery. The asterisk (*) indicates the boundary between media repair and intimal hyperplasia. (e,f) alpha Smooth Muscle Actin staining of a CIA with on close-up a proliferation of myofibroblasts in the gap of the media in continuum with a neo-intimal layer.

DISCUSSION

In this animal study, we sought to validate a proposed pig model for IH. We found it is a predictable model for IH, particularly when a more extensive amount of vascular wall damage is reached. However, due to the difficulty of sizing the CB and due to the extent of vascular wall damage we raise the question whether this model is practicable to study novel (endo-)vascular techniques.

The use of non-, semi- or compliant-balloons to produce endothelial damage and IH have reported mixed results for the extent of damage within the study animal population and current used animal model has not been validated yet[10,11,13,15]. IEL breaks are identified as necessary to create a sufficient amount of intimal hyperplasia. In addition, clinical research indicates that media and adventitia damage predicts restenosis after endarterectomy in PAD and that the extent of arterial wall damage with the use of the CB reflects the depth of wall damage after endarterectomy[18]. This is a clear basis for the use of the current model. In our study, major arterial wall damage was observed with a subsequent (adequate) healing process of the artery, which resulted in a significant amount of IH, intimal area and I/M ratio. However, the damage also caused a fibrotic reaction of the adventitia and in some cases dilatation of the artery on angiography. The distinction between (neo-)intima and (neo-)media and adventitia becomes hard to observe in some of the explanted arteries, because media repair and IH are a continuum.

In addition, the media of a healthy (pig) iliac artery is approximately 0.3mm thick, thus the margins are very slim. The CB only comes in 1.0 mm maximum diameter step sizes (5.0 – 6.0 – 7.0 – 8.0), resulting in a gap where you have to decide whether to oversize or undersize the balloon instead of 1:1 with the diameter of the artery. In a clinical setting, the CB is only inflated in the artery creating damage only over the length of the balloon, and not pulled-back over a longer arterial segment as in the current protocol. According to the instructions for use, the use of the CB is reported to be safe – if oversizing is avoided[19].

Over the course of the artery (proximally to distally), the inflicted damage varies – typically from no damage to maximum damage back to no damage to the artery wall. Similarly, the areas with IH vary throughout the artery segment. As a consequence, it is necessary to measure vascular damage and intimal hyperplasia at sufficient sites in of the explanted artery. Although, it must be noted that the trajectory damaged (from the iliac bifurcation, proximally to the circumflex branch of the common iliac (Figure 1)), was approximately 3 to 4 cm long, and longer than the 1.5 cm damaged by Houballah et al[15]. It was observed that in some cases the Cutting Balloon tended to "dig" itself into the artery wall when pulling it back retrogradely through the artery, which could have increased the amount of vascular damage on one side of the artery. The use of the CB and TB has the clear advantage over other pig models that it is successful in creating IH in most cases, without leaving a stent in the artery[13,20–22], which makes assessment more difficult and could interfere in further measurements. It is an

easy and reproducible model, and the IH is formed within a short amount of time[15]. But, as stated earlier, we think this model has some limitations. First of all, the sizing of the Cutting balloon proves to be difficult in some occasions due to the intervals of available sizes of the CB. In addition, standardised inflation of the TB was impossible and allows for variations between procedures. Second, the amount of damage made is such that, although it correlates with damage after an endarterectomy[18], in some arteries correct measurement of intimal area and hyperplasia becomes difficult. The use of a non-compliant balloon overstretch model does not have these limitations, although this model is not as successful in inducing IH as the current used model, and literature is not unambiguous as to the extent of oversizing, duration of oversizing and the number of times to repeat oversizing[11,14]. Lastly, in the current study the arteries were not pressure fixed after explantation, as the arteries were used for another experiment in which explantation of the artery occurred without pressure. In our opinion it does not change the findings of our study, as the sizing of the CB was not affected. It could explain the lower diameter of the arteries after explantation, but angiography showed no narrowing of the lumen of the arteries before explantation. The areas of the intima, media and adventitia will have decreased as a result of a lack of pressure, but we believe that was not the cause of the difficulty to distinguish between neo-intima and media.

CONCLUSION

Current endovascular technique is an effective model to induce IH in pigs within six weeks. However, due to limited available sizes of the CB and no standardisation to inflate the TB, precise dosing of the extent of vascular wall damage is difficult.

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CHAPTER 5

Synthetic Intimal Coating for prevention of intimal hyperplasia after vascular damage – a novel concept

Manuscript in preparation

ABSTRACT

Background
Intimal hyperplasia (IH) limits the patency after (endo)vascular intervention. Post-revascularization sealing may limit the development of IH. Within a European Consortium, an absorbable Synthetic Intimal Coating (SIC) consisting of recombinant elastin-like polymers and chimeric protein was developed for arterial wall adhesion, with the aim to form a new intimal layer after atherectomy to prevent IH.

Aim
To investigate if the SIC is non-toxic; feasible to applicate, absorbs in the arterial wall after implantation. Our primary aim was to learn if SIC was effective to prevent the occurrence of IH after vascular damage in a minimally invasive animal model.

Method
In 16 pigs, the intima in both common iliac arteries was damaged with the use of a Cutting Balloon (CB)(Boston Scientific) and a Fogarty Balloon (FB)(Edwards Lifesciences). On one side, pre-operatively randomly decided, the SIC was applied with the use of a balloon catheter (CONIC Vascular). After a small feasibility study with immediate explantation (n=4), and a pilot study with a follow-up of respectively six (n=6) and twelve weeks (n=6), all arteries underwent for blinded histological examination to the extent of vascular damage, IH and intima/media (I/M) ratio.

Results
Post-operatively, one pig died due to bleeding resulting from a perforation; this animal was replaced. The extent of vascular damage was equally divided between SIC and non-SIC treated arteries, and was associated with increased IH area and I/M ratio ($p=0.004$). At six and twelve weeks follow-up, the I/M ratio was not-significantly different between arteries with and without SIC placement ($p=0.713$). The SIC was non-toxic. The presence of SIC deposition was confirmed by immunohistochemistry, however SIC was not observed throughout the entire circumference of the artery.

Conclusion
In this pig arterial damage model, only minimal circumferential placement of the SIC on the artery wall was established. The current application of the SIC did not prevent formation of IH. Further studies are needed following improved SIC placement and absorption in the arterial wall.

INTRODUCTION

Treatment for peripheral arterial disease (PAD) yield unsatisfactory long term results with 1-year restenosis rates of up to 30%, often resulting from intimal hyperplasia (IH) or negative remodelling[1–5]. New endovascular treatment modalities and devices are first being tested in animal models; of which the pig model is often the model of choice because of the similarities in anatomy and arterial healing process[6]. Current new developments include drug eluting techniques and bioresorbable stents to treat PAD[7]. Although no medium to long term improvement in clinical endpoints such as: amputation, Rutherford class, quality of life or walking ability has yet been established[8–11].
As such, the search towards an effective method to restenosis after (endo)vascular treatment in PAD patients remains valuable. In this pilot animal study, we present the hypothesis, achievements and future in a minimally invasive vascular damage model to induce IH in pig iliac arteries. The aim was to study whether the SIC was effective to prevent the occurrence of IH in this animal model at six and twelve weeks follow-up. In addition, toxicity, feasibility of application and the absorption of the SIC over time was assessed.

METHODS

Ethical statement

The study protocol was approved by the partners of the European consortium, in line with The Grail study protocols, and the local ethical commission on animal testing (DEC), which consists of an expert panel on animal research and welfare, under protocol number: 2014.II.12.103.

The hypothesis and fabrication of the Synthetic Intimal Coating (SIC)

Within a European Consortium, an absorbable Synthetic Intimal Coating (SIC) was developed, consisting of recombinant elastin-like polymers and silk as a chimeric protein. The SIC is a non-covalent non-reversible protein gel composed of recombinant elastin-like polymer harbouring the fibroin protein gene. The ELASTIN-SILK gel of the SICAR scaffold combines two different but important properties, elasticity and stiffness, as a result of the combination of two different naturally occurring proteins, namely human elastin and silk derived from the Bombyx mori Silkworm. This chimeric protein has been achieved by genetic engineering and recombinant expression, the end product obtaining a 99% pure product suitable for biomedical applications. Its solution in a water-based solvent leads to the formation of stable hydrogels that can be used in different tissue engineering approaches, such as arterial regeneration.
The hypothesis was that the SIC, based on the selected ELASTIN-SILK gel offers the optimal properties to use as an endovascular applicable internal coating of an artery after endarterectomy or PTA, since it possesses the elasticity of elastin in combination

with the stiffness and stability of silk. In addition, the SIC supports cell attachment as a result of the presence of cell adhesion sequences. This increases biocompatibility with the host and the cellularisation of the SIC, which allows the SIC to act as a tissue-like intimal layer while it is being bio-absorbed and replaced by a new intima. The result of these processes would be to limit the formation of intimal hyperplasia during the healing of the artery wall.
The chimeric protein is produced as recombinant protein in E.coli host and purified by temperature reverse transition and purified by ultrafiltration to remove endotoxins.

SIC delivery on the balloon surface
The SIC-gel was delivered onto the inflated balloon surface with the use of a dipping technique inside a manufactured mould. The balloon is subsequently dried and deflated before removal of the mould and insertion into the protective tube. With the use of blue dye mixed with the gel clear gel coverage of the entire balloon service was observed in-vitro.

Ex vivo results
Ex-vivo, in pig iliac and carotid arteries with blood stasis, circumferential placement of the SIC against a healthy artery wall was proven on histological slides (Figure 2).

In vivo results
In-vivo, with the blocking of blood-flow in pig iliac arteries in a hybrid procedure via open approach, endovascular circumferential placement of the SIC was proven. In addition, in a flow-metric device, adhesion properties of the SIC were established.

Study design
In total, 16 female land/Yorkshire pigs from a local qualified supplier (van Beek; Lelystad) were obtained. The weight of the pigs was chosen at 60kg, to maximize the possibility of the common iliac artery (CIA) to be approximately 7mm[12]. Ten days for the start of the experiments, dual anti-platelet aggregation therapy was started, consisting of a daily dose of Ascal (Carbasalate Calcium 80mg) and Clopidogrel (75mg). The 16 pigs were divided into 3 different groups. Group 1 consisted of four pigs in an acute setting with no follow-up (explantation after 30-45 minutes) to test the feasibility of the protocol and the delivery of the SIC. Arterial damage was inflicted in both CIAs and the SIC was also placed in both CIAs. Group 2 consisted of 6 pigs with six weeks follow-up and group 3 consisted of 6 pigs with twelve weeks follow-up. Both group 2 and group 3 received endovascular arterial damage in both CIAs, but were treated with SIC only on one side (which was pre-operatively determined).

Experimental procedures: Arterial damage
The Cutting Balloon (Boston Scientific, Marlborough, Massachusetts, United States) and a Thrombectomy balloon (Fogarty Balloon, Edwards Lifesciences, Irvine, California,

United States) as a minimal invasive vascular injury animal model was used[13]. Via open approach an introducer was placed in the carotid artery, through which a guiding catheter was placed in the distal abdominal artery and the CIA diameters were measured. The CB was used to damage both CIAs (1:1 sizing) from the iliac bifurcation towards the branch of the circumflex artery by slow pull-back of the CB. Subsequently, bilateral pull-back of an inflated FB, with clear flattening of the balloon on angiography, was performed over the same trajectory in the CIAs. Angiography was used to assess for perforation or dissection after each step.

Experimental procedures: SIC placement

After arterial damage was inflicted, the SIC was placed in an unilateral CIA, which was determined before the procedure commenced (right or left) and could not be changed during procedure. The SIC was delivered over the wire with the use of custom made endovascular balloon catheters (CONIC Vascular, Lugano, Switzerland). Inflation of the balloon was standardized at 60 seconds after correct positioning was confirmed on angiography.

Imaging assessment

Before explantation of the arteries, an angiography and rollscan were made with the use of a fixed C-arm (Philips, Amsterdam, The Netherlands). XCelera (Philips) was used to analyse these images (Figure 1).

Histological assessment

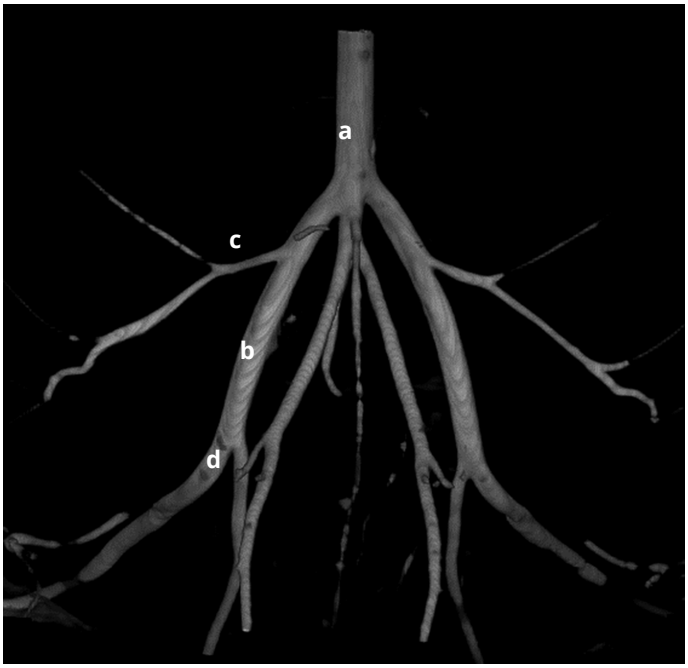
The explanted arteries were fixated in Formalin 4% for a minimum of 48 hours and maximum of 5 days. The CIAs were cut at 5mm interval and embedded in paraffin, after which cross sections were made at 4 micrometer per slide. Hematoxylin & Eosin (H&E) staining was used for general inspection of the artery. An Elastin van Gieson (EvG) stain was used to visualize the IEL and EEL and to determine the I/M ratio. An alpha Smooth Muscle Actin (αSMA) immunostain was performed to stain smooth muscle cells and myofibroblasts in the media and IH. Endothelial cells of the intimal layer were visualized using an ETS related gene (ERG) immunostain. The SIC was labelled with a rabbit GFP antibody, which was visualized with the use of anti-GFP Antibody (B-2) (Santa Cruz Biotechnology). All slides were digitalized with the used of the Aperio scanner (Aperio, Leica Biosystems, Germany), at 20x magnification. Blinded histological analysis and measurements were performed with the use of ImageScope (Aperio).

Artery injury score

Arterial wall damage was assessed with the use of the Artery Injury Score (AIS), based on a previously published scoring list[14]:

0: Internal elastic lamina (IEL), media and external elastic lamina (EEL) intact
1: IEL ruptured, media and EEL intact
2: IEL ruptured, profound media damage, EEL intact
3: IEL, media and EEL ruptured

FIGURE 1 Rollscan of pig iliac vascular anatomy

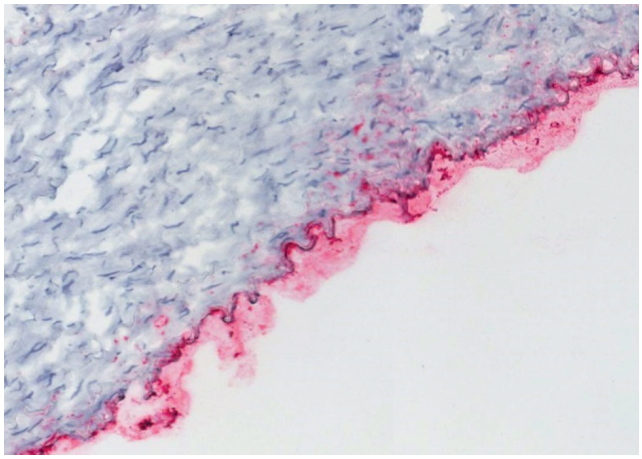


Rollscan of the pic iliac artery system. a: Aorta, b: Common iliac artery (CIA), c: Circumflex artery branch, d: Bifurcation of external and internal iliac artery.

Statistical analysis

The statistical analyses were performed on the pigs with follow-up (24 CIAs in total). The four animals treated in the feasibility study are described separately. Data is shown as median and range or mean standard deviation of the mean. For the final analysis the segment of the artery with the greatest extent of AIS was used. When multiple slides of the same artery had a similar AIS, the slide with the greatest extent of IH was chosen for further analysis. Chi-square tests were used to compare categorical variables and Mann-Whitney U tests to compare continuous variables. A value of $p < 0.05$ was considered statistically significant. SPSS version 22.0 was used for statistical analyses.

FIGURE 2 Ex-vivo SIC placement



Close-up of ex vivo SIC placement on the carotid arterial wall with anti-GFP stain.

RESULTS

Feasibility study

In total, sixteen pigs were included in this study, of which four pigs were included in a feasibility study, with explantation and evaluation after 30-45 minutes. Average weight was 59.5 kg at inclusion. Mean diameter of the CIAs before treatment was similar in both groups at 7.1mm on angiography (range: 6.4-7.8). One pig in the six week follow-up group died due to blood loss as a result of a perforation in the iliac arteries (both sides) as a complication of the procedure. The complication was reported and, after approval, the animal was replaced.

During the acute procedures (n=4), it was noted that the delivery of the SIC on the balloon had to be protected from the blood-flow until it reached the designated area. After adjustments to the CONIC Vascular balloon catheter, adding a protective tube, application of the SIC damaged segments of the arterial wall was proven, but not circumferential application of the SIC to the arterial wall (Figure 3).

SIC vs no-SIC pilot study

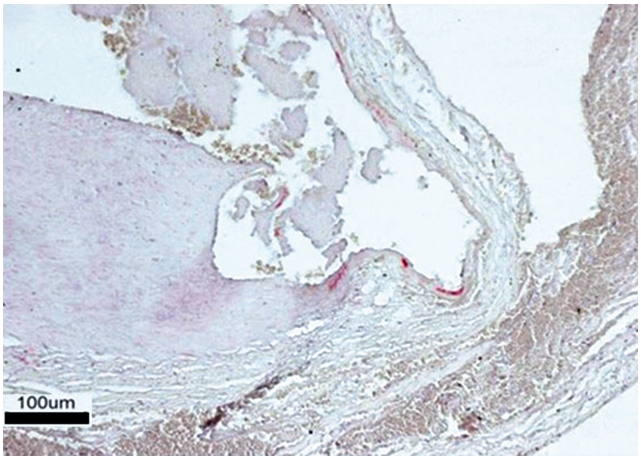
In the follow-up experiments (n=12, with in total 24 CIAs), profound vascular damage was observed in most treated arteries. None of the arteries showed no damage. The amount of damage was similar in both SIC and no-SIC arteries (p=0.744) and was associated with increased IH area and I/M ratio (Table 1). Histological assessment showed no difference in IH area and I/M ratio after six and twelve weeks follow-up in both SIC and no-SIC treated arteries; 2.3mm^2 vs 3.2mm^2 (p=0.590) and 0.42 vs 0.62; (p=0.713), respectively. Complete endothelialisation was observed after six weeks on ERG staining. The lesions itself consisted mostly of media repair and newly formed IH,

consisting of myofibroblasts. The SIC was no longer visible on anti-GFP staining at six weeks follow-up. After six and twelve weeks follow-up, angiography showed 1 significant (>50%) stenosis of an iliac artery in the SIC treated group, no other significant stenosis were observed.

Toxicity assessment

Tissue obtained from different organs (heart, lung, thymus, liver, kidney, adrenal gland, and ovary) and blood samples of all pigs with follow-up was assessed for toxicological alterations. None of the samples showed any damage or tissue alterations.

FIGURE 3 Placement of SIC in acute experiment



Anti-GFP antibody stain of in vivo endovascular SIC placement, close-up of a damaged arterial wall segment. Explantation within 1 hour after procedure. (*) Indicates parts of SIC on the damaged wall segments. (A) Indicates adventitial layer, (M) indicates medial layer which is completely interrupted by the blade of the Cutting Balloon, and (L) indicates the lumen of the artery.

DISCUSSION

In this animal pilot study, a Synthetic Intimal Coating to inhibit the formation of IH in a minimally invasive vascular damage model in pig iliac arteries was investigated. The used animal model allowed for profound vascular damage and produced a significant IH reaction in the healing process. Application of the SIC to the arterial wall in vivo was proven, but were unable to establish circumferential placement. The SIC was absorbed within the study timeframe, and was deemed non-toxic with the use of organ-tissue and blood evaluation. However, no benefit of the SIC in its current form and current application to the arterial wall was observed to reduce IH at six and twelve weeks.
Ex-vivo, in pig iliac arteries with blood stasis, circumferential placement of the SIC was

TABLE 1 Characteristics of SIC vs no-SIC arteries after follow-up

Mean (± SD)	**No SIC arteries**	**SIC arteries**	**p value**
N	**12**	**12**	
Diameter (mm)	3.6 (0.9)	3.7 (1.4)	0.713
Thrombosis, yes	0	0	-
Injury score	2.7 (0.7)	2.8 (0.6)	0.744
Max Injury score per artery	(%)	(%)	
0	0	0	
1	8	8	
2	17	8	
3	75	83	
Lumen area (mm^2)	6.3 (4.0)	8.6 (8.7)	0.478
Intimal area (mm^2)	2.3 (1.9)	3.2 (3.1)	0.590
Intimal thickness in mm, greatest extent	0.7 (0.4)	0.9 (0.8)	0.755
Media area (mm^2)	5.0 (0.8)	5.6 (1.2)	0.219
EEL area (mm^2)	13.6 (6.4)	17.5 (9.5)	0.266
I/M ratio	0.42 (0.31)	0.62 (0.66)	0.713

I/M ratio = intimal/media ratio

proven. In-vivo, with the blocking of blood-flow in pig iliac arteries in a hybrid procedure via open approach, endovascular circumferential placement of the SIC was proven. In addition, in a flow-metric device, adhesion of the SIC has been established. Unfortunately, in-vivo with an endovascular approach only, without the (external or internal) blocking of blood flow, we were unable to proof circumferential placement of the SIC on the artery wall. Bits of the SIC-gel were observed particularly on damaged segments of the arteries in the feasibility study. Therefore, the hypothesis was that this would be enough to reduce the formation of IH in these arteries. The main reason we did not observe a reduction in intimal hyperplasia could stem from 3 causes: 1) circumferential placement of the SIC is necessary for it to work, 2) the animal model created too much wall damage for the SIC to work, or 3) the SIC does not work. Our current study, unfortunately, does not point to one of these causes.

For future experiments, to adequately investigate the working mechanism and effectivity of the SIC, some factors should be established. First of all, circumferential adhesion of the SIC to the artery wall should be established in an endovascular procedure, preferably without blocking the blood flow proximal to the placement of the SIC. If blocking of the blood flow is deemed necessary, a blocking balloon could be placed proximally to the damaged segment of the artery, prior to inflation of the balloon with the SIC. Second, it is questionable whether current used animal model is a correct model to assess the extent of IH. Due to the large extent of vascular wall damage, correct distinction of neo-intimal area and media repair becomes difficult. Although, the amount of damage does correlate with the extent of wall damage after

an endarterectomy[15], it is probably too extensive to answer current research questions. In addition, the CB comes in limited sized and inflation of the FB is not standardisable. To be able to gain more control over arterial wall damage another method, such as balloon over distention, could be used at the cost of somewhat lower security of gaining IH. Third, the follow-up time can be limited to direct, two- and six weeks post-procedure, as complete endothelialisation in all arteries after six weeks was observed. In addition, no traces of the SIC could be found in the arteries after follow-up.

CONCLUSION

In this pig arterial damage model, only minimal circumferential placement of the SIC on the artery wall was established. The current application of SIC did not prevent formation of IH. Further studies are needed following improved SIC placement and absorption in the arterial wall.

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CHAPTER 6

Macroscopic and histologic analysis of vessel wall reaction after mechanochemical endovenous ablation using the ClariVein OC device in an animal model

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ABSTRACT

Introduction

Mechanochemical endovenous ablation (MOCA) has been developed as a tumescentless technique to ablate saphenous veins and to avoid heat-induced complications, and postprocedural pain. The mechanism of action of MOCA is poorly understood. Our experiments were conducted to determine the effect of MOCA on vein wall injury and sclerosis in an animal model.

Methods

A total of 36 lateral saphenous veins (LSVs) were treated in 18 goats according to human protocol. Veins from 9 goats were evaluated 45 minutes after the procedure while in the remaining 9 goats, the treated veins were evaluated 6 weeks later. All treated veins were divided equally over 3 treatment groups: MOCA, mechanical ablation without the sclerosant, and liquid sclerotherapy alone. The histologic effects of treatment on the vein wall were systematically evaluated.

Results

The average diameter of the LSV was 4.0 ± 0.5 mm. Technical success was achieved in all but 1 LSV (35/36, 97.2%), with a median procedure time of 14 minutes (range, 9-22 minutes). In the acute group, histologic examination showed that mechanical ablation (alone or MOCA) induced severe injury to the endothelium in 82% but no damage to other layers of the vein wall. Mechanical ablation led to vasoconstriction. After 6 weeks of follow-up, 4 of 6 MOCA-treated veins were occluded. The occluded segments consisted mainly of fibrotic lesions probably evolved from organized thrombus. No occlusions were observed after sclerotherapy or mechanical treatment alone. No major complications occurred during procedures or follow-up.

Conclusion

MOCA has a significantly higher occlusion rate than its separated components. However, MOCA resulted in occlusion in only two-thirds of the animals. This study underlines the hypothesis that additive use of MOCA increases the effectiveness of liquid polidocanol , delivered by the ClariVein OC, by inducing endothelial damage and probably vasoconstriction.

INTRODUCTION

Lower limb chronic venous insufficiency (CVI) is a common diagnosis with a prevalence of up to 21% in the adult population associated with physical impairment and decreased general and disease-specific quality of life[1–3]. Without treatment venous pathology has a tendency to progress over time[4].
For more than a century, surgical high ligation, with or without stripping or compression therapy, was the only available treatment option of superficial venous insufficiency. The introduction of minimally invasive ablation techniques in recent decades has revolutionized the treatment of varicose veins. As a result, endovenous laser or radiofrequency ablation became the new standard of care due to excellent occlusion rates in both the great and small saphenous vein (GSV/SSV)[5,6]. However, the need for tumescent anesthesia, the risk of heat-induced nerve injury, especially in the SSV and below-the-knee GSV, and postprocedural pain are considered disadvantages to both of these endothermal techniques. To eliminate these cons, a growing attention for nonthermal techniques has developed in recent years.
Mechanochemical endovenous ablation (MOCA) is a nonthermal technique that combines endovenous mechanical injury to the vein wall with simultaneous infusion of a liquid sclerosant. Even though MOCA has proven to be safe for the treatment of GSV and SSV insufficiency, with increasing data available on long-term results, the precise working mechanism and effect on the vein wall remain unknown. To date, experimental histologic studies on MOCA are sparse[7–9]. The goal of this study was to elucidate the mechanism of action of MOCA by analyzing its separate components' effect on the vessel wall histology in 18 goats.

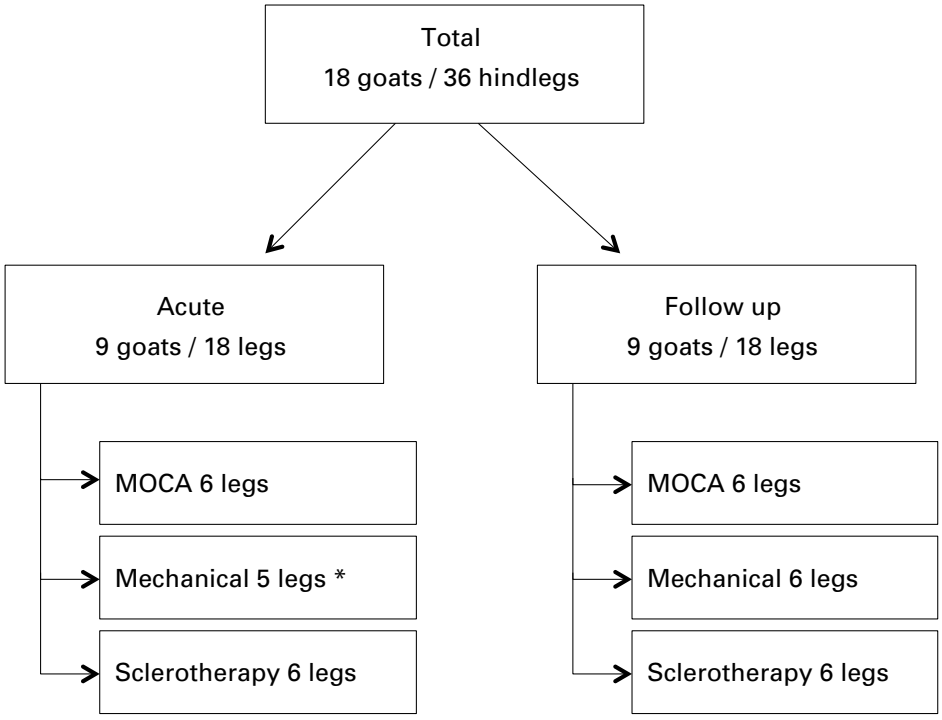
METHODS

Study design

The study included 18 female dairy goats. Half of the goats (9 goats/18 veins) were enrolled into an acute experiment to assess direct effects of the MOCA treatment and its separate components. The remaining 9 animals (18 veins) were treated within the 6-week follow-up protocol. The following experimental groups (6 treated veins each) were formed:

1. Acute experiment: mechanochemical ablation (ClariVein + 2% Aethoxysklerol)
2. Acute experiment: liquid sclerotherapy (2% Aethoxysklerol)
3. Acute experiment: mechanoablation (ClariVein without Aethoxysklerol)
4. Follow-up experiment: mechanochemical ablation (ClariVein + 2% Aethoxysklerol)
5. Follow-up experiment: liquid sclerotherapy (2% Aethoxysklerol)
6. Follow-up experiment: mechanoablation (ClariVein without Aethoxysklerol)

The experiments were approved by the Committee on Animal Experiments (DEC), Utrecht, The Netherlands (Protocol No.: 2012.II.12.185). All procedures were performed by an endovascular specialist with extensive experience with MOCA (over 50 procedures) and were conducted in accordance with good laboratory practice and international guidelines in animal research, under guidance of licensed biotechnicians at the animal laboratory of the Experimental Cardiology Department, University Medical Centre Utrecht, The Netherlands.



* No successful cannulation in 1 vein due to small caliber vein

Animals

After a pilot study was conducted to prove the feasibility of the study protocol, we included a total of 18 female dairy goats that were allocated to the different experimental groups. The sample size of the study groups is in line with previous international publications in this field[7,10]. The fully grown animals were obtained from a local qualified supplier. All animals were given normal chow without supplements, and water was freely available. The animals were housed in pairs, except for preprocedure and postprocedure days, when isolation was maintained to protect the animals.

Experimental Procedures

All animals were treated under general anaesthesia and oxygenated with a mechanical respirator. The animal was placed lateral supine on the operating table. The hind legs were shaved, the skin was disinfected with iodine, and sterile draping was applied. A small transverse skin incision at the level of the ankle was made to visualize the distal lateral saphenous vein (LSV) and to place a 4F sheath (Figure 1). The LSV of the goat was chosen because of similarity to the human saphenous vein regarding diameter and length. The 2.67F ClariVein occlusion catheter (OC) [Vascular Insights LLC, Madison, CT, USA] was introduced via a 5F introducer sheath, and the tip was positioned at approximately 25 cm from insertion into the vein.

The goats were equally divided into the 6 groups, as stated above, and once the allocation was determined, changing the procedure was no longer possible. In groups 1 and 4, the treatment was in line with current treatment in humans[11]. In short, the ClariVein OC was used at maximum rotations per minute (3500 rpm), and after activation for 7 seconds proximally without infusion or withdrawal, the device was pulled back 1 cm every 7 seconds. Aethoxysklerol 2% (Kreussler Pharma, Wiesbaden, Germany) was administered simultaneously through the ClariVein OC. The dosage and infusion rate were chosen according to the dosing table made available by the manufacturer.

In groups 2 and 5, the treatment consisted of solely liquid sclerotherapy. The sclerosant was delivered over 25 cm using a ClariVein catheter, without activation of the motor, with a similar dosage and infusion rate as in MOCA. In the remaining groups 3 and 6, the treatment consisted of the mechanically induced damage without additional sclerosant.

Venous explantation

In the acute experiments, a pressure bandage was applied at the puncture site to control bleeding. The treated LSV was harvested between 30 and 45 minutes after the procedure was finished. The veins were surgically exposed over the total length of treatment. The exposed vein was studied macroscopically for occlusion or any complication (perforation, rupture, or vein wall hematoma). Proximally and distally the vein was ligated with Vicryl 3-0 (Johnson & Johnson, New Brunswick, NJ, USA) suture. Long ends of the suture were used for identification of the proximal end. Large side branches were ligated with a similar suture or with a titanium clip. The veins were fixed in formaldehyde solution 4% for 48 hours before further histologic processing. After the veins were harvested, the animals in the acute experiment were killed directly with a lethal overdose of potassium. In goats randomized to the follow-up experiments, the puncture site was closed with a Prolene 6-0 (Johnson & Johnson) suture. The skin was closed with Monocryl 3-0 (Johnson & Johnson). After monitored recovery, the animals were placed in group housing for 6 weeks. All follow-up animals were administered Augmentin (10 mg/kg intravenous; GlaxoSmithKline, London, UK) before treatment and Depomycine (1 mL/kg intramuscular; Intervet, Boxmeer, The Netherlands) at the end of the procedure. No anticoagulants or platelet aggregation inhibitors were administered.

At 6 weeks of follow-up, general anesthesia was initiated similar to the first procedure. The hind legs were studied for ecchymosis, wound infection, and discolouration. After inspection, the treated veins were explanted, as described above, studied macroscopically, and stored in 4% formalin. Thereafter, the animals were killed with intravenous potassium.

Experimental outcomes

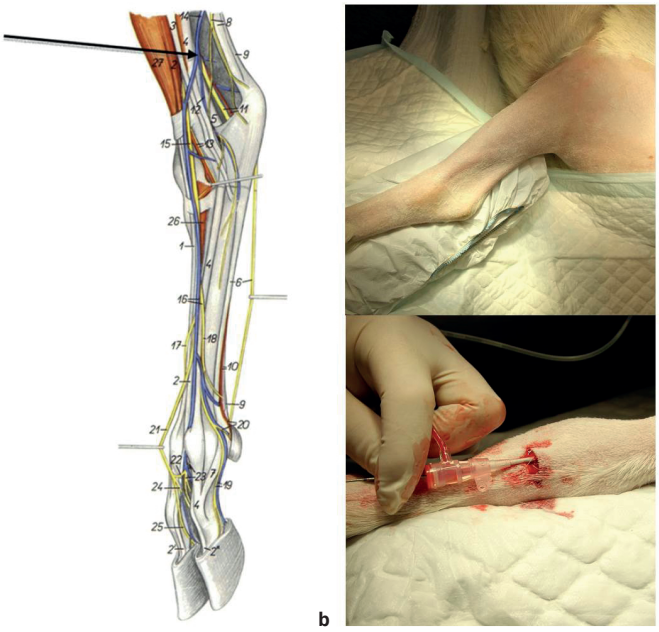
The acute group was designed to assess the aspect and severity of the damage inflicted by the treatment. Macroscopically, the vein was inspected for perforations and surrounding hematoma. Microscopically, the degree of intimal damage and injury to other layers of the vein wall was evaluated. The vein wall thickness was measured to quantify vasoconstriction.

In the 6-week follow-up group, the veins were macroscopically and microscopically studied to assess venous occlusion (anatomic success). Microscopically, the veins were further analysed to describe the histology and components of the occluded segments.

Histological analysis

The treated LSVs were fixed in formalin 4%. All veins were segmented into 5-mm pieces every 2 cm starting at 1 cm from the proximal end (1 cm, 3 cm, 5 cm, etc.) and processed

FIGURE 1 Goat hind leg anatomy



a b

(a) Anatomy of the hind leg of a goat, arrow: LSV. (b) level of puncture. (c) placement of sheath.

into standard paraffin blocks. Slides were made of 4-µm-thick sections and stained with hematoxylin and eosin for general observations and with Elastin van Gieson for microscopic evaluation and assessment of fibrosis. Alpha smooth muscle actin (α-SMA) immunostains were used to assess vein medial damage, and quantatively scored with the use of cellSens (Olympus Lifesciences, Tokyo, Japan) in all sections.
In the acute experiments, the intimal layer and the entire vein wall were assessed for damage. To visualize the endothelial cells, von Willebrand factor and ERG (ETS related gene) immunostains were performed. The percentage of the circumference with injured or absent endothelium was measured and categorized as mild (<10% damage), moderate (20% to 50%), and severe (>50%).[8] The section of each vein with the highest degree of injury was scored**,** in which the circumference of endothelium was measured and divided by the total circumference of the lumen of the vein. To score the injury, this percentage was deducted from 100%.To measure possible venous constriction, a ratio between diameter and vein wall thickness was calculated. The mean of the vein wall thickness measured on each quadrant of the vein was divided by the radius of the vein. Total occlusion or remaining lumen area and area of intimal hyperplasia and the aspect of the vein wall were assessed at 6 weeks. Additional α-smooth muscle actin immunostain and Perl's iron stain were performed to further study the components of intimal lesions in selected slides.

Statistical analysis
Final analysis was performed on the 9 acute goats (18 veins) and 9 goats with 6 weeks of follow-up (18 veins) separately. Mean data are presented with the ± standard deviation. We used median and range to present numeric data. SPSS 21.0 software (IBM Corp, Armonk, NY) was used for all analyses.

RESULTS

Procedure
The goats were an average weight of 59 ± 7 kg. The average diameter of the LSV at the level of introduction was 4.0 ± 0.5 mm. The median skin-to-skin treatment duration was 14 minutes (range, 9-22 minutes). Cannulating and treating the LSV according to plan was feasible in all but 1 hind leg (technical success rate, 97.2%). The unsuccessful procedure was in a vein with the smallest diameter of all (2 mm) and was planned for mechanoablation without sclerosant in an acute experiment (group 3). After reviewing this case, no replacement for the vein was deemed necessary. In all remaining acute experiments, harvesting the vein was feasible within the designated time of 30 to 45 minutes after treatment. No other perioperative problems or complications were noted. No complications were observed during the follow-up, especially no signs of deep venous thrombosis or wound infection.

A pilot study of 2 animals was first conducted to evaluate the feasibility of the study protocol. This pilot study revealed no technical difficulties and showed that MOCA treatment could be executed as planned. Because the initial protocol was feasible and no changes were made to surgical procedures, the samples of these 2 animals were included in the analysis of group 1.

Macroscopic evaluation

All treated veins in the acute experiments were surgically exposed. No hematoma or perforation of the vein wall was observed (Figure 2a). All veins were compressible and filled with blood. No thrombus or occlusion was observed. The diameter varied between and within the veins (range 1,5 to 9mm).

The wounds in all follow-up animals healed without signs of infection or local hematoma. Hyperpigmentation of the overlying skin was seen in the 3 hindlegs (1 after MOCA and 2 after liquid sclerotherapy), The most distinct cases were present in a leg treated with liquid sclerotherapy (Figure 2b). No ecchymosis over the treated trajectory was noted.

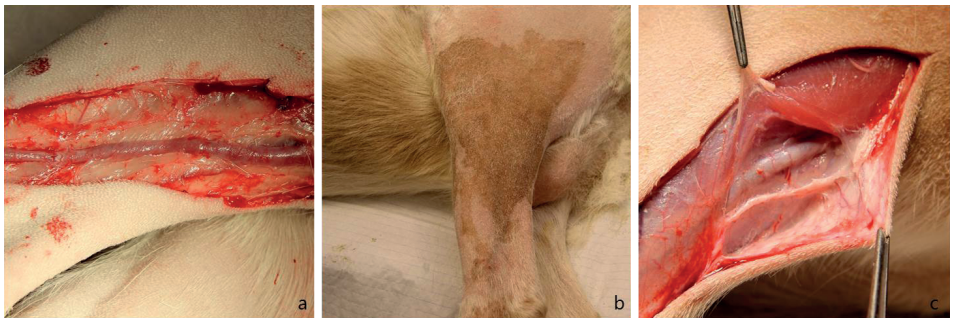
All veins treated with liquid sclerotherapy or mechanoablation (groups 5 and 6) were patent and compressible over the entire length. No macroscopic signs of perforation or total vein destruction were present. Of 6 veins treated with MOCA (group 4), 4 were macroscopically occluded, were fibroticly though, non-compressible, and no efflux of blood was seen (Figure 2c). Some of the smaller side branches of this part of the LSV were macroscopically occluded over the first millimeters. A major side branch was present in all animals a few centimeters above the puncture zone. Distal to this major side branch, all LSVs were patent. The remaining 2 veins treated with MOCA (group 4) showed no macroscopic changes. The veins were patent and compressible over the entire length.

Histological evaluation

In the acute experiments, no histologic evidence of damage beyond the endothelium layer of the vessel wall was found in any of the treatment groups. In 82% (9 of 11) of the veins treated with MOCA and mechanical action (groups 1 and 3), at least one segment showed severe endothelial injury compared to 17% (1 of 6) in the veins treated with sclerotherapy (group 2). The endothelial damage differed greatly within the veins: segments with (nearly) total endothelium abrasion and segments with totally intact endothelium were seen within single veins (Figure 3). Veins with intact venous valves were seen in all acute groups. Quantitative measurement of α-SMA staining in the media showed no (significant) differences between the treatment groups ($p = 0.654$). In the groups treated with mechanical ablation or MOCA, the vein wall thickness and the vein wall–to–vein radius ratio was increased compared with Aethoxysklerol, indicating venous constriction. The absolute measurements are reported in Table 1.

In the follow-up experiments, we only observed occlusion in veins treated with MOCA, of which total occlusion was observed in 4 of 6 veins (Table 2). The veins with total

FIGURE 2 Macroscopic evaluation



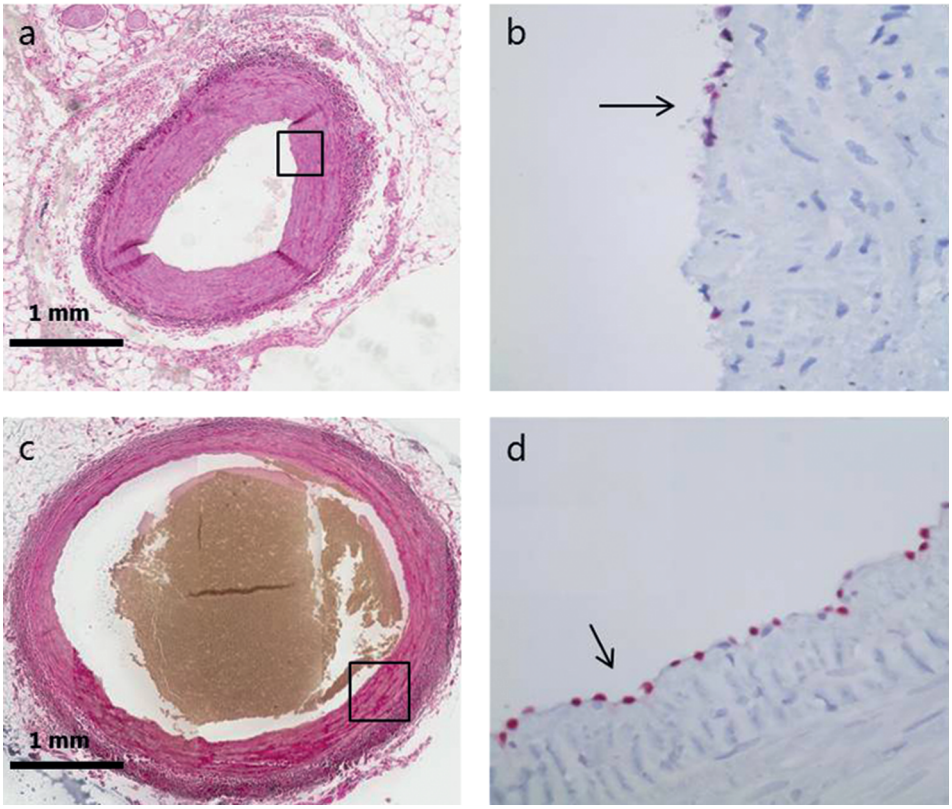
(a) Exposed LSV after acute experiment without hematoma or perforation. (b) hyperpigmentation after MOCA. (c) thickened, fibrotic LSV with full occlusion after MOCA.

occlusion showed a cellular fibrotic lesion with α-SMA–positive myofibroblasts and microvessels. The observed fibrosis extended in the medial and adventitial layers, but showed no signs of earlier perforation of the veinwall. Abundant iron pigment was observed within the lesions, suggesting that these have evolved from organized thrombus (Figure 4). One segment in 1 of these veins showed a 50% stenosis, showing intimal hyperplasia consisting of loose connective tissue with myofibroblasts, a few deposits of iron pigment, and the presence of neovascularization. The media layer of this section showed no fibrotic alterations. Elastica van Gieson staining showed an increase of fibrosis in the media/adventitia of totally occluded veins, which was not present in the nonoccluded veins. The 2 remaining MOCA-treated veins were open, with limited intimal hyperplasia of no more than 20% of the luminal area. Quantitative measurement showed a (significant) decrease in α-SMA positive area in the media of MOCA treated veins as compared to mechanical or aetoxysklerol treated veins ($p = 0.001$) (Figure 5). The open segments within the MOCA group showed higher percentages of α-SMA areas as compared to occluded segments in the MOCA group($p < 0.001$).

As also reported in the macroscopic evaluation, only the segments proximal to the large side branch were occluded. In line with the macroscopic data, no occlusions were seen in groups 5 and 6. In 1 of 6 veins treated with Aethoxysklerol, limited intimal thickening was noted of up to 20% of the vein lumen. The remaining 5 veins were fully open. The occlusion rate in veins treated with MOCA was higher than in the other 2 treatment groups ($p = 0.036$). We observed no histologic signs of damage or fibrosis to other layers of the vein walls in the nonoccluded veins, including the 2 nonoccluded veins treated with MOCA.

The ClariVein OC got stuck in 2 veins several times during treatment, and debris was tangled around the tip of the device. One vein was in group 4 (MOCA) and was totally occluded at 6 weeks of follow-up, the other vein was in group 6 (mechanical only) and was fully patent at 6 weeks, with no signs of vein wall damage on histologic examination.

FIGURE 3 Histology of acute experiment



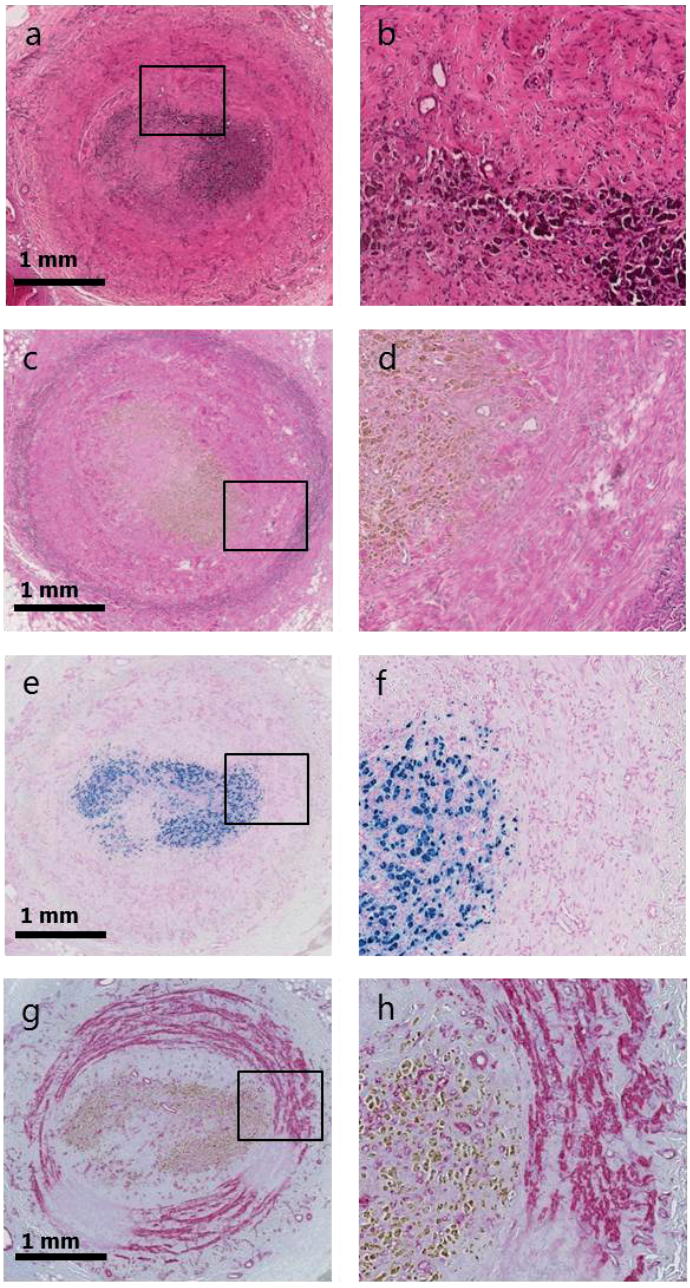
(a and b) mechanical treatment. (a) Venous constriction with reduction of the lumen. Elastica van Gieson stain. (b) Endothelial damage with loss of endothelial cells. Endothelial cells are stained red and indicated with arrow. ERG immunostain. (c and d) Aethoxysklerol treatment. (c) No venous constriction with preservation of the lumen. Elastica van Gieson stain. (d) No loss of endothelial cells. ERG immunostain.

TABLE 1 Histological aspects of veins in the acute experiments

Acute experiment	**MOCA (n = 6)**	**Mechanical (n = 5)**	**Sclerotherapy (n = 6)**
Maximal endothelial damage ERG*			
Minor / moderate (<50%)	2 (33.3%)	0 (0%)	5 (83.3%)
Severe (>50%)	4 (66.7%)	5 (100%)	1 (16.7%)
Wall thickness, µm	376 (127-426)	277 (206-383)	170 (85-379)
Vein diameter, mm	2.5 (1.8-4.9)	2.9 (1.4-4.0)	4.0 (1.8-7.7)
Wall-to-radius ratio	0.31 (0.05-0.44)	0.25 (0.10-0.40)	0.10 (0.03-0.42)

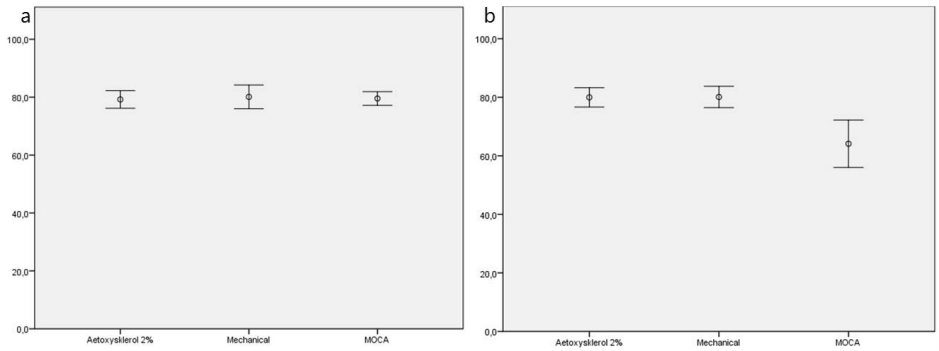
Categoric data are reported as number, and continuous data are reported as mean ± standard deviation or median (range).
*Segment with highest percentage of endothelial damage scored.

FIGURE 4 Histology at long-term follow-up



Histology of total occlusion after mechanochemical endovenous ablation: a and b) Hematoxylin and eosin stain showing cellular fibrotic lesion ingrowth of microvessels and presence of iron pigment. b and c) Elastica van Gieson stain confirming the presence connective tissue in the lesion (purple). d and e) Perl's iron stain shows abundant presence of iron pigment, suggesting an organized thrombus. g and h) α-Smooth muscle actin stain (red) shows the smooth muscle cells in the media and the myofibroblasts in the occlusive lesion.

FIGURE 5 Quantitative measurement of α-SMA positive area in the media



a) No difference between groups in acute experiment (groups 1-3). b) significant decrease in MOCA treated veins as compared to mechanical or aetoxysklerol treated veins (p = 0.001)

TABLE 2 Histological aspects of veins in the follow-up experiments

Follow-up 6 weeks	**MOCA (n = 6)**	**Mechanical (n = 6)**	**Aethoxysklerol 2% (n = 6)**
Aspect lumen			
Occlusion	4 (66.7%)	0 (0%)	0 (0%)
Open*	2 (33.3%)	6 (100%)	6 (100%)
Vessel diameter, mm	3.2 (1.7-4.8)	4.0 (2.0-5.5)	4.7; 3.3-6.0)
Lumen diameter, mm	1.1 (0-4.4)	3.6 (1.7-5.1)	4.5; 1.7-5.8)
Lumen area, mm^2	1.2 (0-11.9)	6.7 (2.3-15.2)	14.9; 0.8-18.6)
Intimal hyperplasia	5 (83.3)	0 (0)	2 (33.3)
Intimal hyperplasia area, mm^2	1.1 (0.0-5.9)	0.0 (0.0-0.1)	0.01 (0.0-0.7)

Categoric data are presented as number (%) and continuous variables as median (range). *Limited intimal hyperplasia up to 20% may be present.

DISCUSSION

The present study shows the effects of MOCA and its separate components in an acute and follow-up animal experiment. The experiments revealed that the mechanical action inflicts damage to the endothelium without signs of injury to the other layers of the vein wall. We observed a significant narrowing of the veins after MOCA or mechanical ablation compared with sclerotherapy. After 6 weeks of follow-up, the results of MOCA were superior to mechanical ablation only and sclerotherapy: we observed occlusion in 4 of 6 veins treated with MOCA compared with no occlusions in the veins treated with mechanical action or Aethoxysklerol separate.

The results of our study confirm the hypothesis that the ClariVein leads to venous occlusion by inflicting mechanical injury to the endothelial barrier, permiting the liquid sclerosans to induce an increased chemical reaction to the deeper layers of the vein

wall. In the acute experiment severe endothelium injury was seen in 82% (9 of 11) of veins treated with MOCA or solely mechanical action, significantly more than after sclerotherapy. In line with results of an earlier small ex vivo study[8], there was a large spread in degree of injury within a single vein. This is an interesting finding to discuss, because this could be the cause for occurrence of partial recanalization, which is relatively frequently seen in humans studies and is usually without clinical consequences[11,14,16]. Increasing endothelium injury might be the key in further optimizing treatment results. Decreasing the speed of pull-back of the ClariVein might lead to more injury by prolonged exposure to the mechanical action and, thus, potentially induce more vasoconstriction. Evaluation of techniques to increase endothelium damage and its effect on anatomic success could be relevant subjects for future studies.

Another important finding from this study is the observation that veins were significantly constricted after MOCA and solely mechanical treatment than after sclerotherapy alone. This might be induced by direct contact of the "stirring wire" to the vessel wall or by shear stress of whirling intraluminal fluid. This narrowing might be a contributor to the overall effect in MOCA by further increasing the effect of sclerosant on the vein wall. Vasoconstriction will result in the sclerosant reaching a higher concentration in the vein as a result of a decreased amount of intraluminal blood and, possibly, stasis. Furthermore, vasoconstriction will theoretically limit the washout of liquid sclerosant and thereby lead to prolonged exposure. As recently published, prolonging the exposure to liquid sclerosant leads to increased chemically induced injury to the vein wall[17].

In contrast to histological studies with endovenous laser ablation in which acute and total destruction of venous wall is seen[10], our results suggest that all occlusions seemed to originate from organized thrombus with fibrotic alterations. This might also give insight in the reason for recanalisation of initially MOCA occluded veins, especially when neovascularisation arises within these occlusive lesion. This phenomenon is seen in almost all clinical cohort studies published to date.

Compared with the anatomic success of 88.2% to 96.7% occlusion rates in human cohort studies[11–16], the occlusion rate in this animal study is less than expected. This leads to the discussion whether the current model is adequate to evaluated the working mechanism of MOCA. Even when the potential factors of influence on the anatomical success rate are evaluated the reason remains without clear explanation. The procedure was performed exactly as the standard procedure in clinical practice. To avoid bias due to experience, the procedures were performed by a dedicated team with vast experience in MOCA. Furthermore, the concentration and dosage of Aethoxysklerol was determined according to the human dosingtable.

Furthermore, the goat as the model to induce and study venous damage may be questioned. The main reason this animal model was chosen is that previous experiments in endothermal ablation[10] and MOCA[7] used goats and the size of the LSV was another major reason: with an average diameter of 4 ± 0.5 mm, the veins are

within the lower range of varicose veins included in humans studies[11]. In contrast to the current study the occlusion rate in the study form Tal *et al.* was 100%. There two important differences compared with that study: 1. sodium tetradecyl sulfate 1.5% was used instead of Aethoxysklerol (polidocanol) 2% and 2. compression stockings were applied. Although sodium tetradecyl sulfate (trademarks: Sotradecol or Fibrovein) has been shown to be a more potent sclerosant than polidocanol in a in-vitro experiment[18], no differences in anatomical success were observed in men.

Similar to our study, Tal et al. described no occlusion in the treatment with only mechanical ablation or sclerotherapy[7]. Finally, it is important to appreciate that the results from the discussed study described only selected data of a larger experiment (11 of 18 goats)[7].

Only one aspect differs between this study or the clinical setting and our experiments: the MOCA procedure is directly followed by the use of compression stockings for at least the first 24 hours[11,14,16]. We were unable to apply stockings during follow-up for practical reasons and animal welfare. Compression therapy might be a beneficial addition measure in MOCA.

Finally, even though this study gives an important insight into the tissue reaction to MOCA and its separate components, we could not retrieve significant data on end points to draw indisputable conclusions owing to the small sample size.

CONCLUSION

MOCA is associated with an increased occlusion rate compared to its separated components of mechanical ablation or sclerotherapy. However, MOCA in the animal model resulted in occlusion in only two-thirds of the animals. The occlusion consists of cellular fibrotic material likely to be evolved from organized thrombus. This study underlines the hypothesis that the additive use of MOCA increases the effectiveness of sclerosants alone by inducing endothelium damage and probably vasoconstriction.

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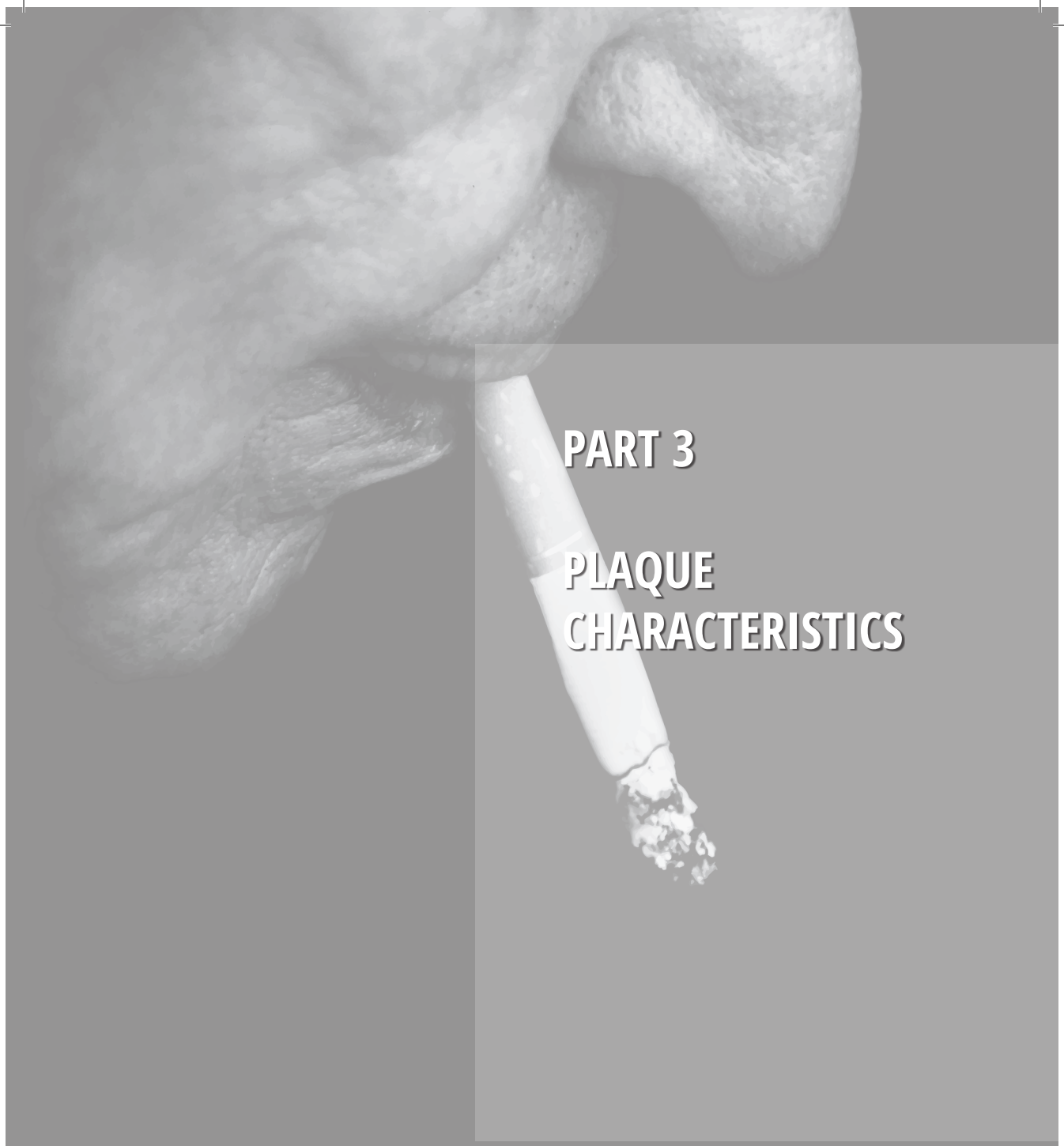

PART 3

PLAQUE CHARACTERISTICS

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CHAPTER 7

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

Atherosclerosis. 2016

ABSTRACT

Aim

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

Methods

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

Results

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

Conclusions

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

INTRODUCTION

The prevalence of peripheral arterial disease (PAD) is increasing worldwide, with a current estimated prevalence of 200 million people in the world. Expanding obesity rates, and a higher incidence of diabetes and smoking, will only increase the number of affected people, especially in high and middle income countries[1]. Although vascular risk factor management and improved (endovascular) treatment for atherosclerotic disease have lowered the disease burden and prolonged the survival of PAD patients, the disease is still associated with high morbidity and mortality risks[2,3]. Nonetheless, recent reports have shown that in patients presenting with claudication complaints, there is a sharp decline in the number of lower leg amputations[4,5]. Several explanations for this decline have been proposed, such as the importance of early screening, preventive (medical) care and the increased use of endovascular revascularization after 20005.

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

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

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

METHODS

Patient population

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

In and exclusion criteria

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

Sample collection

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

Histological assessment

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

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

Statistical analysis

To evaluate whether plaque characteristics differed over time we split the cohort into groups of two subsequent operation years. The analyses were based on methods previously described6. The data were imputed using single imputation. We used chi-square tests to compare categorical baseline characteristics of patients across the different time cohorts and Kruskall-Wallis tests for continuous, non-normally distributed variables. Logistic regression models were used to study the association of operation year with binary plaque characteristics and linear regression models for continuous plaque characteristics. For multivariable analyses, we added the time-dependent baseline characteristics that associated ($p<0.1$) with the plaque characteristic to the multivariable model for each plaque characteristic separately (for calcification: operation type, for collagen: total cholesterol, for lipid core: stenosis grade, for IPH: hypertension and anticoagulant use, for smooth muscle cells: hypertension and operation type, and for macrophages: hypertension, stenosis grade, operation type, total cholesterol and LDL cholesterol). Values with a $p<0.05$ were considered statistically significant. The R computing platform version 3.0.2 was used to carry out single imputation. SPSS version 21.0 was used for all other analyses.

RESULTS

Patient population

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

TABLE 1 Baseline characteristics

	Cohort (years)					
	2003-2004	**2005-2006**	**2007-2008**	**2009-2010**	**2011-2012**	**P value**
Number of patients	87	127	71	82	118	
Male sex	62 (71.3)	100 (78.7)	49 (69.0)	55 (67.1)	90 (76.3)	0.917
Age in years (median; IQR)	67 (59-74)	68 (59-76)	68 (62-74)	69 (63-73)	70 (63-75)	0.459
BMI (median; IQR)	26.5 (22.9-29.0)	25.5 (23.3-28.3)	25.3 (22.9-29.0)	25.8 (23.4-28.9)	26.1 (23.1-27.8)	0.977
Current smoker	44 (50.6)	55 (44.0)	25 (36.2)	36 (44.4)	51 (43.6)	0.448
Diabetes	25 (28.7)	44 (34.6)	25 (35.2)	26 (31.7)	32 (27.1)	0.531
Hypertension	62 (72.1)	75 (59.1)	50 (72.5)	62 (77.5)	92 (80.7)	0.005 *
Hypercholesterolaemia	59 (68.6)	76 (60.3)	46 (73.0)	43 (69.4)	73 (70.2)	0.337
History of CAD	31 (35.6)	50 (39.4)	31 (43.7)	28 (34.1)	48 (40.7)	0.729
History of stroke	16 (18.6)	22 (17.6)	7 (9.9)	9 (11.3)	13 (11.3)	0.059 *
History of PAD	38 (43.7)	50 (39.4)	27 (38.0)	32 (39.0)	45 (38.1)	0.494
History of Amputation	3 (3.5)	7 (5.5)	1 (1.6)	2 (2.9)	4 (3.4)	0.607
Amputation during 3-year FU	3 (3.5)	6 (4.8)	7 (10.1)	6 (7.6)	5 (4.4)	0.636
Duration of CI, years (median; IQR)	5 (2-8)	5 (1-10)	3 (1-9)	3 (1-8)	4 (2-9)	0.451
Fontaine Classification						0.014 *
Fontaine IIb	52 (61.9)	75 (60.0)	33 (54.1)	30 (51.7)	43 (46.7)	
Fontaine III	18 (21.4)	27 (21.6)	13 (21.3)	20 (34.5)	21 (22.8)	
Fontaine IV	14 (16.7)	23 (18.4)	15 (24.6)	8 (13.8)	28 (30.4)	
Stenosis grade						0.015 *
50-70%	2 (2.7)	8 (8.5)	1 (2.1)	1 (1.3)	24 (22.4)	
70-99%	33 (31.1)	23 (24.5)	17 (36.2)	20 (26.0)	27 (25.2)	
Occlusion	49 (66.2)	63 (67.0)	29 (61.7)	56 (72.7)	56 (52.3)	
Stenose contralateral						0.233
0-50%	23 (43.4)	9 (33.3)	3 (60.0)	8 (20.0)	27 (35.5)	
50-100%	30 (56.6)	18 (66.7)	2 (40.0)	32 (80.0)	49 (64.5)	
Operated Artery						0.002 *
Femoral	75 (86.2)	108 (85.7)	62 (91.2)	77 (95.1)	108 (95.6)	
Iliac	12 (13.8)	18 (14.3)	6 (8.8)	4 (4.9)	5 (4.4)	
Operation Type						<0.001 *
REA	40 (46.0)	46 (36.5)	16 (27.1)	15 (18.8)	20 (17.1)	
TEA	47 (54.0)	80 (63.5)	43 (72.9)	65 (81.3)	97 (82.9)	
Ankle-brachial index (median; IQR)	0.62 (0.48-0.72)	0.60 (0.45-0.71)	0.62 (0.46-0.80)	0.58 (0.43-0.70)	0.56 (0.42-0.72)	0.465
eGFR in mL/min/1.73 m^2 (median; IQR)	73 (55-91)	78 (59-104)	80 (57-102)	73 (59-95)	78 (65-111)	0.329
Mean arterial pressure (median; IQR)	105 (97-116)	100 (90-107)	103 (92-112)	100 (92-107)	100 (93-111)	0.119
Triglycerides in mmol/L (median; IQR)	2.1 (1.3-2.9)	1.5 (1.1-2.2)	1.9 (1.1-3.0)	1.5 (1.0-2.2)	1.5 (1.2-2.2)	0.015 *

TABLE 1 Continued

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

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

Patient characteristics alter over time

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

Ilio-femoral plaque characteristics alter over time

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

Multivariable analyses

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

TABLE 2 Plaque characteristics for two-year cohorts

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

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

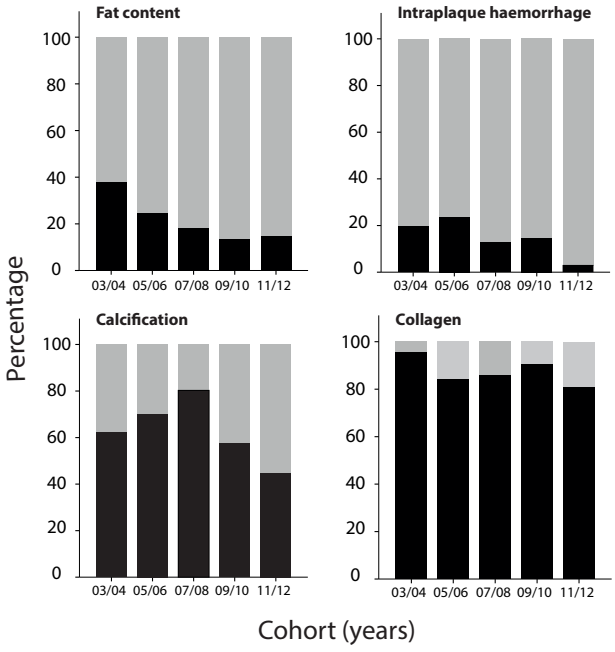
DISCUSSION

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

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

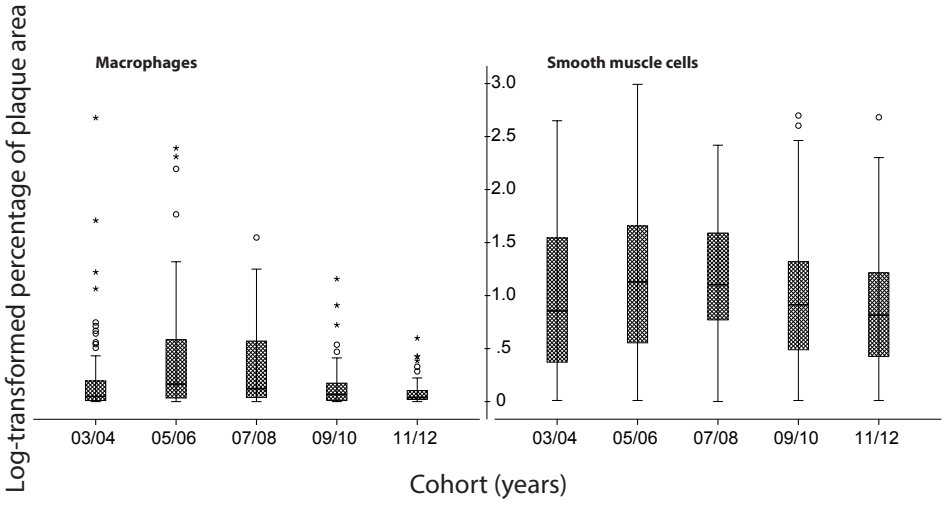
The observed changes in fat and IPH over time resembles earlier research from the same biobank in carotid arteries[6]. This is of particular interest, when taking into account the differences between ilio-femoral and carotid plaques. While extracted carotid plaques are generally more unstable and removed in an acute setting within days to

FIGURE 1 Percentage of plaque characteristics per two-year cohort



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

FIGURE 2 Boxplots of continuous plaque characteristics per two-year cohort

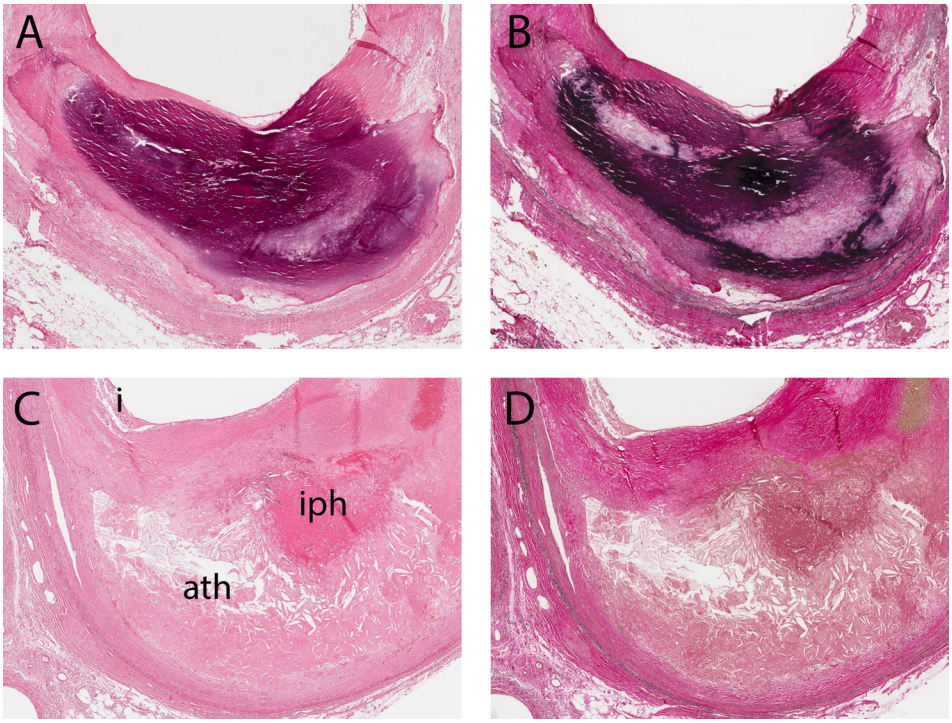


Overview of computerized measurements of ilio-femoral plaquecharacteristics over time. Bullet: 1.5 times IQR outlier, Asterisk: 3 times IQR outlier.

weeks after the initial event, ilio-femoral plaques are often the end product of years of remodelling, which is the result of conservative therapy or postponed surgery due to improved endovascular options, and therefore often display a more stable plaque type. Yet, even when these differences are taken into account, plaques in ilio-femoral arteries show time dependent changes comparable with the previously reported observed changes in the carotid artery. This strengthens the idea that the alteration of atherosclerotic plaque characteristics is a more widespread and systemic phenomenon.

Possible explanations for the consistent decrease of lipid-rich plaques and plaques with IPH over time are multifold. For example, the use of statins increased significantly during the course of this biobank from 64.4% to 79.7% of patients, and statin use is known to affect atherosclerotic plaques[26]. Indeed, during the same period, blood lipid and cholesterol levels dropped significantly. Still, the decline in vulnerable plaque characteristics were also seen in the patient group that did not use statins (data not

FIGURE 3 Example of stable and unstable plaque



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

TABLE 3 Multivariable analysis

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

Continuous computerized plaque characteristics	beta of 2005-2006 vs 2003-2004 (95% CI)	P value	beta of 2007-2008 vs 2003-2004 (95% CI)	P value	beta of 2009-2010 vs 2003-2004 (95% CI)	P value	beta of 2011-2012 vs 2003-2004 (95% CI)	P value
Macrophages	0.16 (0.05-0.27)	0.004*	0.13 (0.00-0.26)	0.05	-0.07 (-0.19-0.06)	0.32	-0.10 (-0.22-0.02)	0.11
Smooth Muscle Cells	0.21 (0.03-0.39)	0.02*	0.25 (0.03-0.47)	0.03*	0.07 (-0.13-0.27)	0.49	0.02 (-0.17-0.22)	0.81

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

shown). Moreover, we corrected for these changing patient characteristics in the multivariable analyses. Further improvements in preventive and conservative care could have contributed towards more stabilizing plaques, as the ilio-femoral plaque matures and remodels into a more stable plaque[27]. A shift towards longer conservative treatment before surgical intervention may also explain the observed increase in the patients operated with a higher disease severity, as measured by Fontaine classification and higher stenosis grades. Yet, the duration of complaints of intermittent claudication before surgical intervention did not change over time. In addition, one could speculate that the decrease of anticoagulant therapy might contribute towards plaque alterations, predominantly IPH, but we found no evidence for this[26]. Furthermore, the increasing prevalence of hypertension in this patient group could have caused plaque changes, but we found no significant effect in our cohort. Better treatment of hypertension could lead to relative hypoperfusion of the lower extremity, which could cause earlier complaints and therefore less stable plaques upon examination due to earlier resection of the plaque. Although we cannot exclude better compliance, we found no increased subscription of antihypertensive medication in more recent years and no lower mean arterial pressure. Furthermore, in the Dutch governmental smoking policy has changed significantly over the last decade, leading to a significant reduction in passive smoking. It has been established that such policies result in a decline in myocardial infarction in non-smokers[28]. Although, whether this also applies to peripheral arterial disease is

unknown. Finally, over the course of the years, the biobank protocol for handling and analysis of the plaques and cross-sections has not changed. The histological plaque analysis has been performed by the same dedicated technician and algorithms for computerized measurements were not adjusted over the years. Moreover, our methods have undergone substantive quality control. The phenotyping was validated by an independent observer, and have shown excellent intra and extra-observer variability measures[17,18].

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

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

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

CONCLUSION

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

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Conflict of interest
None.

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SUPPLEMENTAL

SUPPLEMENTAL TABLE 1 Baseline characteristics of the whole cohort

	All patients n = 497 (%)
Gender	
Female	135/497 (27.2)
Male	362/497 (72.8)
Age in years (median; IQR)	69; 61-75
BMI (median; IQR)	25.8; 23.1-28.4
Current smoker, yes	217/497 (43.7)
Diabetes, yes	154/497 (31.0)
Hypertension, yes	356/497 (71.6)
Hypercholesterolaemia, yes	334/497 (67.2)
History of CAD, yes	194/497 (39.0)
History of stroke, yes	26/497 (5.2)
History of peripheral intervention, yes	199/497 (40.0)
History of Amputation, yes	17/497 (3.4)
Duration of CI, years (median; IQR)	4 (1-9)
Fontaine Classification	
Fontaine IIb	276/497 (55.5)
Fontaine III	112/497 (22.5)
Fontaine IV	109/497 (21.9)
Stenosis grade	
50-70%	42/497 (8.5)
70-99%	141/497 (28.4)
Occlusion	314/497 (63.2)
Stenose contralateral	
0-50%	178/497 (35.8)
50-100%	319/497 (64.2)
Operated Artery	
Femoral	441/486 (90.7)
Iliac	45/486 (9.3)
Operation Type	
REA	139/481 (28.9)
TEA	342/481 (71.1)
Alcohol intake	
None	94/497 (18.9)
1-10	207/497 (316)
>10	196/497 (39.4)
Ankle-brachial index (median; IQR)	0.60; 0.45-0.72
eGFR in mL/min/1.73 m^2 (median; IQR)	77.8; 58.6-98.7

SUPPLEMENTAL TABLE 1 Continued

	All patients n = 497 (%)
Triglycerides in mg/dL (median; IQR)	1.6; 1.1-2.3
Total cholesterol in mg/dL (median; IQR)	4.8; 4.0-5.5
HDL in mg/dL (median; IQR)	1.2; 0.9-1.4
LDL in mg/dL (median; IQR)	2.7; 2.1-3.3
Statin use, yes	359/497 (72.2)
Antiplatelet use, yes	414/497 (83.3)
Anti-coagulant use, yes	82/497 (16.5)

IQR: interquartile range, CAD: coronary artery disease, CI: claudication intermittens, REA: remote endarterectomy, TEA: thrombendarterectomy, HDL: high density lipoprotein, LDL: low density lipoprotein

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CHAPTER 8

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

Br J Surg. 2017

ABSTRACT

Background

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

Methods

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

Results

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

Conclusion

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

INTRODUCTION

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

The pathophysiological mechanisms that could explain the decline in myocardial infarction and stroke due to cardiovascular disease in the general population may relate to changes in nutritional status, lifestyle changes, cessation of smoking[8], a steady decline in the percentage of smokers in the Dutch population[9], and medication use (statins, antihypertensive and antiplatelet therapy). Furthermore, several studies have[10–18] investigated plaque characteristics to determine which vascular lesions are more prone to rupture, one of the underlying mechanisms in stroke and myocardial infarction. Interestingly, recent studies[19,20] within the Athero-Express biobank revealed a significant decline in what are considered to be vulnerable plaque characteristics in patients undergoing carotid endarterectomy (CEA) or iliofemoral endarterectomy (IFE) over the past decade (Figures 1 and 2). These findings are important in light of the present follow-up study as it has been established previously that patients with plaque characteristics reflecting a measure of instability, such as intraplaque haemorrhage (IPH), have a poorer outcome during 3-year follow-up than patients with plaques that do not contain intraplaque haemorrhage[15].

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

METHODS

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

for analysis in the present prospective observational cohort study. The year 2012 was chosen as a cut-off point for inclusion, so that 3-year follow-up data could be analysed for every included patient. The whole cohort was divided into 2-year strata based on time of inclusion. Indication for surgery was based on international guidelines for carotid and iliofemoral atherosclerotic disease[22–25], and standardized treatment protocols and operative techniques were applied. The medical ethics committees in both participating centres approved the study. All patients provided written informed consent.

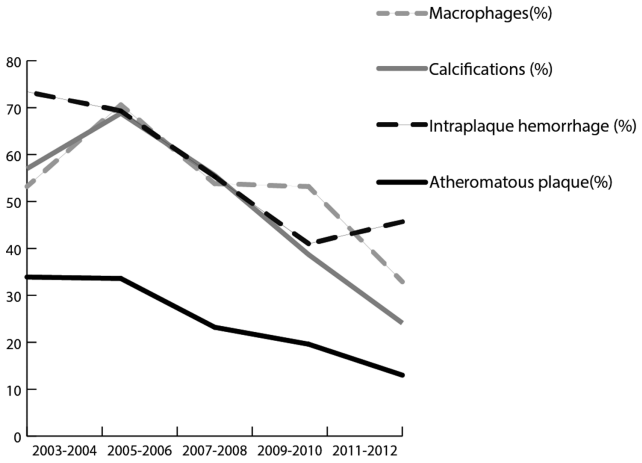
Inclusion and exclusion criteria

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

Follow-up

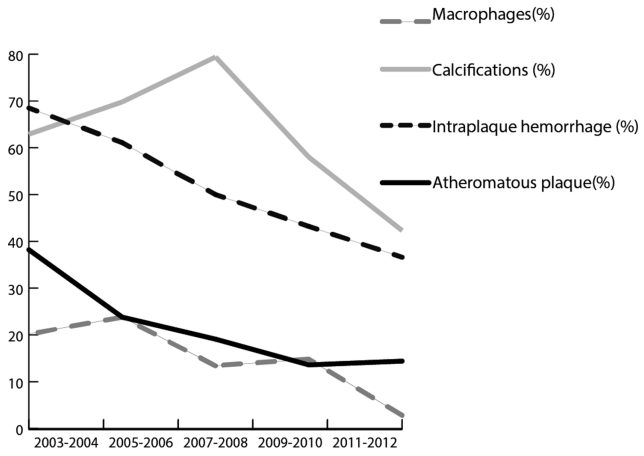
All patients were followed for 3 years after the initial procedure with a follow-up questionnaire at 1, 2 and 3 years after intervention. If the patient indicated that a cardiovascular event had occurred, this was validated through health records kept by the general practitioner. If the questionnaire was not returned, the general practitioner was contacted directly for follow-up information.

FIGURE 1 Changes in plaque characteristics with time after carotid endarterectomy



Plaques dissected from patients operated on in 2011–2012 show less heavy staining for macrophages than those from patients operated on in earlier years, with fewer heavy calcifications (P = 0.001), intraplaque haemorrhages (P = 0.001) and large lipid cores (P < 0.001)

FIGURE 2 Changes in plaque characteristics with time after iliofemoral endarterectomy



Plaques dissected from patients who had surgery in 2011–2012 show less heavy staining for macrophages than those from patients operated on in earlier years, with fewer heavy calcifications ($P = 0.001$), intraplaque haemorrhages ($P < 0.001$) and large lipid cores ($P < 0.001$)

Endpoints

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

Subanalyses

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

Statistical analysis

Patient characteristics at baseline across the different time cohorts were compared using Pearson c^2 test for dichotomous variables and one-way ANOVA for normally distributed continuous variables. The Mann–Whitney *U* test and Kruskal–Wallis test were applied for continuous variables that showed a non-parametric distribution. Possible confounders that were added to the multivariable analyses were based on both empirical evidence and changes occurring in baseline characteristics.

For patients who had CEA, the following variables were added to the model: age, sex, kidney function and contralateral stenosis. For those who had IFE, age, sex, type of operation, Fontaine classification, kidney function and diabetes were added. These confounders were added to the multivariable model of operation year strata and secondary cardiovascular outcomes, in which $P < 0.050$ was deemed significant. To avoid the limitation of complete-case analyses, single imputation with R computing platform version 3.0.2 (R Project for Statistical Computing, Vienna, Austria) was performed to calculate missing values. SPSS® version 21.0 (IBM, Armonk, New York, USA) was used for all statistical analyses.

RESULTS

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

Patient characteristics over time

The time to CEA surgery after the index event decreased from a median of 92 (range 34–145) days in 2003–2004 to 16 (9–30) days in 2011–2012. In most recent years, fewer asymptomatic patients had surgery than in earlier years: 9.4 per cent of patients in 2011–2012 compared with 16.2 per cent in 2003–2004. The percentage of symptomatic patients presenting with a stroke increased from 27.8 per cent in 2003–2004 to 34.7 per cent in 2011–2012, and those with ocular symptoms from 12.2 to 19.3 per cent, respectively. The number of patients presenting with a transient ischaemic attack remained stable throughout the years. The percentage of patients with a contralateral stenosis, an important predictor of cardiovascular events during follow-up[28], did not change over time. In patients having IFE, the Fontaine class was worse in recent years (23.5 per cent Fontaine IV in 2011–2012 *versus* 16 per cent in 2003–2004). Conversely, the stenosis grade of the culprit lesion decreased in the same interval, especially in the last 2-year cohort. In more recent years, surgical procedures were increasingly performed in the femoral arteries instead of the iliac arteries, and thromboendarterectomy (TEA) was more often undertaken instead of remote endarterectomy (REA) (TEA from 53 to 84.9 per cent, and REA from 47 to 15.2 per cent, in 2003–2004 and 2011–2012 respectively).

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

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

TABLE 1 Continued

	2003–2004	2005–2006	2007–2008	2009–2010	2011–2012	
	(*n* = 345)	(*n* = 382)	(*n* = 294)	(*n* = 326)	(*n* = 337)	*P*‡
Beta-blocker use†	154 (45.0)	159 (41.6)	134 (45.6)	145 (44.5)	133 (39.5)	0.454
Oral glucose inhibitor use†	53 (15.5)	62 (16.2)	45 (15.3)	54 (16.6)	58 (17.2)	0.965
Insulin use†	20 (5.8)	30 (7.9)	9 (3.1)	25 (7.7)	24 (7.1)	0.091

Values in parentheses are percentages unless indicated otherwise; values are median (i.q.r.). †Some data were missing for these variables. CAD, coronary artery disease; PAD, peripheral artery disease; TIA, transient ischaemic attack; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; RAAS, renin–angiotensin–aldosterone system. ‡Pearson c^2 test, except §one-way ANOVA and ¶Kruskal–Wallis test.

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

	2003–2004	2005–2006	2007–2008	2009–2010	2011–2012	
	(*n* = 93)	(*n* = 128)	(*n* = 73)	(*n* = 104)	(*n* = 132)	*P*‡
Age (years)*	67	68	68	69	70	0.269§
	(60–74)	(59–75)	(62–75)	(63–74)	(64–75)	
Men	67 (72)	100 (78.1)	51 (70)	67 (64.4)	94 (71.2)	0.245
BMI (kg/m^2)*†	26	26	26	25	27	0.925§
	(23–29)	(24–28)	(23–29)	(23–28)	(23–28)	
Current smoker†	45 (48)	56 (44.4)	25 (36)	46 (45.1)	53 (40.5)	0.322
Diabetes†	26 (28)	44 (34.4)	24 (33)	32 (31.1)	40 (30.3)	0.968
Hypertension†	66 (72)	76 (59.4)	51 (72)	78 (77.2)	101 (79.5)	0.006
Hypercholesterolaemia†	63 (68)	78 (61.4)	47 (72)	52 (65.0)	82 (70.7)	0.455
History of CAD†	35 (38)	52 (40.6)	30 (41)	33 (32.0)	56 (42.4)	0.885
History of stroke	4 (4)	10 (7.8)	2 (3)	6 (5.8)	3 (2.3)	0.059
History of peripheral intervention†	40 (43)	51 (39.8)	29 (40)	44 (42.7)	53 (40.2)	0.868
History of amputation†	3 (3)	7 (5.5)	1 (1)	2 (2.3)	4 (3.1)	0.495
Fontaine class						0.044
IIb	60 (65)	78 (60.9)	42 (58)	58 (55.8)	66 (50.0)	
III	18 (19)	27 (21.1)	16 (22)	31 (29.8)	35 (26.5)	
IV	15 (16)	23 (18.0)	15 (21)	15 (14.4)	31 (23.5)	
Stenosis grade (%)						< 0.001
0–49	2 (2)	12 (9.4)	4 (5)	2 (1.9)	33 (25.0)	
50–99	28 (30)	31 (24.2)	23 (32)	31 (29.8)	34 (25.8)	
100 (occlusion)	63 (68)	85 (66.4)	46 (63)	71 (68.3)	65 (49.2)	
Contralateral stenosis 50–100%	58 (62)	82 (64.1)	48 (66)	71 (68.3)	77 (58.3)	0.598
Operated artery						0.001
Femoral	80 (86)	110 (85.9)	67 (92)	98 (94.2)	126 (95.5)	
Iliac	13 (14)	18 (14.1)	6 (8)	6 (5.8)	6 (4.5)	
Operation type						< 0.001
REA	44 (47)	47 (36.7)	19 (26)	19 (18.3)	20 (15.2)	
TEA	49 (53)	81 (63.3)	54 (74)	85 (81.7)	112 (84.9)	

TABLE 2 Continued

	2003–2004 (*n* = 93)	2005–2006 (*n* = 128)	2007–2008 (*n* = 73)	2009–2010 (*n* = 104)	2011–2012 (*n* = 132)	*P*‡
eGFR (ml per min per 1.73 m²)*†	74 (54–91)	78 (59–103)	84 (60–107)	73 (59–96)	78 (61–107)	0.310§
Systolic BP (mmHg)*†	150 (140–170)	144 (130–163)	145 (130–167)	145 (130–154)	147 (132–167)	0.137§
Diastolic BP (mmHg)*†	80 (75–90)	80 (70–85)	80 (71–87)	76 (70–85)	76 (69–85)	0.052§
Triglycerides (mg/dl)*†	1.8 (1.2–3.0)	1.6 (1.0–2.4)	1.8 (1.0–2.5)	1.7 (1.0–2.2)	2.0 (1.4–3.2)	0.044§
Total cholesterol (mg/dl)*†	5.1 (4.3–5.7)	4.5 (3.9–5.2)	4.8 (3.8–5.5)	4.8 (4.0–5.5)	4.8 (4.0–5.6)	0.023§
HDL (mg/dl)*†	1.2 (0.9–1.5)	1.2 (0.9–1.4)	1.2 (0.9–1.3)	1.2 (1.0–1.5)	1.1 (0.9–1.3)	0.198§
LDL (mg/dl)*†	2.9 (2.1–3.5)	2.5 (1.8–3.1)	2.7 (1.9–3.3)	2.6 (2.1–3.2)	2.6 (1.9–3.2)	0.083§
Statin use†	59 (63)	90 (70.3)	59 (81)	72 (69.9)	105 (79.5)	0.018
Antiplatelet use†	79 (85)	101 (78.9)	57 (78)	95 (93.1)	113 (85.6)	0.137
Anticoagulant use†	17 (18)	30 (23.4)	16 (22)	7 (6.8)	13 (9.8)	0.001
Dual antiplatelet use†	30 (32)	11 (8.6)	11 (15)	18 (17.6)	21 (15.9)	0.115
RAAS medication use†	56 (60)	76 (59.4)	51 (70)	61 (59.2)	79 (59.8)	0.934
Beta-blocker use†	37 (40)	60 (46.9)	31 (42)	37 (35.9)	67 (50.8)	0.409
Oral glucose inhibitor use†	18 (19)	29 (22.7)	13 (18)	24 (23.3)	33 (25.0)	0.341
Insulin use†	12 (13)	14 (10.9)	8 (11)	8 (7.8)	12 (9.1)	0.262

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

Measures of established risk factors and medication use over time

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

Secondary cardiovascular outcome

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

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

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

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

TABLE 4 Clinical outcomes after iliofemoral endarterectomy in 2-year cohorts

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

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

Secondary cardiovascular outcome over time

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

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

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

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

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

Subgroup analyses: major cardiovascular endpoint

Some 209 patients (13.0 per cent) in the CEA group and 49 (9.5 per cent) in the IFE group had a major cardiovascular event during follow-up (Tables 3 and 4). Correcting for possible confounders, this did not decline significantly between 2003–2004 and 2011–2012 in patients who underwent CEA (HR 0.95, 95 per cent c.i. 0.59 to 1.52; *P* = 0.833) (Table S1, supporting information). The number of events was too small in the IFE population to determine changes over the 2-year strata statistically, but the percentage of patients reaching a major cardiac endpoint did not change over time (Table 4).

DISCUSSION

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

Several studies[1,2,6] have investigated population-wide trends in atherosclerotic disease and its variety of manifestations over time. Most pointed towards a decrease in major

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

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

Among patients who had IFE, the observed decrease in composite secondary cardiovascular events in the 2011–2012 cohort was mainly due to a decrease in peripheral reinterventions during follow-up and not to a decrease in major secondary cardiovascular events, which remained stable over time. After correcting for possible confounders, this decrease in peripheral reinterventions was observed only in the last 2-year cohort. A possible explanation could be that collagen-rich plaques were observed less often in recent years. A recent publication[20] reported a significant decline in collagen-rich plaques over time. As high collagen content is associated with restenosis after endarterectomy[29], this could explain the decrease in peripheral interventions in the last 2 years (from 36 per cent in 2009–2010 to 26.0 per cent in 2011–2012). Another explanation for the decrease in peripheral reinterventions could be that guidelines for IFE treatment have changed over the course of this study. This could have resulted in altered indications for surgical treatment, or improved postprocedural care, such as improved medical treatment or structured exercise therapy[30]. It was not possible to test for these effects in the present study. In further analyses, no changes in the incidence of other possible markers of more advanced atherosclerotic disease, such as ankle : brachial pressure index, duration of symptoms or history of atherosclerotic disease in other vascular beds, were found over time among patients undergoing IFE.

In patients with established manifest atherosclerotic disease, it is not fully understood what risk determinants explain the occurrence of subsequent cardiovascular events. A possible explanation for the lack of decline in major secondary cardiovascular events over time could be that, with current optimal preventive care, the average patient at cardiovascular risk less often develops symptomatic atherosclerotic plaques considered for surgery. This indicates that over time there has been natural selection of patients least responsive to current medical treatment. In patients who do develop symptoms despite best preventive care, the risk of secondary cardiovascular events may be less likely to change. Still, major changes in the underlying pathological substrate of the

dissected culprit lesions have been observed[19,20]. The changes in atherosclerotic plaque characteristics that underlie symptomatic disease could thus point to the selection of a poorly understood patient group with a different risk profile, and for whom different risk prevention and treatment strategies may have to be considered[18,31].

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

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

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

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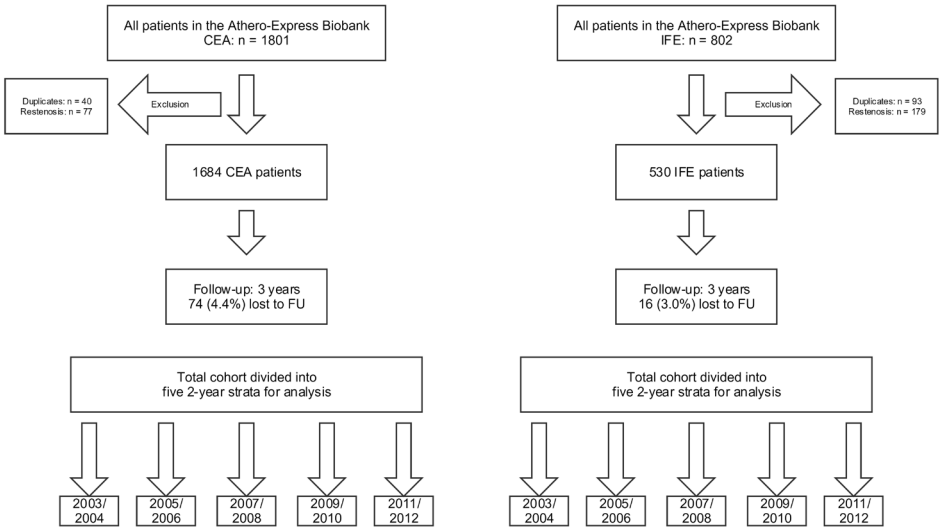
SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

SUPPLEMENTAL FIGURE 1 Study flow chart (Word document)



SUPPLEMENTAL TABLE 1 Baseline characteristics of CEA patients that associate with secondary cardiovascular events during follow-up

	No composite endpoints (CE); n = 1205(74.8%)	**Patients with CE; n = 405 (25.2%)**	**Risk of primary outcome HR [95% CI]**	**P-value**
Male sex, n(%)	813 (67.5)	296 (73.1)	1.24 [0.99-1.55]	0.053*
Age in years (median; IQR)	70 (62-76)	69 (63-76)	1.00 [0.99-1.01]	0.538
BMI (median; IQR)	26 (24-28)	26 (24-29)	1.03 [1.00-1.05]	0.036*
Current smoker, yes	412 (34.7)	132 (33.3)	0.97 [0.78-1.20]	0.783
Diabetes, yes	261 (21.7)	108 (26.7)	1.30 [1.04-1.61]	0.021*
Hypertension, yes	840 (72.0)	298 (76.0)	1.22 [0.97-1.54]	0.092*
Systolic blood pressure (median; IQR)	150 (135-170)	154 (137-172)	1.00 [0.99-1.01]	0.299
Diastolic blood pressure (median; IQR)	80 (73-90)	80 (73-90)	1.00 [0.99-1.01]	0.339
Hypercholesterolaemia, yes	735 (65.8)	263 (72.3)	1.27 [0.95-1.70]	0.111
History of CAD, yes	322 (26.7)	168 (41.6)	1.76 [1.45-2.15]	<0.001*
History of peripheral intervention, yes	136 (11.3)	106 (26.2)	2.36 [1.89-2.95]	<0.001*
Operation indication				
Asymptomatic	134 (11.2)	68 (16.8)	1.42 [1.09-1.84]	0.009*
Occular	189 (15.8)	53 (13.1)	0.80 [0.60-1.07]	0.131
TIA	531 (44.3)	184 (45.5)	1.06 [0.87-1.29]	0.564
Stroke	345 (28.8)	99 (24.5)	0.86 [0.68-1.07]	0.175
Stenose contralateral				
50-100%	450 (42.3)	208 (55.8)	1.63 [1.33-2.00]	<0.001*
Time between last event and operation (median; IQR)	33 (15-75)	37 (13-86)	1.00 [0.99-1.00]	0.527
eGFR in mL/min/1.73 m^2 (median; IQR)	73.3 (60.4-86.4)	69.3 (54.5-83.5)	0.99 [0.99-0.99]	<0.001*
Triglycerides in mg/dL (median; IQR)	1.4 (1.0-1.9)	1.4 (1.0-2.1)	1.11 [0.99-1.24]	0.066 *
Total cholesterol in mg/dL (median; IQR)	4.6 (3.8-5.5)	4.5 (3.7-5.3)	0.90 [0.83-0.97]	0.009*
HDL in mg/dL (median; IQR)	1.1 (1.0-1.4)	1.1 (0.9-1.3)	0.67 [0.50-0.90]	0.007*
LDL in mg/dL (median; IQR)	2.7 (2.0-3.5)	2.5 (2.0-3.2)	0.87 [0.79-0.96]	0.004*
Statin use, yes	920 (76.5)	320 (79.2)	1.15 [0.90-1.46]	0.260
Antiplatelet use, yes	1068 (88.9)	352 (87.1)	0.81 [0.60-1.08]	0.146
Anti-coagulant use, yes	124 (10.3)	62 (15.3)	1.52 [1.16-1.99]	0.003*
Dual antiplatelet, yes	676 (56.3)	197 (48.8)	0.75 [0.62-0.91]	0.004*
RAAS medication use, yes	584 (48.5)	236 (58.4)	1.41 [1.16-1.72]	0.001*
β-blocker, yes	482 (40.1)	207 (51.1)	1.49 [1.24-1.81]	<0.001*
Oral glucose inhibitor, yes	182 (15.1)	79 (19.6)	1.31 [1.02-1.67]	0.033*
Insulin, yes	68 (5.7)	34 (8.4)	1.52 [1.07-2.15]	0.021*

Logistic regression analysis of baseline characteristics from CEA patients with secondary cardiovascular events during follow-up. CAD: coronary artery disease. PAD: peripheral arterial disease. RAAS medication: Renin-angiotensin-aldosterone-system medication.*p<0.1.

SUPPLEMENTAL TABLE 2 Baseline characteristics of IFE patients that associate with secondary cardiovascular events during follow-up

	No composite endpoints (CE); n = 278 (54.1%)	**Patients with CE; n = 236 (45.9%)**	**Risk of primary outcome HR [95% CI]**	**P-value**
Male sex	198 (71.2)	169 (71.6)	0.98 [0.74-1.30]	0.900
Age in years (median; IQR)	68; 61-74	69; 62-74	1.01 [0.99-1.02]	0.271
BMI (median; IQR)	26; 23-28	26; 23-29	1.00 [0.97-1.03]	0.968
Current smoker, yes	122 (44.7)	95 (40.6)	0.87 [0.67-1.14]	0.312
Diabetes, yes	77 (27.7)	87 (36.9)	1.41 [1.08-1.84]	0.011 *
Hypertension, yes	195 (71.4)	169 (73.2)	1.06 [0.79-1.42]	0.698
Hypercholesterolaemia, yes	166 (64.6)	148 (70.5)	1.23 [0.92-1.66]	0.170
History of CAD, yes	98 (35.3)	98 (41.5)	1.27 [0.98-1.65]	0.068 *
History of stroke, yes	7 (2.5)	18 (7.6)	2.12 [1.31-3.43]	0.002 *
History of peripheral intervention, yes	93 (33.5)	115 (48.7)	1.59 [1.23-2.05]	<0.001 *
History of Amputation, yes	5 (1.9)	11 (5.0)	1.90 [1.04-3.49]	0.038 *
Fontaine Classification				
Fontaine IIb	172 (61.9)	124 (52.5)	- ref -	
Fontaine III	60 (21.6)	61 (25.8)	1.35 [0.99-1.83]	0.058 *
Fontaine IV	46 (16.5)	51 (21.6)	1.44 [1.05-2.00]	0.028 *
Stenosis grade				
50-70%	34 (12.2)	18 (7.6)	- ref -	
70-99%	82 (29.5)	61 (25.8)	1.22 [0.72-2.06]	0.467
Occlusion	162 (58.3)	157 (66.5)	1.52 [0.93-2.47]	0.095 *
Stenose contralateral				
50-100%	171 (61.5)	154 (65.3)	0.93 [0.81-1.06]	0.273
Operated Artery				
Femoral	251 (90.3)	214 (90.7)	- ref -	
Iliac	27 (9.7)	22 (9.3)	1.02 [0.66-1.59]	0.919
Operation Type				
REA	70 (25.2)	76 (32.2)	- ref -	
TEA	208 (74.8)	160 (67.8)	0.76 [0.58-0.99]	0.047 *
Systolic blood pressure (mm/Hg)	145; 130-160	150; 134-168	1.01 [1.00-1.01]	0.056 *
Diastolic blood pressure (mm/Hg)	79; 70-85	80; 70-88	1.00 [0.99-1.01]	0.652
eGFR in mL/min/1.73 m^2 (median; IQR)	81; 60-101	74; 57-99	0.99 [0.99-1.00]	0.086 *
Triglycerides in mg/dL (median; IQR)	1.8; 1.2-2.8	1.7; 1.1-2.4	0.93 [0.84-1.02]	0.126
Total cholesterol in mg/dL (median; IQR)	4.8; 4.0-5.6	4.7; 3.9-5.7	0.96 [0.85-1.07]	0.440
HDL in mg/dL (median; IQR)	1.2; 0.9-1.4	1.2; 0.9-1.4	1.00 [0.71-1.40]	0.985
LDL in mg/dL (median; IQR)	2.6; 2.0-3.3	2.6; 2.0-3.3	1.03 [0.90-1.19]	0.646
Statin use, yes	193 (69.4)	183 (77.5)	1.34 [0.99-1.82]	0.058 *
Antiplatelet use, yes	234 (84.2)	199 (84.7)	0.97 [0.68-1.38]	0.842
Anti-coagulant use, yes	40 (14.4)	39 (16.5)	1.19 [0.84-1.67]	0.333
Dual antiplatelet, yes	40 (14.4)	49 (20.9)	1.50 [1.01-2.06]	0.012*
RAAS medication use, yes	160 (57.6)	157 (66.5)	1.33 [1.02-1.75]	0.037*
β-blocker, yes	119 (42.8)	104 (44.1)	1.09 [0.85-1.41]	0.495
Oral glucose inhibitor, yes	56 (20.1)	61 (25.8)	1.28 [0.96-1.72]	0.093*
Insulin, yes	26 (9.4)	27 (11.4)	1.28 [0.86-1.91]	0.228

Logistic regression analysis of baseline characteristics of IFE patients with secondary cardiovascular events during follow-up. CAD: coronary artery disease. PAD: peripheral arterial disease. REA: remote endarterectomy, TEA: trombendarterectomy. RAAS medication: Renin-angiotensin-aldosterone-system medication. *p<0.1.

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CHAPTER 9

Atherosclerotic plaque characteristics are not associated with future cardiovascular events in patients undergoing ilio-femoral endarterectomy

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ABSTRACT

Background
Plaque characteristics such as intraplaque hemorrhage (IPH) have been associated with secondary cardiovascular events (CVE) in patients undergoing carotid endarterectomy. In addition, carotid plaques containing macrophage infiltration or a large lipid core size were associated with less restenosis. It is currently unknown whether iliofemoral plaque histopathological characteristics are predictive for secondary CVE in peripheral arterial disease (PAD) patients undergoing iliofemoral endarterectomy.

Objective
To examine the association between iliofemoral atherosclerotic plaque characteristics and secondary CVE in patients undergoing iliofemoral endarterectomy.

Methods
497 patients with iliofemoral atherosclerotic disease underwent primary endarterectomy of the iliac or femoral artery from 2002 to 2013 were included. All specimen were uptaken in the Athero Express biobank and seven histological plaque characteristics were analyzed: calcification, collagen, fat content, IPH, macrophages, smooth muscle cells, and vessel density. The composite CVE consisted of: myocardial infarction, cerebrovascular accident, peripheral (re-)interventions and cardiovascular death. Multivariate Cox regression models were used to examine the association between plaque and the composite endpoint during a follow-up period of three years.

Results
Of the 497 patients, 225 patients (46.4%) experienced a composite CVE within 3 years after the initial surgery. Calcified plaques were univariably associated with composite CVE (HR 1.32 (95%CI 1.00-1.73), P=.049). After correction for confounders, multivariable analyses showed no significant association between calcified plaques and composite CVE (HR 1.13 (95%CI 0.85-1.50), P=.413). IPH was not predictive of secondary CVE (HR 1.02 (95%CI 0.79-1.33, P=.867).

Conclusion
In this cohort of PAD patients undergoing iliofemoral endarterectomy, investigated atherosclerotic plaque characteristics were not independently associated with secondary CVE during follow-up.

INTRODUCTION

Patients with peripheral arterial disease (PAD) have an increased risk of cardiovascular events and death[1–3]. Current population trends show a predicted increase in PAD patients worldwide due to the ageing population and increased prevalence of diabetes[4,5]. Although patients often suffer from well recognized clinical risk factors to develop primary cardiovascular events (CVE)[6], plaque characteristics may contain additional valuable information regarding the risk to develop secondary CVE or the need for re-interventions[7].

Several plaque characteristics are associated with plaque vulnerability and subsequent CVE[8], although this theory is not undisputed[9]. In carotid endarterectomy (CEA) plaques, it has been established that plaques containing intraplaque hemorrhage (IPH) are associated with an increased risk of stroke and adverse outcomes during follow-up[10,11]. Similarly, carotid plaques removed after a cerebrovascular event often show plaque inflammation and cap rupture[12]. On the other hand, carotid plaques containing much inflammation or a large lipid core were associated with less restenosis[13]. In lower limb atherosclerosis it is still unknown whether local plaque characteristics are associated with an increased risk of secondary CVE during follow-up. Several smaller studies have investigated plaques of PAD patients, but so far no large study into iliofemoral atherosclerotic plaque characteristics has been conducted[14–19]. In an earlier study, our group has compared plaque characteristics of patients with and without diabetes, but not whether certain iliofemoral plaque characteristics are of predictive value for secondary CVE[20]. If certain plaque characteristics are identified as predictors for secondary CVE, radiological studies could benefit from this knowledge in a clinical setting or in future research[21–23].

The aim of this study was to examine whether an association between iliofemoral plaques characteristics and secondary CVE exists in patients undergoing iliofemoral endarterectomy. To achieve this, we analyzed the plaque characteristics and follow-up of all patients included in the Athero-Express biobank from 2002 to 2013 undergoing iliofemoral endarterectomy in two large Dutch tertiary referral hospitals.

METHODS

Patient population

The Athero-Express biobank is a prospective ongoing biobank study that includes blood and plaque specimens of patients undergoing carotid and/or iliofemoral endarterectomy in two large tertiary referral hospitals in the Netherlands: the St. Antonius Hospital in Nieuwegein and the University Medical Center in Utrecht. Plaque removal was conducted according to local and international guidelines[2]. This study was approved by medical ethics boards of both hospitals and is conducted in accordance with the declaration of Helsinki. Patients provided written informed consent prior to participation in the study.

In and exclusion criteria

All consecutive patients undergoing iliofemoral endarterectomy from 2002 to 2013 with available plaque characteristics in the AtheroExpress study were included. Clinical data were extracted from patient files and standardized questionnaires. Only primary endarterectomies were included, as research indicates that restenotic plaques have different characteristics[19,24]. Follow-up data during a three-year period were obtained through questionnaires sent to patients. Secondary cardiovascular events were validated using health records kept by general practitioners. In case patients did not respond, general practitioners were directly contacted for follow-up information.

Endpoints

The composite cardiovascular endpoint was determined by either one of the following events during follow-up: (1) cardiovascular death, (2) major CVE (i.e. patients diagnosed by a specialist with either a cerebrovascular accident (CVA) or myocardial infarction (MI)), (3) leg amputation or (4) percutaneous transluminal angioplasty (PTA) or (endo) vascular (re-)intervention. Time to first occurrence of any of the events was scored as time to the composite endpoint, while patients could have reached two or more endpoints. A new peripheral procedure was only counted as reintervention when it occurred more than 6 months after the initial procedure. Target vessel revascularization (TVR) was defined as any intervention in the same artery as was treated in the original inclusion endarterectomy and was assessed to determine whether a re-intervention had occurred.

To differentiate between patients undergoing a (re-)intervention of the lower limb arteries as compared to systemic 'major' secondary CVE. A subanalysis was performed with the peripheral endpoint (e.g. PTA, peripheral (re-)intervention and amputation) and with the major secondary CVE, including; CVA, MI and cardiovascular death.

Sample collection

An extensive depiction of the Athero-Express study protocol and sample collection is provided elsewhere25. We provide a brief explanation here. Blood was retrieved prior to surgery and stored at -80 degrees. The plaques were collected immediately during surgery and the culprit lesion was identified. Subsequently, the culprit lesion was stored in 4% formaldehyde, decalcified and embedded in paraffin for histological assessment. The rest of the plaque was cut into segments of 5mm each and stored at -80 degrees.

Histological assessment

Histological assessment of cross-section slides of the plaques were also described earlier[26]. Plaque specimens were stained to examine the earlier mentioned plaque characteristics as follows: CD68 for macrophages, α-actin to identify smooth muscle cells (SMCs), picro-sirius red for collagen, and CD34 for microvessels (CD34). Hematoxylin-eosin staining and the presence of fibrin by Mallory's phosphotungstic acid-hematoxylin staining was used to identify the presence of intraplaque haemorrhage

(IPH). Polarized light was used to assess the area of the lipid core of the plaque, with a cut-off point of 10%. Macrophages and SMCs were quantified as percentage of plaque area with the use of computerized analyses[27]. After morphological identification, microvessels were counted in three hotspots of the plaque and subsequently averaged per slide. Semi-quantitative scoring at 40x magnification was performed for collagen and calcification by a technician into four categories: no (1), minor (2), moderate (3) or heavy (4) staining in the plaque. In the current study, these categories were grouped into binary variables (no/minor and moderate/heavy). All histological slides were assessed by the same dedicated technician, who was blinded for patient characteristics and outcomes. Good inter and intra-observer similarities have previously been confirmed[28].

Statistical analysis

Baseline characteristics were compared between patients with and without composite endpoints during three-year follow-up. Cox proportional hazards models were used to determine the relation between plaque characteristics and secondary CVE during three-year follow-up, for which a cut-off point of $p<0.1$ was chosen to add plaque characteristics to the multivariable model. Covariates were defined as baseline characteristics with a p-value <0.1 for the relation with the composite endpoint, and which were also associated in univariable analysis ($p<0.25$) with any of the plaque characteristics associated with the composite endpoint. These covariates were added to the final multivariable model of the association between plaque characteristics and composite endpoints. For multivariable analyses, missing data on baseline characteristics were imputed using single imputation. Values with a $p <0.05$ were considered statistically significant. Single imputation was carried out with the R computing platform, version 3.0.2. All other analyses were carried out in SPSS version 22.1.

RESULTS

Baseline characteristics

In total, 651 patients (73.4% male) undergoing iliofemoral endarterectomy were included in the Athero-Express biobank study from 2002 to 2013. Of these, 154 (75.3% male) were treated for a restenotic lesion and were excluded from the current dataset. Of the remaining 497 patients, 12 patients were lost to follow-up (2.4%). Most patients were male (n = 362; 72.8%) and the median age was 69 years (IQR 61-75). In the majority of patients the femoral artery was treated (90.7%), for an occlusive lesion (62.8%). Thrombendarterectomy (TEA) (71.1%) was more often performed than a remote endarterectomy (REA) (28.9%). All patient characteristics are shown in Table 1.

TABLE 1 Baseline characteristics

	All patients N = 497[a]	No CE n = 260 (52.6%)	Patients with CE n = 225 (46.4%)	Risk of primary outcome HR [95% CI]	P-value
Clinical characteristics					
Sex					
Female	135/497 (27.2)	70 (26.9)	64 (28.4)	- ref -	
Male	362/497 (72.8)	190 (73.1)	161 (71.6)	0.90 [0.67-1.20]	0.465
Age in years (median; IQR)	69; 61-75	69; 61-75	69; 62-74	1.00 [0.99-1.02]	0.579
BMI (median; IQR)	25.9; 23.1-28.4	25.8; 23.2-28.3	26.2; 23.1-28.9	1.00 [0.97-1.03]	0.904
Current smoker, yes	215/490 (43.9)	117 (45.7)	92 (41.4)	0.88 [0.67-1.14]	0.332
Diabetes, yes	154/497 (31.0)	68 (26.2)	83 (36.9)	1.51 [1.15-1.98]	0.003 *
Hypertension, yes	347/487 (71.3)	182 (71.1)	159 (72.6)	1.04 [0.78-1.41]	0.778
Hypercholesterolaemia, yes	302/450 (67.1)	154 (64.2)	142 (71.0)	1.27 [0.93-1.72]	0.131
Statin use, yes	359/497 (72.2)	179 (68.6)	174 (77.3)	1.35 [0.99-1.85]	0.057 *
Antiplatelet use, yes	413/496 (83.3)	218 (83.8)	188 (83.9)	0.92 [0.65-1.32]	0.665
Anti-coagulant use, yes	82/497 (16.5)	39 (15.0)	38 (16.9)	1.16 [0.82-1.64]	0.410
History of CAD, yes	194/497 (39.0)	89 (34.2)	96 (42.7)	1.34 [1.03-1.75]	0.029 *
History of stroke, yes	26/488 (5.3)	8 (3.1)	18 (8.1)	1.91 [1.18-3.09]	0.009 *
History of peripheral intervention, yes	199/497 (40.0)	87 (33.5)	106 (47.1)	1.51 [1.16-1.96]	0.002 *
History of Amputation, yes	17/473 (3.6)	4 (1.6)	11 (5.2)	2.11 [1.15-3.87]	0.016 *
Alcohol intake					
None	85/439 (19.4)	47 (20.3)	36 (18.2)	- ref -	
1-10	175/439 (39.9)	83 (35.8)	88 (44.4)	1.19 [0.81-1.76]	0.377
>10	179/439 (40.8)	102 (44.0)	74 (37.4)	0.87 [0.59-1.30]	0.500
Years of CI complaints	4; 1-9	4; 1-8	4; 2-10	1.01 [0.99-1.03]	0.281
Arterial characteristics					
Fontaine Classification					
Fontaine IIb	239/430 (55.6)	132 (58.7)	104 (53.0)	- ref -	
Fontaine III	100/430 (23.3)	51 (22.7)	46 (23.5)	1.18 [0.83-1.67]	0.352
Fontaine IV	91/430 (21.2)	42 (18.6)	46 (23.5)	1.35 [0.95-1.91]	0.092 *
Stenosis grade					
50-69%	37/411 (9.0)	24 (11.2)	12 (6.3)	- ref -	
70-99%	116/411 (28.2)	64 (29.9)	51 (27.0)	1.35 [0.72-2.53]	0.349
Occlusion	258/411 (62.8)	126 (58.9)	126 (66.7)	1.59 [0.88-2.87]	0.125
Contralateral Stenosis					
0-49%	75/208 (36.1)	48 (40.0)	27 (32.5)	- ref -	
50-100%	133/208 (63.9)	72 (60.0)	56 (67.5)	1.34 [0.85-2.12]	0.212
Operated Artery					
Femoral	441/486 (90.7)	229 (90.2)	200 (90.9)	- ref -	
Iliac	45/486 (9.3)	25 (9.8)	20 (9.1)	1.00 [0.63-1.58]	0.989
Operation Type					
REA	139/481 (28.9)	65 (25.8)	71 (32.7)	- ref -	
TEA	342/481 (71.1)	187 (74.2)	146 (67.3)	0.75 [0.57-0.99]	0.048 *

TABLE 1 Continued

	All patients N = 497[a]	No CE n = 260 (52.6%)	Patients with CE n = 225 (46.4%)	Risk of primary outcome HR [95% CI]	P-value
Ankle-brachial index (median; IQR)	0.60; 0.46-0.71	0.60; 0.46-0.71	0.60; 0.45-0.71	1.06 [0.51-2.21]	0.873
Lab results					
eGFR in mL/min/1.73 m^2 (median; IQR)	78.0; 58.8-98.8	80.9; 61.9-102.9	73.1; 55.2-96.5	0.99 [0.99-0.99]	0.004 *
Triglycerides in mg/dL (median; IQR)	1.6; 1.1-2.2	1.7; 1.2-2.2	1.5; 1.1-2.3	1.00 [0.85-1.18]	0.981
Total cholesterol in mg/dL (median; IQR)	4.7; 4.0-5.5	4.8; 4.0-5.7	4.6; 4.0-5.4	0.91 [0.77-1.08]	0.277
HDL in mg/dL (median; IQR)	1.2; 0.9-1.4	1.1; 0.9-1.4	1.2; 0.9-1.4	0.89 [0.53-1.48]	0.643
LDL in mg/dL (median; IQR)	2.6; 2.0-3.3	2.6; 2.0-3.4	2.6; 2.0-3.2	1.00 [0.80-1.25]	0.999

CE: composite endpoints. - ref -: reference value. CAD: coronary artery disease. REA: remote endarterectomy. TEA: thrombendarterectomy. CI complaints: claudication complaints. eGFR: estimated glomerular filtration rate. [a] 12 patients with missing follow-up. *P<.1

A total of 225 patients (46.4%) had a composite endpoint within the three years follow-up after initial surgery, of which 34 patients died due to cardiovascular causes (7.0%), and 187 (38.6%) had a peripheral intervention during follow-up. TVR was performed in 61/171 patients (35.7%, 16 missing cases) (Table 2). Several clinical baseline characteristics were associated with the composite cardiovascular endpoint, such as diabetes, worse kidney function, and a history of cardiovascular disease (Table 1). In addition to these characteristics, female sex and increased age were associated with major CVE during follow-up.

Most plaques were rich in collagen (85.1% moderate/heavy) and calcification (60.8% moderate/heavy) but low in fat content (21.5% of patients had a lipid core above 10% of plaque surface). Figure 1 shows an example of a retrieved femoral atherosclerotic plaque. IPH was prevalent in 51.7% of included plaques.

Association of plaque characteristics with secondary cardiovascular events

IPH was not associated with the composite CVE (HR 1.02 (95%CI 0.79-1.33), P=.867). Plaques containing moderate to heavy calcification (HR 1.32 (95%CI 1.00-1.73), P=.049) and plaques rich in collagen (HR 1.43 (95%CI 0.94-2.16), P=.095) were univariably associated with the composite CVE (Table 3). Collagen was univariably associated with peripheral (re-)intervention (HR 1.68 (95%CI 1.03-2.73), P=.037), but not with TVR (P=1.000). There was a trend between calcification and major CVE in univariable analysis (HR 1.58 (95%CI 0.98-2.54), P=.061).

Multivariable analyses of the composite cardiovascular endpoint

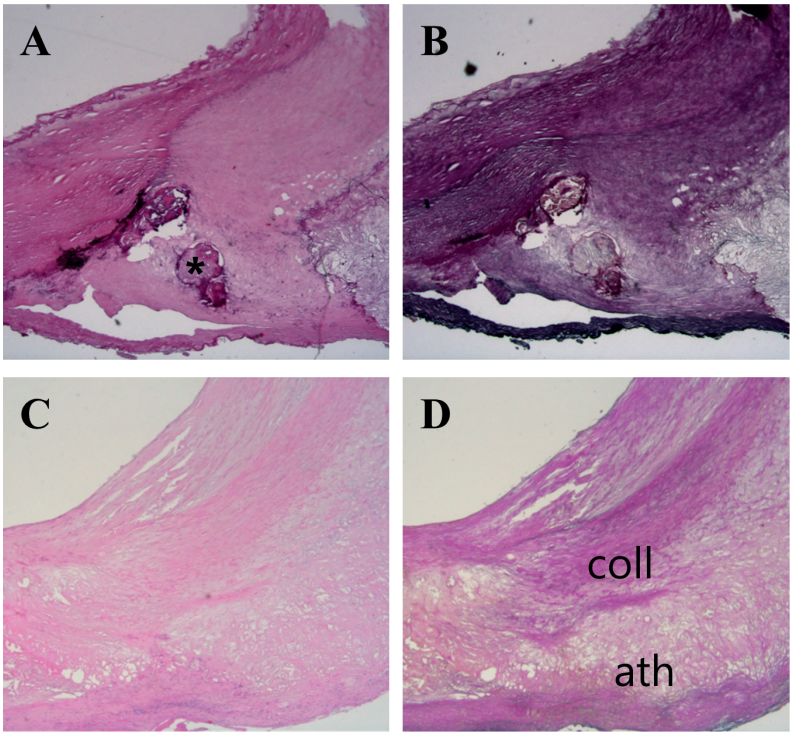
After correction for possible confounders, no association was found between plaque calcification and plaque collagen with the composite cardiovascular endpoint (HR 1.13

TABLE 2 Clinical outcomes at 3 year follow-up

Secondary clinical outcomes	n/total (%)
Composite CVE	225/485 (46.4%)
Of which:	
Cardiovascular death	34/483 (7.0%)
Major cardiovascular event	47/485 (9.7%)
Leg amputation	27/485 (5.6%)
Peripheral intervention	187/485 (38.6%)
Of which: TVR	61/171 (35.7%)[a]

Composite CVE consists of: cardiovascular death, major cardiovascular events and peripheral intervention. Major cardiovascular event consists of: CVA/TIA, cardiovascular death, myocardial infarction or coronary artery disease. Peripheral intervention also includes leg amputation. TVR: target vessel revascularization. [a]n = 16 missing cases.

FIGURE 1 Histologic samples of atherosclerotic plaques retrieved from the femoral artery



(A and B) Atherosclerotic plaque containing calcified nodules (*) and fibrous connective tissue.
(C and D) Atherosclerotic plaque with an atheroma (ath), in the process of remodeling with collagen (coll) infiltration of the atheroma.

(95%CI 0.85-1.50), P=.413) and (HR 1.27 (95%CI 0.84-1.94), P=.263), respectively (Table 4). Stratified for sex, no specific association between plaque characteristics and the composite outcome was found in male patients. In female patients, an univariable association between calcified plaques and the composite CVE was observed (HR 1.62 (95%CI 0.98-2.67), P=.060). After correction for possible confounders, this association was no longer observed (HR 1.35 (95%CI 0.80-2.26), P=.259) (Supplemental Table 1).
After correction for possible confounders, collagen was not associated with peripheral (re-)intervention during follow-up (HR 1.44 (95%CI 0.87-2.40), P=.161) (Supplemental Table 2).
Calcification was not associated with the major secondary cardiovascular endpoint during follow-up after correction for possible confounders (HR 1.12 (95%CI 0.61-2.09), P.712) (Supplemental Table 3).

TABLE 3 Plaque characteristics vs composite cardiovascular endpoint

Plaque characteristic vs composite CVE	Patients without Endpoint	Patients with Endpoint	HR [95% CI]	P-value
Fat content				
Fat <10%	202/258 (78.3)	175/223 (78.5)		
Fat >10%	56/258 (21.7)	48/223 (21.5)	1.00 [0.73-1.38]	0.983
Calcification				
Calc no/minor	108/256 (42.2)	78/224 (34.8)	-	-
Calc mod/heavy	148/256 (57.8)	146/224 (65.2)	1.32 [1.00-1.73]	0.049 *
Collagen				
Collagen no/minor	41/254 (16.1)	25/223 (11.2)	-	-
Collagen mod/heavy	213/254 (83.9)	198/223 (88.8)	1.43 [0.94-2.16]	0.095 *
Intraplaque hemorrhage				
IPH no	125/258 (48.4)	105/224 (46.9)	-	-
IPH yes	133/258 (51.6)	119/224 (53.1)	1.02 [0.79-1.33]	0.867
Quantified characteristics				
SMC				
Median (IQR)	1.43; 0.52-3.33	1.90; 0.73-3.48	1.00 [0.96-1.04]	0.969
Macrophages				
Median (IQR)	0.09; 0.02-0.38	0.08; 0.02-0.33	1.04 [0.93-1.16]	0.483
Vessel density (n=176)				
Median (IQR)	5.83; 2.83-10.59	5.67; 2.83-8.00	0.97 [0.92-1.01]	0.156

IPH: intraplaque hemorrhage. SMC: smooth muscle cells. *P<.1

TABLE 4 Multivariable analysis

Calcification	Risk of CVE HR [95% CI]	P value	Collagen	Risk of CVE HR [95% CI]	P-value
Operation year	0.94 [0.90-0.99]	0.013 *	Operation year	0.94 [0.90-0.98]	0.005 *
Diabetes	1.38 [1.03-1.84]	0.030 *	Leg amputation	1.89 [1.03-3.50]	0.041 *
Operation type	0.76 [0.57-1.02]	0.071	Operation type	0.83 [0.62-1.11]	0.213
History of CAD	1.25 [0.95-1.65]	0.112	Collagen rich plaque	1.27 [0.84-1.94]	0.263
Calcified plaque	1.13 [0.85-1.50]	0.413			

CVE: cardiovascular event. CAD: coronary artery disease. * P<.05

DISCUSSION

In total, the plaques of 497 PAD patients undergoing iliofemoral endarterectomy were analyzed. The association between plaque characteristics and secondary cardiovascular outcomes during 3-year follow-up was examined. No association between the investigated plaque characteristics and the composite cardiovascular endpoint was found. As the composite endpoint consisted for a large part of peripheral (re-) interventions, in subanalyses the endpoint was split into major CVE and peripheral events during follow-up. No association between plaque characteristics and major CVE or peripheral events was observed.

The lack of any association of the investigated plaque characteristics with secondary outcome in our cohort are in contrast to earlier observations in CEA plaques, and could result from multiple causes. First of all, little variation in plaque characteristics was observed within the iliofemoral tract, and the observed plaque characteristics mostly reflected stable plaque characteristics. Patients undergoing surgery for PAD in the lower limb often underwent a long trajectory of conservative and medical treatment prior to surgery, in which time the plaque has had time to remodel and stabilize[29]. Therefore, it could be that when investigating iliofemoral plaque characteristics, one is actually investigating end stage atherosclerotic plaques which does not necessarily represent plaques in other arteries and therefore does not predict clinically important secondary cardiovascular outcomes. Which is in contrast to CEA retrieved plaques as these patients are treated early after the initial cerebrovascular event[30]. This means CEA plaques are retrieved at another disease-stage, namely after a thromboembolic event causes symptoms, and the plaque may therefore reflect a more acute situation[31,32]. The removed iliofemoral plaques could represent a local more stable remodeling stage of atherosclerotic disease with most problems caused by a hemodynamic misbalance caused by narrowing of the lumen. In addition, it has been shown that asymptomatic CEA patients have more stable plaque characteristics as compared to symptomatic CEA patients[33,34].

Furthermore, PAD patients often present with multiple comorbidities and other risk factors, as is the case in our cohort (shown in Table 1). One would expect that patients with Fontaine IV complaints would have a worse prognosis compared to Fontaine IIb

or III. Some association between Fontaine IV and the composite endpoint was observed, but not significant (p=0.092). The reason no association was observed could be the fact that only a smaller amount of patients had major CVE during follow-up, whereas more patients had peripheral (re-)interventions – which was not associated with level of Fontaine classification in our cohort. The high prevalence of comorbidities and other risk factors could mean that the investigated plaque characteristics do not add additional information to the patient with already advanced atherosclerosis. However, a prospective study analyzing the association between plaque characteristics and the development of restenosis during follow-up after endarterectomy of the common or superficial femoral artery found an association between the removal of a collagen rich plaque and the incidence of peripheral restenosis[14]. In the current study, we did not observe an association between plaque characteristics and peripheral (re-)intervention in our cohort (Supplemental Table 2). There are several explanations for this discrepancy. First of all, the definitions of the endpoints "restenosis" and "peripheral (re-)intervention" differs. The measurement of restenosis was confirmed by echo duplex (peak systolic velocity (PSV) rate >2.5 or >50% lumen narrowing), which does not mean the patient underwent a re-intervention. In the protocol of this study cohort, no routine follow-up echo duplex was performed to assess patency of the treated artery. Furthermore, the peripheral endpoint is subject to changes in procedural protocols and the shift towards preventive medical treatment and guided walking therapy for patients with post-procedural leg complaints. This could also have led to a postponed re-intervention or a suspension of re-intervention. Collagen-rich plaques could therefore predict restenosis, but not necessary indicate an increased risk of re-intervention within 3 years of follow-up in our cohort.

No association between plaque characteristics and major CVE was observed in multivariable analysis, but the actual number of patients with a major CVE during follow-up was low which could have resulted in a lack of power for this subanalysis.

This study suffers from some limitations. First of all, as stated earlier, we studied the effect of plaque characteristics on secondary cardiovascular outcomes in an end-stage disease patient population with long-standing, and therefore probably remodeled and stabilized plaques. As a result, we cannot exclude effects of early plaque characteristics, rate of plaque progression or acute symptomatic plaque characteristics on secondary cardiovascular events.

Second, some of the plaque characteristics were scored in a semi-quantitative manner. The use of cut-off points for no/minor to moderate/heavy could have contributed to the observation that most plaques have similar stabilized characteristics. The use of quantitative data for all plaque characteristics is favorable, but unfortunately not available in our cohort. Although, great inter- and intra-observer similarities in the scoring of plaque characteristics were proved, and the same dedicated technical technician examined all slides[28].

Lastly, as stated earlier, the composition of the plaques has changed over the years. Similarly, protocols and indication for surgery have changed over the years as well,

with greater emphasis on conservative and (secondary) preventive treatment. Which could mean that when comparing secondary cardiovascular events with plaque characteristics, one must use contemporary data, and cohorts over a shorter period of time than for the current study.

Implication

Recently it has been shown that PAD patients have worse clinical prognosis when compared with cardiac or carotid disease[35]. These patients often have chronic complaints or invalidating symptoms, leading to a high disease burden for both patients and society[35]. Both for the surgeon and especially the patient, removing a certain plaque could possibly hold information regarding the prognosis of this patient. In addition, for example radiology studies could assess plaque characteristics when examining patients' plaques. Unfortunately, our study revealed no plaque characteristics to be used for this purpose. Further research should focus on other markers of atherosclerotic plaques, for example erosions on the plaque surface[36], in a continuous effort to establish possible risk factors in PAD patients.

CONCLUSION

In this cohort of PAD patients operated on for iliofemoral atherosclerotic disease, investigated atherosclerotic plaque characteristics were not associated with composite cardiovascular events during follow-up.

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SUPPLEMENTAL

SUPPLEMENTAL TABLE 1 Multivariable analysis on calcification and composite endpoint stratified for female sex

Multivariable analysis Stratified for female sex	Risk of primary outcome HR [95% CI]	P-value
Diabetes	1.76 [1.02-3.05]	0.044 *
Operation year	0.92 [0.85-1.00]	0.063
Current smoker	0.69 [0.40-1.19]	0.186
Calcification	1.35 [0.80-2.26]	0.259

* P<.05

SUPPLEMENTAL TABLE 2 Multivariate analysis on collagen and peripheral (re-)intervention

Multivariate analysis Total cohort	Risk of primary outcome HR [95% CI]	P-value
Operation type (TEA)	0.59 [0.42-0.82]	0.002 *
Previous leg amputation	2.74 [1.43-5.26]	0.002 *
Renal function (GFR)	0.99 [0.99-0.99]	0.016 *
Collagen	1.44 [0.87-2.40]	0.161

TEA: thrombendarterectomy. GFR: Glomerulation filtration rate. * P<.05

SUPPLEMENTAL TABLE 3 Multivariable analysis on calcification and major secondary CVE

Multivariate analysis Total cohort	Risk of primary outcome HR [95% CI]	P value
Diabetes	2.32 [1.29-4.17]	0.005 *
Sex	0.50 [0.27-0.89]	0.020 *
Age	1.03 [0.99-1.06]	0.132
Calcified plaque	1.12 [0.61-2.09]	0.712

CVE: Major cardiovascular endpoint (myocardial infarction, cerebrovascular event, cardiovascular-death). * P<.05

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CHAPTER 10

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

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ABSTRACT

Objective

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

Methods

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

Results

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

Conclusions

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

INTRODUCTION

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

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

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

METHODS

Patient population

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

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

For this study, the first entry of iliac and femoral endarterectomy for each unique patient was included, without any exclusion criteria. This enabled us to investigate the effect of one of the most prevalent comorbidities in PAD on plaque histology and to compare this with existing literature on other plaque domains such as carotid and coronary plaques.

Presence of DM was defined as presence of one of the following: 1) diabetes in medical history extracted from the patient file, 2) the use of either insulin or oral glucose inhibitors extracted from the patient file or 3) self-reported diabetes in the patient questionnaire. Duration of the disease was extracted from the patient questionnaire. A restenotic artery at baseline was defined as surgery on an artery already treated in the past, either by percutaneous intervention or by surgery.

Follow-up data during a three-year period was obtained through questionnaires sent to patients. Secondary cardiovascular events were validated using health records kept by general practitioners. Composite secondary cardiovascular events were defined as myocardial infarction (MI), stroke, cardiovascular death or peripheral intervention. Cardiovascular death was defined as one of the following: fatal MI, fatal stroke (either bleeding or ischemic), fatal ruptured abdominal aneurysm, fatal heart failure, sudden death. Stroke was defined as neurological symptoms lasting >24 hours and diagnosed by a neurologist as a stroke. A history of coronary artery disease was defined as either one of the following: MI, percutaneous coronary intervention or coronary artery bypass grafting surgery. Peripheral interventions consisted of either surgery or percutaneous events, including thrombolysis, in any artery other than the coronary arteries or the aorta. Patients could have had more than one event, but only the first occurrence of any of the secondary endpoints was used for the survival analyses on composite endpoints. To determine a measure of patency, target vessel revascularization (TVR) was defined during a three-year follow-up as peripheral reintervention on the same operation side and artery as the entry surgery. If patients suffered from more than one TVR, the first was used as an endpoint for the survival analysis of TVR-free survival.

The study was approved by respective medical ethics boards of both hospitals. The study is conducted in accordance with the declaration of Helsinki. All patients provided written informed consent prior to participation in this study.

Sample collection

A detailed description of the sample collection within the Athero-Express biobank can be found elsewhere[21]. In short, blood is collected preoperatively from the radial artery and subsequently stored at -80 degrees. The plaque is processed immediately after surgical removal. The culprit lesion is identified, stored in 4% formaldehyde, decalcified

(softening the calcification in the plaque for handling purposes without fully dissolving it) and embedded in paraffin for histological analysis. The rest of the plaque is cut into pieces of 5 mm and stored at -80 degrees.

Histological assessment

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

Statistical analysis

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

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

RESULTS

Baseline characteristics

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

Plaque phenotype

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

TABLE 1 Baseline characteristics

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

TABLE 1 Continued

	Patients without diabetes mellitus n = 453 n (%)	Patients with diabetes mellitus n = 198 n (%)	p - value
Use of antiplatelets	379 (84.0)	157 (79.3)	.14
Use of anti-coagulants	72 (15.9)	41 (20.7)	.14
Use of oral antidiabetics	-	136/198 (68.7)	*NA*
Use of insulin	-	64/198 (32.3)	*NA*
Years since diagnosis DM (median; IQR)		9.5 (4.0-15.3)	*NA*

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

TABLE 2 Association of diabetes with plaque phenotype

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

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

TABLE 3 Occurrences of clinical outcomes in patients with and without diabetes

	Patients without diabetes n = 453 (%)	Patients with diabetes n = 198 (%)
Composite endpoints[a]	198/439 (45.1)	112/196 (57.1)
Cardiovascular death	27/439 (6.2)	21/196 (10.7)
Coronary artery disease	35/439 (8.0)	15/196 (7.7)
Stroke	11/439 (2.5)	6/196 (3.1)
Peripheral intervention		
PTA or TEA	162/439 (36.9)	90/196 (45.9)
of which TVR[b]	*51/162 (31.5)*	*27/90 (30.0)*
Leg amputation	19/439 (4.3)	24/196 (12.2)
of which ipsilateral	*16/19 (84.2)*	*18/24 (75.0)*

CAD: coronary arterial disease, PTA: percutaneous transluminal angioplasty, TEA: thrombendarterectomy. Model corrected for hypercholesterolaemia, history of CAD, previous leg amputation, Fontaine classification and statin use.

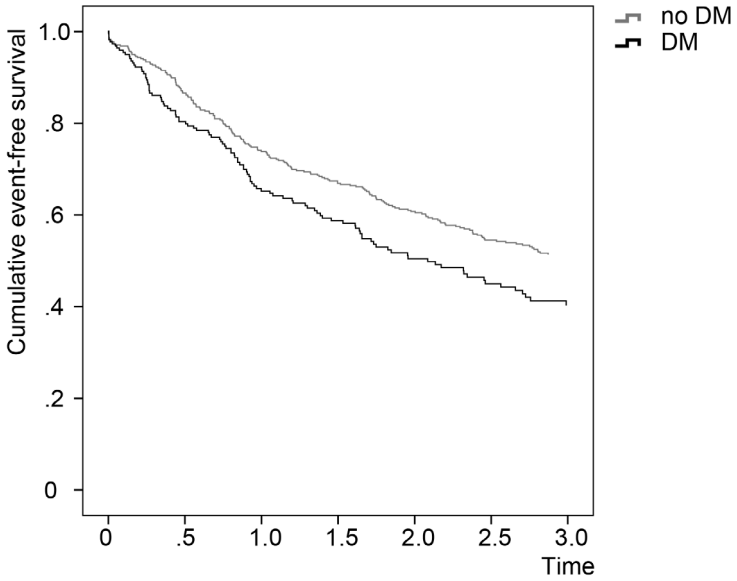
[a] Composite cardiovascular endpoints include cardiovascular death, coronary artery disease, stroke and peripheral intervention

[b] TVR: target vessel revascularization, intervention (either surgical or endovascular) on the artery that was operated on during the index surgery.

Clinical outcomes

To assess whether DM influences clinical outcome in patients with PAD, the incidence of composite secondary cardiovascular outcome rate during three-year follow-up was analyzed. Three-year follow-up information was available on 635/651 (97.5%) of patients (Table 3, Supplemental Figure 2). After correcting for possible confounders with the use of a multivariable cox regression model, DM was an independent predictor for secondary manifestations of cardiovascular disease (HR DM 1.36 (95% CI 1.07-1.72), P = .01) (Figure 1). In patients undergoing peripheral reintervention, no difference in TVR between DM patients (31.5%) and non-DM patients (30.0%) was observed, (Kaplan Meier mean survival estimate 2.16 years (1.88-2.43) in DM patients vs 2.16 years (1.97-2.35) in non-DM patients, P-log rank = .86 (Supplemental Figure 3)).

FIGURE 1 Cox survival plot for event-free survival for composite cardiovascular events of patients with and without diabetes



HR DM = 1.36 (95% CI 1.07-1.72), P=.01

Effect of restenosis

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

P = .06) as in the de novo operated patients (74.5% vs 55.7% moderate/heavy calcification in patients with and without diabetes respectively, P < .01).
Furthermore, analysis showed a similar effect of diabetes on composite event-free survival in patients suffering from restenosis (mean survival time estimate 1.5 (95% CI: 1.0-2.0) vs 1.9 (95% CI: 1.6-2.2) years for patients with and without diabetes respectively, log-rank test P = .17). Patients with diabetes who were operated on de novo lesions had a decreased composite event-free survival (mean survival estimate 1.8 (95% CI: 1.7-2.0) vs 2.2 (95% CI: 2.1-2.3) years for patients with and without diabetes respectively, log-rank test P = < .01, Supplemental Figure 4).

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

DISCUSSION

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

carotid arteries[24], further research into the effect of plaque characteristics on secondary clinical outcome is needed in PAD patients.

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

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

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

This study has some limitations. First, all patients underwent surgical therapy for PAD, which is most often preceded by a period of optimal conservative therapy. We are, therefore, most likely observing the remodelling phase of the peripheral plaque and cannot make any statements about plaque initiation. In fact, this study still observes large differences between DM and non-DM patients, maybe even pointing out that a more calcified plaque in patients with diabetes is a mere reflection of disturbed plaque

remodelling due to endothelial dysfunction in this patient group[8,9]. This mechanism could also be the explanation of the absence of DM-specific characteristics in plaques derived from semi-acutely operated patients with carotid artery disease[3]. Second, TVR was used as a proxy for patency rates, which is normally used to measure durability of vascular reconstruction. Unfortunately the current study protocol and ethical approval allow only for prospective data collection of events during follow-up with the use of questionnaires. Furthermore, as the UMC Utrecht and the St. Antonius Hospital Nieuwegein are tertiary referral centers, many of the patients return to their peripheral hospital after the surgery for postoperative follow-up. As a result, no systematic duplex follow-up for our patients is available, and patency, including patency rates, are hard to define in this cohort. The use of TVR could be an incomplete measure. Therefore, our results might underestimate restenosis. However, this effect is most likely equally divided between both patients with and without diabetes. Similarly, data regarding concomitant endovascular procedures, which could have a positive effect on the number of secondary peripheral procedures, were not available in the Athero-Express biobank study, so we were not able to correct for this effect. Yet, we do not think this effect differs between patients with and without diabetes and therefore cannot confound the observed effect. Furthermore, the Athero-Express Biobank Study unfortunately does not contain information regarding the type of diabetes of which patients suffer from. This prohibits us from making any claims as to what extent patients with type I diabetes differ from patients with type II diabetes. In addition, arteries below the knee were not covered by our biobank as these are either treated conservatively or with percutaneous or endovascular procedures. The large effect of DM on below the knee arteries could explain the increased rate of leg amputations[4,11]. Next, the Athero-Express Biobank Study does not collect information regarding previous radiation therapy for pelvic or inguinal malignancies. Although we think the occurrence of these therapies are evenly distributed between patients with and without diabetes, patients with diabetes have a higher chance of developing malignancies and as radiation therapy gives rise to arterial occlusive disease, this could have confounded the relationship between more diabetes and a more calcified plaque. Last, although our biobank has been collecting plaque material since 2002, a number of 198 patients with diabetes remains modest. Still our analysis holds, as far as we know, the first comparison of plaque characterization in a cohort of patients undergoing ilio-femoral endarterectomy. As the search for modifiable causative predictors of secondary cardiovascular outcome is ongoing, we would encourage other researchers to analyze large patient groups with a variety of cardiovascular risk factors and to collect long-term follow-up.

Overall, patients with diabetes, presenting with PAD requiring surgical revascularization belong to a very vulnerable subgroup of an already threatened group of patients. PAD patients often present with multiple other comorbidities and often do not achieve as adequate risk factor control as other (cardiovascular) patients[32]. We observed more secondary cardiovascular events during follow-up in the patient group with DM and

PAD, which leads to increased disease burden. Therefore, these patients need to be under tight surveillance to optimize disease control and (early) treatment of possible comorbidities[3,8].

CONCLUSION

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

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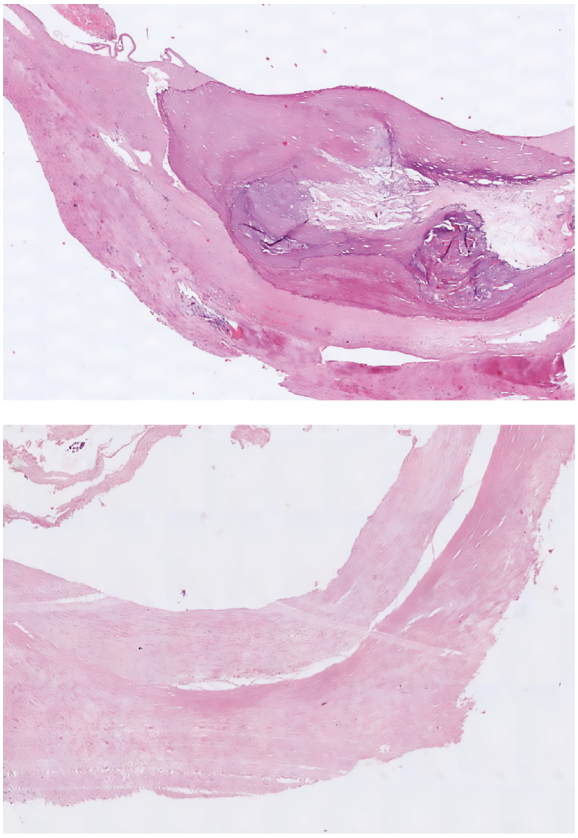
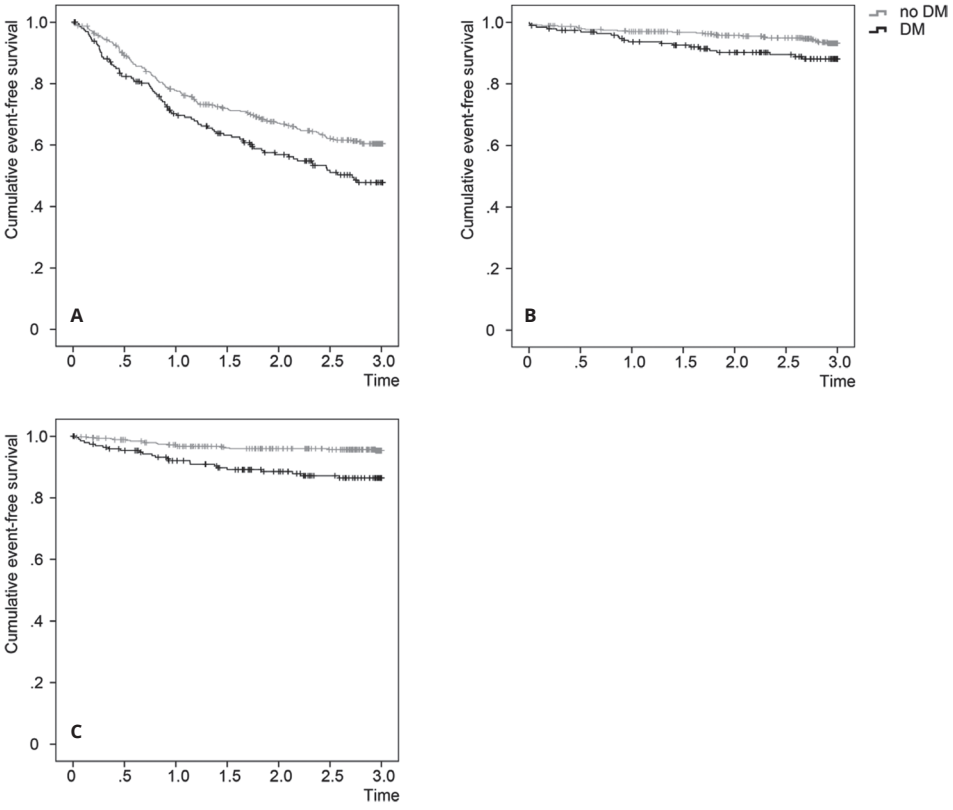
SUPPLEMENTAL

SUPPLEMENTAL FIGURE 1



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

SUPPLEMENTAL FIGURE 2



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

A. PERIPHERAL INTERVENTION

Patients at risk	t = 0	t = 0.5	t = 1	t = 1.5	t = 2	t = 2.5	t = 3
DM	192.5	149.5	118	99.5	80	57.5	22
No DM	432	375.5	316.5	276	245.5	185	71

P-log rank = <.01

B. CV DEATH

Patients at risk	t = 0	t = 0.5	t = 1	t = 1.5	t = 2	t = 2.5	t = 3
DM	434.5	419	399.5	378	358	291.5	114.5
No DM	192.5	182	169	158	140	113	46.5

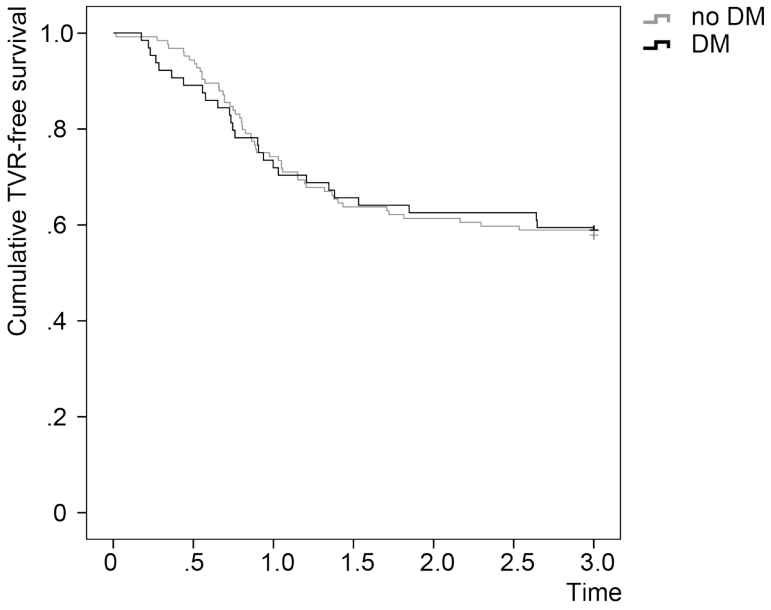
P-log rank = .03

C. AMPUTATION

Patients at risk	t = 0	t = 0.5	t = 1	t = 1.5	t = 2	t = 2.5	t = 3
DM	192.5	174.5	159.5	144.5	128.5	100.5	41
No DM	431.5	414	391	366.5	348	281.5	111.5

P-log rank = <.01

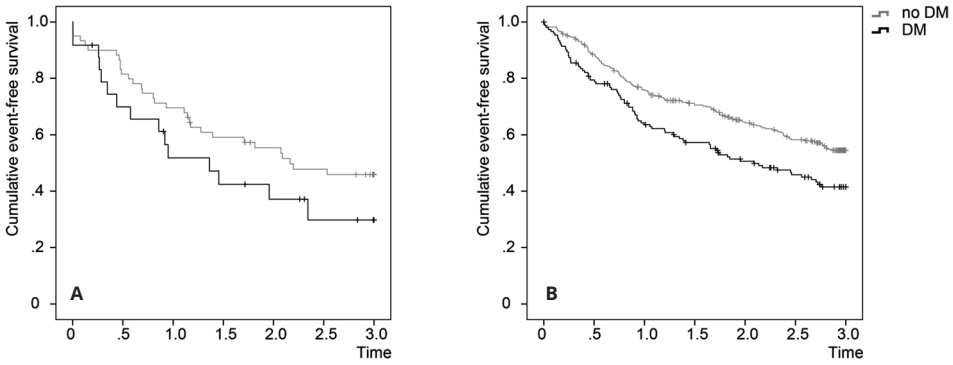
SUPPLEMENTAL FIGURE 3 Kaplan-Meier survival curve for TVR-free survival in patients undergoing peripheral revascularization with and without diabetes



Patients at risk	t = 0	t = 0.5	t = 1	t = 1.5	t = 2	t = 2.5	t = 3
DM	64	57	46	42	40	40	19.5
No DM	124	117	92	79	76	74	36.5

P-log rank = .86

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



A. RESTENOSIS

Patients at risk	t = 0	t = 0.5	t = 1	t = 1.5	t = 2	t = 2.5	t = 3
DM	23.5	15.5	11	8.5	6	3	1
No DM	59	48	40	32	29	22.5	9.5

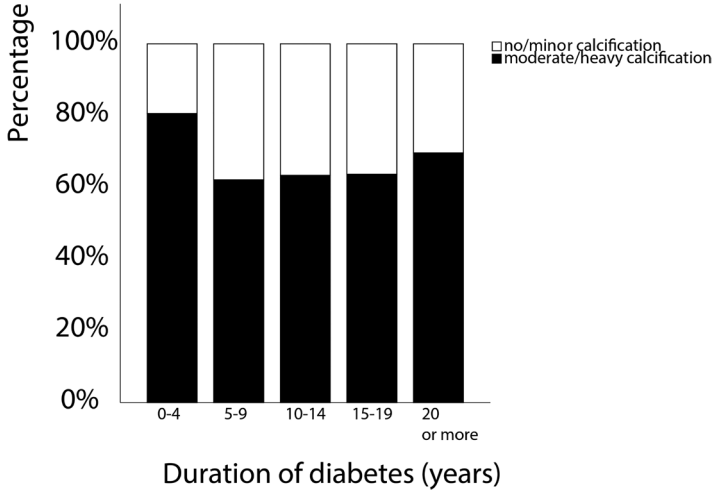
P-log rank = 0.17

B. DE NOVO

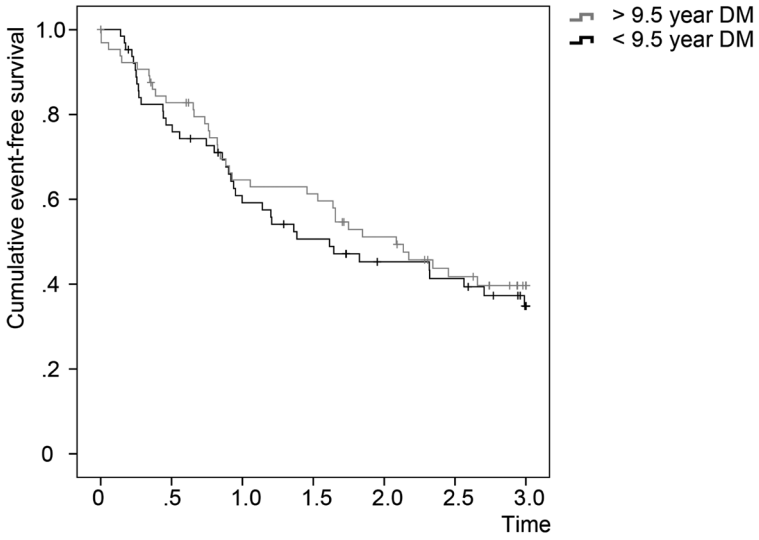
Patients at risk	t = 0	t = 0.5	t = 1	t = 1.5	t = 2	t = 2.5	t = 3
DM	150.5	116.5	90.5	77.5	63.5	48.5	18.5
No DM	330.5	288	238	210	184	136	49

P-log rank = <.01

SUPPLEMENTAL FIGURE 5 Distribution of no/minor calcification and moderate/heavy calcification for duration of diabetes in years



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



Patients at risk	t = 0	t = 0.5	t = 1	t = 1.5	t = 2	t = 2.5	t = 3
=< 9.5 y DM	63	48	35	29	23	21	13
> 9.5 y DM	65	52	39	37	29	21	12

P log-rank = .57

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CHAPTER 11

Sex-specific trends in the prognosis of patients with intermittent claudication and critical limb ischemia in The Netherlands in the period 1998-2010

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ABSTRACT

Objective

To evaluate absolute mortality risks and whether changes in mortality risks occurred in patients with intermittent claudication (IC) or critical limb ischemia (CLI) in the Netherlands between 1998-2010.

Methods

Data were obtained from Dutch nationwide registers between 1998 and 2010; Hospital Discharge Register, Population Register, and Cause of Death Register. The registers were used to obtain information regarding IC and CLI hospitalisations, comorbidities, demographic factors, date and cause of death. The cohort was split into two time periods for comparison: 1998-2004 (period A) and 2005-2010 (period B). 30-day mortality was excluded to exclude per-admission complications. One- and 5-year cardiovascular (CV) and all-cause mortality rates were compared to a representative sample of the general Dutch population (n=28,494) by Cox proportional hazard models.

Results

In total, 47,548 patients were included, of which there were 34,078 patients with IC and 13,470 with CLI. In patients with IC, age-adjusted 5-year CVD-mortality risk significantly decreased in period B (14%), compared with period A (16%) in male patients only (5-year adjusted HR: 0.76; 95%CI 0.69-0.83, p<0.001). In patients with CLI, CV-mortality risks significantly decreased only in female patients; from 31% in period A to 29% in period B (5-year adjusted HR: 0.84; 95%CI 0.74-0.94, p<0.01). Compared to the general population, patients with IC or CLI of both sexes had a 1.70 (1.58-1.83) to 5.19 (4.30-6.26) times increased mortality risk.

Conclusion

The risk of premature mortality for both patients with intermittent claudication and critical limb ischaemia significantly declined in the Netherlands, in a sex-specific manner over the period 1998-2010. Absolute risk of cardiovascular death remains high in patients with intermittent claudication and critical limb ischaemia.

INTRODUCTION

Peripheral artery disease (PAD) is a common atherosclerotic disease that presents with intermittent claudication (IC) or critical limb ischemia (CLI)[1]. Previous studies in PAD patients showed a 2 to 6-fold increase in risk of other cardiovascular manifestations of atherosclerotic disease, such as myocardial infarction (MI) and stroke, as compared to the general population. Similarly, a decreased Ankle Brachial Index (ABI) of <0.90 is associated with an increased mortality risk, compared with an ABI of 1.11-1.40 (HR 3.33). PAD patients also have a lower quality of life[2–4].

In the past two decades, endovascular techniques have been developed which have increased treatment options in PAD patients[5–8]. Simultaneously, advancements in the secondary prevention of cardiovascular disease and PAD have been made, including statin use, antiplatelet therapy, optimized blood pressure control and supervised exercise therapy[9–11]. Government policies such as the prohibition of smoking in public spaces and supported lifestyle adjustments for the total population may also improve outcomes[12–14].

Despite these developments, the estimated prevalence and disease burden of PAD has increased, in part due to the increased prevalence of diabetes and an ageing population[15–18]. In this study, mortality rates and age at first diagnosis or admission of men and women with IC or CLI in the Netherlands were analysed over a twelve year time frame. Analyses of men and women were performed separately, due to reported sex-dependent differences in cardiovascular disease severity in PAD patients[19], and as different time trends in the prevention of cardiovascular disease have been reported between both sexes[20]. The hypothesis was that advancements in revascularisation techniques and improved risk factor treatment have caused mortality rates to decline over time, possibly in a sex-specific manner.

METHODS

Cohort enrolment

Data were obtained from Dutch nationwide registers between 1998 and 2010: Hospital Discharge Register (HDR), Population Register (PR), and Cause of Death Register (CDR)[21]. The HDR contains data on hospital admissions, such as: admission and discharge data, diagnoses, surgical procedures, and the medical specialties concerned, which are recorded at discharge. The PR contains baseline information of all residents of the Netherlands. The CDR registers all causes of death in the Netherlands. The registers were used to obtain information regarding IC and CLI hospitalizations, comorbidities, demographic factors, and date and cause of death. By linking previous registers, all patients with a first hospitalization (multiple day admission to clinical ward: inpatient) or day-clinic visit (i.e. 1-day hospital admission) with primary diagnoses IC (ICD-9 code 443.9 or ICD-10 code I73.9) or CLI (ICD-9 code 785.4 and ICD-10 code I96 or I70.1)

between 1998 and 2010 were included. Patients who are diagnosed by primary care physicians and subsequently not referred to a hospital were not included in this study. Collected cases were linked with the PR by using the record identification number assigned to each resident in the Netherlands. The use of the unique record identification number enables the identification of different admissions, even in different hospitals, from the same person. Patients with IC or CLI with a previous admission for IC or CLI in the prior three years were excluded. To exclude per-admission complications, patients who died within 30 days after hospital admission were excluded from the cohort. Patients younger than 45 years old were excluded from the database, as a low percentage of correct ICD-codes within this age group was noted in a validation study, details of which are presented below. After 2004, the number of participating hospitals in the HDR declined, leading to an increase in missing cases. As a result, the percentage of missing cases varied between 3% to 14% between 2005 and 2010.
From the date of their first admission, patients were followed for 1-year and 5-year CV and all-cause mortality. CV death was defined by the following ICD-codes: I00-I99, R00-R012, Q20-Q289. These include fatal MI, fatal stroke, fatal ruptured aneurysm, and fatal heart failure. Patients were censored in case of death, emigration, or the end of the study period on December 31, 2010. To study time trends, the cohort was divided in two time periods, 1998-2004 (period A), and 2005-2010 (period B). To compare mortality rates with the general Dutch population (GDP), a representative sample from the PR was drawn. Age-strata of one year were used and 500 random individuals per age-sex stratum ≥45 years were selected from all persons living at January 1th 2005 in the Netherlands.

Data governance
All linkages and analysis were performed in a secure environment of Statistics Netherlands and in agreement with the privacy legislation in the Netherlands[22]. Only anonymized records and data sets are involved. The study did not have to be assessed according to the regulations of the Research complying with the Dutch law on Medical Research in Humans.

Comorbidity
Presence and extent of comorbidity were determined with the Charlson comorbidity index (CCI) score, based on discharge diagnoses in the HDR from 1995 to the date of hospitalization. The Charlson index score ranges from zero to six (with zero representing no comorbidity). It proved to be a reliable and valid method to measure comorbidity in clinical research[23].

Validation study
A validation study was performed to examine the correctness of the IC and CLI diagnostic ICD-9 and ICD-10 codes used. This was done by cross checking these codes with patient files in the University Medical Center Utrecht (UMCU) by S.H.[24].

Data analyses

Continuous data were summarized as mean and standard deviation (SD), or as median and interquartile range where appropriate. Categorical data were summarized as whole percentages. Trends in mean age were tested by independent t-tests. Analyses were conducted separately for men and women. Absolute mortality risks in 1998-2004 and 2005-2010 were computed by age and sex according to the actual life table method and expressed as percentages. Hazard ratios with 95% confidence intervals (95% CI) from Cox regression models were used to estimate the differences in prognosis between period A and period B and for differences in mortality risks between patients with IC or CLI and the general population in 2005-2010. Survival was determined at 1- and 5-years after inclusion. Models were adjusted for age, sex, marital status, ethnic origin and CCI. Statistical analysis was performed with SPSS software version 20.0 and SAS version 9.2. A two sided p-value <0.05 was considered statistically significant.

RESULTS

Baseline characteristics

A total of 47,548 patients were included between 1998 and 2010, consisting of 34,078 patients with IC and 13,470 with CLI. 64% of patients with IC and 56% of those with CLI were male. Mean age was higher in patients with CLI compared to patients with IC and in female patients compared to male patients, as shown in table 1. More patients with CLI were admitted to the inpatient ward than those with IC (93% vs 62%). The CCI score was higher in patients admitted to the hospital and in patients with CLI. The sample of the general population consisted of 11,029 men and 17,465 women (table 1).

TABLE 1 Baseline characteristics of patients with a first hospitalization or day clinic visit for Intermittent Claudication and Critical Limb Ischemia in the Netherlands between 1998 and 2010

	Intermittent Claudication				Critical Limb Ischemia				General Population	
	Day clinic visit		Inpatient		Day clinic visit		Inpatient			
	Men	Women	Men	Women	Men	Women	Men	Women	Men	Women
Number of patients	8,334	4,461	13,408	7,875	496	423	7,069	5,482	11,029	17,465
Age (mean ± SD)	66 ± 10	67 ± 11	67 ±10	69 ±11	66 ± 12	68 ± 13	70 ± 11	74 ± 12	68 ± 15	76 ±17
Marital status (% married or living together)	68	40	67	35	63	43	56	28	64	31
Ethnic origin (native %)	88	88	89	89	88	89	89	88	88	89
Charlson comorbidity index (%)										
0	67	73	62	67	57	59	43	50	82	84
1-2	28	23	31	28	35	36	44	39	16	15
≥3	5	4	7	5	8	5	13	11	2	1
Median follow up years (Interquartile Range)	5.4 (2.5-8.4)	5.6 (2.7-8.6)	4.2 (1.7-7.7)	4.3 (1.8-7.8)	2.9 (1.1-6.4)	3.6 (1.3-6.7)	2.2 (0.7-4.8)	2.2 (0.6-4.8)	5.9 (4.5-5.9)	5.9 (2.6-5.9)

Trends in the prognosis of patients with IC

Mortality

In men, 1-year CV mortality was significantly lower in period B (4%) compared with period A (5%) (adjusted HR 0.68; 95%CI 0.59-0.79; p<0.001). In women, no significant change over time was observed in 1-year CV mortality (6% and 5%, respectively) (adjusted HR 0.92; 95%CI 0.78-1.10; p=0.32) (table 2.1 and figure 1).

Similarly, 5-year CV mortality in male patients was significantly lower in period B (14%) as compared to period A (16%) (adjusted HR 0.76; 95%CI 0.69-0.83; p<0.001). In female patients, a non-significant increase in 5-year CV mortality (15% to 16%) was observed (adjusted HR 0.90; 95%CI 0.80 to 1.01; p=0.06) (table 2.2 and figure 1).

Age stratified analyses showed a 1-year CV mortality decline mainly in men aged 55 to 84, and a 5-year CV mortality decline in men aged 65 to 84 years. Age- and sex-stratified Kaplan Meier curves for all-cause mortality in patients with IC are provided as supplemental material (figure 2 and supplemental figure 1).

FIGURE 1

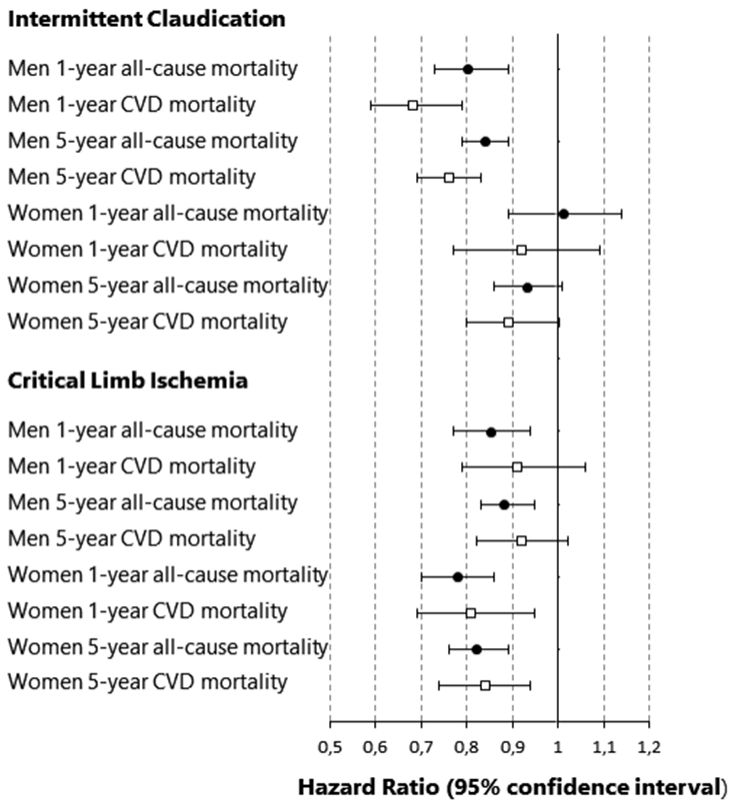


TABLE 2.1 Time trend in 1-year cardiovascular disease (CVD) and all-cause mortality in patients with a first hospitalization or day clinic visit for Intermittent Claudication (IC) in the Netherlands between 1998 and 2010, by age, sex and period

	All-cause mortality			CVD mortality		
	1998-2004 (%)	**2005-2010 (%)**	**Adjusted HR[a] (95% CI)**	**1998-2004 (%)**	**2005-2010 (%)**	**Adjusted HR[a] (95% CI)**
Men						
45-54 years	2	1	0.49 (0.23-1.05), P=0.07	1	1	0.72 (0.23-2.21), P=0.56
55-64 years	4	4	0.95 (0.71-1.26), P=0.71	2	2	0.58 (0.36-0.92), P=0.02
65-74 years	10	8	0.70 (0.58-0.83), P<0.001	5	3	0.56 (0.42-0.73), P<0.001
75-84 years	18	16	0.81 (0.69-0.94), P<0.01	11	8	0.68 (0.54-0.84), P<0.01
≥85 years	34	38	1.05 (0.78-1.42), P=0.73	18	24	1.23 (0.81-1.86), P=0.33
Total	**9**	**9**	**0.80 (0.73-0.89), P<0.001**	**5**	**4**	**0.68 (0.59-0.79), P<0.001**
Women						
45-54 years	2	2	1.02 (0.46-2.29), P=0.95	1	1	1.17 (0.39-3.53), P=0.78
55-64 years	3	4	0.96 (0.60-1.52), P=0.84	2	1	0.61 (0.28-1.32), P=0.21
65-74 years	7	8	1.02 (0.79-1.32), P=0.87	4	3	0.81 (0.55-1.20), P=0.30
75-84 years	14	15	1.02 (0.83-1.25), P=0.84	8	9	1.08 (0.83-1.41), P=0.57
≥85 years	34	33	0.92 (0.72-1.18), P=0.53	23	21	0.81 (0.59-1.11), P=0.18
Total	**9**	**11**	**1.01 (0.89-1.14), P=0.94**	**5**	**6**	**0.92 (0.77-1.09), P=0.32**

[a] Hazard Ratio's (HR) adjusted for age, marital status, ethnic origin, Charlson Index and type of care (day clinic visit or inpatient).

Mean age of patients with IC

Patients with IC first admitted to the hospital in period A had a mean age of 66.4 (male) and 68.3 years (female). This significantly increased in period B to 67.4 (p<0.001) and 69.7 years (p<0.001), respectively. Patients attending hospital as a day-case or for a clinic appointment (not admitted) had a mean age of 65.6 (male) and 66.6 years (female) in period A, which significantly increased to 66.3 years in men (p<0.01), and non-significantly increased to 67.2 years in women (p=0.10) (Supplemental table 2).

Trends in the prognosis of patients with CLI

Mortality

In men, no difference in 1- and 5-year CV mortality was observed over time (11% and 30%, respectively). In female patients, there was a significant decline in both 1-year and 5-year CV mortality. The 1-year CV mortality decreased from 13% to 12% (adjusted

HR 0.81; 95%CI 0.69-0.95; p=0.01), and 5-year CV mortality decreased from 31% to 29% (adjusted HR 0.84; 95%CI 0.74-0.94; p<0.01). In both male and female patients, 1- and 5-year all-cause mortality significantly declined in period B compared with period A (table 2.3, table 2.4 and figure 1).
Age stratified analyses in female patients showed no significant decrease in CV mortality in any particular age group. Age-stratified all-cause mortality showed a significant decline across several age groups in both sexes (figure 2 and supplemental figure 1).

Mean age of patients with CLI

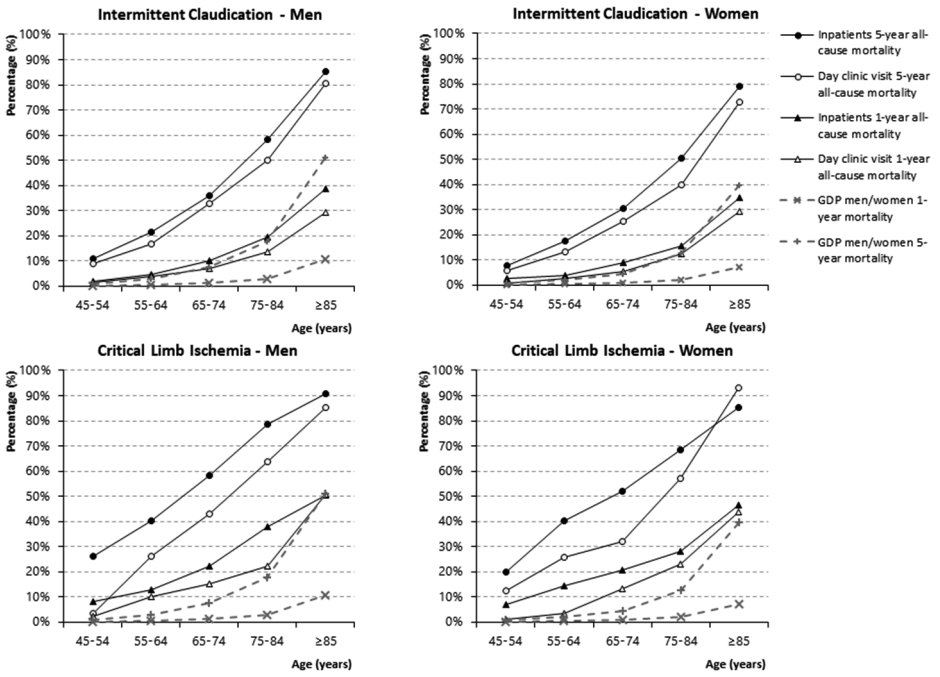
The mean age of patients with CLI first admitted to the hospital in period A was 69.2±11 years in men and 73.6±12 years in women, which did not change over time (supplemental table 2).

TABLE 2.2 Time trend in 5-year cardiovascular disease (CVD) and all-cause mortality in patients with a first hospitalization or day clinic visit for Intermittent Claudication (IC) in the Netherlands between 1998 and 2010, by age, sex and period

	All-cause mortality			**CVD mortality**		
	1998-2004 (%)	**2005-2010 (%)**	**Adjusted HR (95% CI)**	**1998-2004 (%)**	**2005-2010 (%)**	**Adjusted HR (95% CI)**
Men						
45-54 years	11	8	0.58 (0.39-0.84), P<0.01	4	3	0.55 (0.29-1.05), P=0.07
55-64 years	20	18	0.89 (0.77-1.04), P=0.13	8	7	0.81 (0.63-1.03), P=0.08
65-74 years	36	32	0.79 (0.71-0.87), P<0.001	17	14	0.70 (0.60-0.82), P<0.001
75-84 years	56	54	0.87 (0.79-0.95), P<0.01	32	28	0.76 (0.65-0.87), P<0.001
≥85 years	84	77	0.95 (0.77-1.18), P=0.65	55	48	1.01 (0.75-1.38), P=0.93
Total	**33**	**32**	**0.84 (0.79-0.89), P<0.001**	**16**	**14**	**0.76 (0.69-0.83), P<0.001**
Women						
45-54 years	7	6	0.87 (0.54-1.39), P=0.56	3	3	1.01 (0.49–2.12), P=0.97
55-64 years	16	15	0.93 (0.73-1.20), P=0.58	7	5	0.73 (0.49-1.09), P=0.12
65-74 years	28	29	0.96 (0.83-1.12), P=0.62	13	14	0.89 (0.70-1.12), P=0.31
75-84 years	47	47	0.88 (0.78-1.00), P=0.06	26	27	0.91 (0.76-1.08), P=0.28
≥85 years	78	76	0.92 (0.78-1.10) P=0.35	55	47	0.87 (0.69-1.10), P=0.24
Total	**30**	**32**	**0.93 (0.86-1.01), P=0.08**	**15**	**16**	**0.89 (0.80-1.003), P=0.06**

[a] Hazard Ratio's (HR) adjusted for age, marital status, ethnic origin, Charlson Index and type of care (day clinic visit or inpatient).

FIGURE 2



Comparison to the general population

Patients with IC or CLI of both sexes had a 2 to 5-fold increase in 1- and 5-year CV and all-cause mortality risk, as compared to the general population. A higher 1-year CV mortality risk was observed in female patients with IC (HR 3.20, 95% CI 2.69-3.81) than in male patients with IC (HR 2.75, 95% CI 2.23-3.40). Five-year mortality risk increased equally in both sexes as compared to the general population.

Compared to the general population, 1- year CV mortality risks in patients with CLI were higher in men (HR 5.19, 95% CI 4.30-6.26) than in women (HR 3.74, 95% CI 3.19-4.38). Five-year mortality risks remained significantly higher in men than in women. Mortality risk of day clinic patients with IC or CLI were consistently lower than that of patients admitted to the hospital (table 3).

Validation Study

Between the years 1998-2010, a total sample of n=832 patients from the UMCU were examined[24]. In 73% of patients with IC, the discharge diagnosis ICD-9 code 443.9 was correct, 20% of patients were actually suffering from CLI, and 7% of the patients did not present with atherosclerotic disease. The percentage of misclassification was high in patients younger than 45 years old (>50%); we therefore decided to exclude these patients from the cohort (5% of total cohort). In the remaining cohort, we observed

that misclassification increased in patients with older age and differed between period A (26%) and period B (14%).
In 72% of patients with CLI, the discharge diagnosis ICD-9 code 785.4 was correct, and 25% of the patients were not found to have PAD. Of these, most were treated for a skin infection (12%) or necrosis of a surgical wound (6%). The percentage of misclassification was especially high in patients younger than 45 years old (>50%) and it was therefore decided to exclude these patients from the cohort (10% of total cohort) (supplementary table 1).

TABLE 2.3 Time trend in 1-year cardiovascular disease (CVD) and all-cause mortality in patients with a first hospitalization or day clinic visit for Critical Limb Ischemia (CLI) in the Netherlands between 1998 and 2010, by age, sex and period

	All-cause mortality			CVD mortality		
	1998-2004 (%)	**2005-2010 (%)**	**Adjusted HR (95% CI)**	**1998-2004 (%)**	**2005-2010 (%)**	**Adjusted HR (95% CI)**
Men						
45-54 years	8	8	0.87 (0.51-1.47), P=0.60	1	2	1.48 (0.50-4.44), P=0.48
55-64 years	12	13	0.98 (0.73-1.31), P=0.90	4	5	1.44 (0.87-2.38), P=0.16
65-74 years	23	20	0.81 (0.67-0.99), P=0.04	9	9	0.87 (0.64-1.19), P=0.39
75-84 years	38	37	0.89 (0.77-1.03), P=0.13	18	18	0.88 (0.70-1.10), P=0.26
≥85 years	54	47	0.80 (0.64-0.99), P=0.048	30	27	0.87 (0.64-1.19), P=0.39
Total	**26**	**25**	**0.85 (0.77-0.94), P=0.001**	**11**	**11**	**0.91 (0.79-1.06), P=0.22**
Women						
45-54 years	6	6	1.01 (0.51-2.01), P=0.97	1	1	0.96 (0.19-4.78), P=0.96
55-64 years	15	10	0.63 (0.4-0.98), P=0.04	5	2	0.50 (0.20-1.27), P=0.14
65-74 years	21	19	0.76 (0.59-0.99), P=0.04	9	8	0.80 (0.53-1.20), P=0.28
75-84 years	29	26	0.78 (0.65-0.94), P<0.01	15	13	0.81 (0.62-1.06), P=0.12
≥85 years	50	42	0.84 (0.74-0.95), P<0.01	28	25	0.86 (0.67-1.09), P=0.21
Total	**27**	**24**	**0.78 (0.70-0.86), P<0.001**	**13**	**12**	**0.81 (0.69-0.95), P=0.01**

[a] Hazard Ratio's (HR) adjusted for age, marital status, ethnic origin, Charlson Index and type of care (day clinic visit or inpatient).

TABLE 2.4 Time trend in 5-year cardiovascular disease (CVD) and all-cause mortality in patients with a first hospitalization or day clinic visit for Critical Limb Ischemia (CLI) in the Netherlands between 1998 and 2010, by age, sex and period

	All-cause mortality			CVD mortality		
	1998-2004 (%)	**2005-2010 (%)**	**Adjusted HR (95% CI)**	**1998-2004 (%)**	**2005-2010 (%)**	**Adjusted HR (95% CI)**
Men						
45-54 years	24	23	0.92 (0.66-1.27), P=0.60	7	10	1.40 (0.80-2.45), P=0.24
55-64 years	40	39	0.94 (0.79-1.13), P=0.52	16	19	1.21 (0.91-1.63), P=0.20
65-74 years	59	54	0.86 (0.76-0.98), P=0.03	30	26	0.85 (0.69-1.04), P=0.12
75-84 years	79	78	0.88 (0.79-0.99), P=0.03	45	43	0.88 (0.74-1.05), P=0.16
≥85 years	90	92	0.89 (0.75-1.06), P=0.18	66	56	0.86 (0.67-1.11), P=0.26
Total	**60**	**58**	**0.88 (0.83-0.95), P<0.001**	**30**	**30**	**0.92 (0.82-1.02), P=0.11**
Women						
45-54 years	21	16	0.69 (0.44-1.07), P=0.10	6	2	0.34 (0.11-1.02), P=0.06
55-64 years	39	42	0.88 (0.67-1.16), P=0.36	13	16	0.77 (0.45-1.33), P=0.35
65-74 years	52	48	0.76 (0.59-0.99), P=0.01	26	23	0.79 (0.59-1.06), P=0.12
75-84 years	69	66	0.82 (0.68-0.98), P=0.03	39	37	0.87 (0.72-1.05), P=0.16
≥85 years	87	84	0.74 (0.62-0.88), P=0.001	56	55	0.88 (0.72-1.07), P=0.19
Total	**60**	**57**	**0.82 (0.76-0.89), P<0.001**	**31**	**29**	**0.84 (0.74-0.94), P<0.01**

DISCUSSION

In this nationwide, hospital-based population study, including 47,548 patients with PAD, a significant decline in mortality rates over time was observed (period B; 2005-2010 compared with period A; 1998-2004). This decline was markedly different in patients with IC or CLI, in men and women, and across age groups. The absolute 1- and 5-year CV and all-cause mortality rates of patients with IC or CLI remained high in period B. Currently, the mortality risks of patients with IC or CLI are 2 to 5 times higher compared to the general population.

In patients with IC, overall 1- and 5-year CV mortality rates in period B were 4% and 14% in male and 6% and 16% in female patients, respectively. This is in line with a recent meta-analysis, which found a 5-year cumulative CV mortality of 13%[25]. A significant decrease in mortality rates was only observed in male patients. Several

mechanisms may have resulted in the improved prognosis of male patients, including advances in preventive, medical, and interventional treatment[5,6,9,13]. A recent study observed that four out of six major risk factors for coronary heart disease showed a favourable or stable trend, but also noted a rise in BMI and increased prevalence of diabetes in both men and women[26]. However, the reason why mortality rates of the female patient population did not improve remains unclear. Several factors could underlie this observation. First, a larger proportion of the female population started smoking during the second half of the 20th century[27].This could have led to an increase in the total number of female PAD patients and worsened their prognosis. On the other hand, smoking cessation is associated with decreased mortality in PAD patients[14], and in the last two decades the percentage of female smokers has decreased at the same rate as male smokers, except for women aged 65 and older[26]. Second, decreased mortality in male patients may also be secondary to an increase in physician awareness of early stage PAD in men, giving them the benefit of earlier secondary prevention. In addition, female patients more often suffer from atypical symptoms or asymptomatic PAD[19,28–30]. This may result in delayed or under-treatment of women. Lastly, the differences in sex could result from yet unidentified risk factors in PAD women, possibly due to a differential effects on the microvasculature[31].

In patients with CLI, 1- and 5-year CV mortality rates in period B were 11% and 30% in male and 12% and 29% in female patients, respectively. Female patients with CLI had a significant decrease in CV mortality in period B compared with period A, but not male

TABLE 3 Difference in risk on 1- and 5-year CVD and all-cause mortality in IC and CLI patients compared to the Dutch general population between 2005-2010

		Adjusted HR (95% CI) Intermittent Claudication patients vs. Dutch general population		Adjusted HR (95% CI) Critical Limb Ischemia patients vs. Dutch general population	
	Type of care	**All-cause mortality**	**CVD mortality**	**All-cause mortality**	**CVD mortality**
Men					
1-year mortality	Day clinic visit	1.51 (1.23-1.84)*	2.02 (1.45-2.82)*	2.99 (2.08-4.30)*	4.55 (2.60-7.95)*
	Inpatients	2.06 (1.79-2.36)*	3.08 (2.47-3.84)*	3.62 (3.23-4.07)*	5.24 (4.33-6.32)*
	Total patients	1.85 (1.63-2.11)*	2.75 (2.23-3.40)*	3.57 (3.19-4.01)*	5.19 (4.30-6.26)*
5-year mortality	Day clinic visit	1.53 (1.37-1.71)*	1.87 (1.56-2.26)*	2.68 (2.08-3.46)*	3.77 (2.56-5.54)*
	Inpatients	1.93 (1.78-2.09)*	2.64 (2.32-3.01)*	3.08 (2.86-3.32)*	4.03 (3.56-4.55)*
	Total patients	1.76 (1.63-1.89)*	2.34 (2.08-2.64)*	3.04 (2.83-3.28)*	3.99 (3.53-4.50)*
Women					
1-year mortality	Day clinic visit	1.84 (1.46-2.31)*	2.78 (2.00-3.87)*	2.23 (1.43-3.47)*	2.51 (1.24-5.06)*
	Inpatients	2.31 (2.03-2.63)*	3.51 (2.91-4.23)*	2.88 (2.59-3.19)*	3.83 (3.27-4.49)*
	Total patients	2.11 (1.87-2.38)*	3.20 (2.69-3.81)*	2.81 (2.53-3.11)*	3.74 (3.19-4.38)*
5-year mortality	Day clinic visit	1.58 (1.37-1.81)*	2.18 (1.78-2.68)*	1.82 (1.35-2.45)*	1.83 (1.10-3.05)*
	Inpatients	1.81 (1.69-1.97)*	2.52 (2.23-2.85)*	2.28 (2.12-2.45)*	2.81 (2.52-3.15)*
	Total patients	1.70 (1.58-1.83)*	2.36 (2.10-2.64)*	2.24 (2.09-2.40)*	2.74 (2.46-3.07)*

[a] Hazard Ratio's (HR) adjusted for age, marital status, ethnic origin, Charlson Index. *P-value <0.05

patients with CLI, which is opposite to the trend observed in patients with IC. Also, CV mortality was higher in female patients compared with male patients in period A, and decreased to similar levels in period B. We are unable to fully explain why the mortality rates in female patients were higher in period A, but might be due to factors stated in the previous section. Male patients with CLI in the current cohort presented with more comorbidities compared with female patients, reflected in a higher CCI score (table 1), and may present with more severe systemic atherosclerotic disease[32]. Consequently, one would expect somewhat higher mortality rates in males as opposed to females, but this was not observed in the current cohort. However, several female-specific risk factors for PAD have been identified which may not be represented in these CCI scores[33]. This could have led to an underestimation of disease burden in female patients.

Patients with IC or CLI of both sexes had a 2 to 5-fold increase in 1- and 5-year all-cause and CV mortality risk compared to the general population (table 3). The 5-year mortality risks were somewhat lower than the 1-year mortality risks, but still considerable. In our opinion, these results reflect the high mortality risks of PAD patients within the first year after diagnosis. Symptomatic PAD patients may present with a much more advanced disease burden than one might expect based on a patient symptoms. It may be difficult to prevent a secondary cardiovascular event from occurring when a patient is already severely affected by systemic atherosclerotic disease. In addition, optimizing secondary preventive care proves difficult in these patients[5,13,34].

The main strengths of this study are its large sample size due to the use of nationwide registries, a standard and verified methodology to analyse all-cause and CV mortality, and a comparison with a sample of the Dutch population. A clear overview of trends in mortality in the PAD population over the period 1998-2010 is provided.

The use of current methodology has some limitations. First, patients were identified using hospital discharge codes which may contain errors. Nonetheless, earlier analysis revealed a high overall quality of recording these codes in the Netherlands[21,24]. To determine the accuracy of the ICD-codes used for PAD patients, these were cross-checked within the UMCU hospital. The main observation was that 73% of IC-codes and 72% of CLI-codes were accurate (supplemental table 1). In total, 20% of the patients with a discharge diagnosis for IC actually presented with CLI complaints. This could have resulted in overestimation of disease severity in IC patients. Furthermore, the percentage of patients with CLI complaints in period B was lower than in period A. This could explain at least some of the improvement in mortality rates observed in period B compared to period A, especially in patients aged >85 years.

Some 25% of the patients with a discharge diagnosis for CLI did not have PAD. Most of these patients were admitted for skin infection (13%) or necrosis of a surgical wound (6%). The long-term survival of these patients is probably higher compared to patients with CLI, leading to a possible underestimation of disease severity in CLI patients.

The digital database has patient data from 1995 onwards. To determine 'first' event PAD patients, a cut-off point of three years without PAD diagnosis was chosen, which

allowed analysis of first events in patients from 1998 onwards. However, it is possible that some patients would have been admitted for peripheral arterial disease more than three years before 1998. These patients may have a poorer prognosis than those patients with a true first admission, with potential overestimation of disease burden. Patients with IC or CLI have been compared with available data of the general population from 2005-2010. An increase in life-expectancy in the general population, including people aged 65 and older (which did occur for both sexes in 2005-2010 compared with 1998-2004[35]), could have increased the mortality risks in patients in period A, as they were compared with the general population in 2005-2010. This could have resulted in a slight overestimation of mortality risks in the total cohort of patients with IC or CLI in period A.

There was a decline in the number of hospitals participating in the HDR (between 2005 and 2010 the percentage of hospitals not participating varied between 3% and 14%, before 2005 it was 1%). Consequently, the incidence of PAD in period B may be underestimated compared to period A. The hospitals no longer participating in the HDR represent a variety of hospitals both in size and geography; therefore, it is unlikely that any selection bias has occurred. The decline in participating hospitals did not alter the possibility to follow current included patients over time and the mortality percentages of included patients in both periods will probably not have been affected as a result.

The current study shows a significant decreasing trend in mortality risks in this vulnerable patient group which may reflect an improvement in secondary preventive, conservative and interventional care. Nonetheless, absolute mortality risks remain high, especially when compared to the general population. When diagnosing PAD, clinicians should base their additional cardiovascular workup on the severity of PAD. This might lead to earlier detection and treatment of other affected vascular beds and potential risk factors, and may help to improve the outcome in PAD patients in the future.

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SUPPLEMENTAL

SUPPLEMENTAL TABLE 1 Validation of ICD-codes in the UMCU

CLI Age groups	Total n	CLI %	CI %	No PAD %
45-54	24	54	0	46
55-64	26	65	0	35
65-74	55	76	7	16
75-84	30	87	0	13
>85	6	67	0	33
Total:	**141**	**72**	**3**	**25**

CI Age groups	Total n	CI %	CLI %	No PAD %
45-54	87	83	7	10
55-64	170	79	14	7
65-74	207	75	20	5
75-84	116	63	30	7
>85	29	38	52	10
Total:	**609**	**73**	**20**	**7**

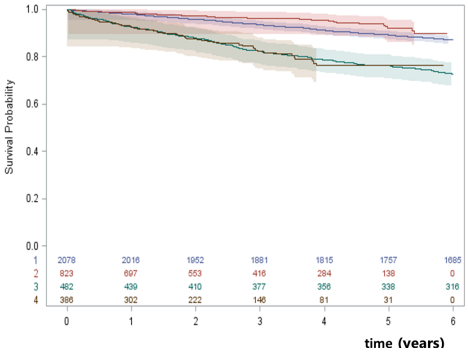
SUPPLEMENTAL TABLE 2 Trends in mean age of patients with a first hospitalization or day clinic visit for Intermittent Claudication and Critical Limb Ischemia in the Netherlands between 1998-2004 and 2005-2010

	Intermittent Claudication				Critical Limb Ischemia			
	Day clinic visit		Inpatient		Day clinic visit		Inpatient	
	Men	Women	Men	Women	Men	Women	Men	Women
Age (mean ± SD)	65.7 ± 10	66.8 ± 11	66.7 ± 10	68.8 ± 11	65.7 ± 12	67.5 ± 13	69.2 ± 11	73.5 ± 12
Age (mean ± SD) 1998 - 2004	65.6 ± 10	66.6 ± 11	66.4 ± 10	68.3 ± 11	66.2 ± 12	67.0 ± 13	69.2 ± 11	73.6 ± 12
Age (mean ± SD) 2005 - 2010	66.3 ± 10	67.2 ± 12	67.4 ± 10	69.7 ± 11	65.1 ± 12	68.0 ± 14	69.3 ± 11	73.5 ± 13
1998-2004 vs. 2005-2010 P-value	<0.01*	0.10	<0.001*	<0.001*	0.35	0.46	0.60	0.82

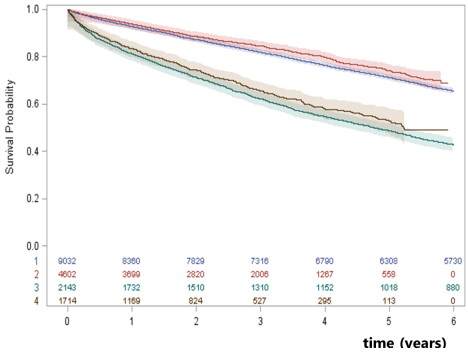
* P-value <0.05

SUPPLEMENTAL FIGURE 1

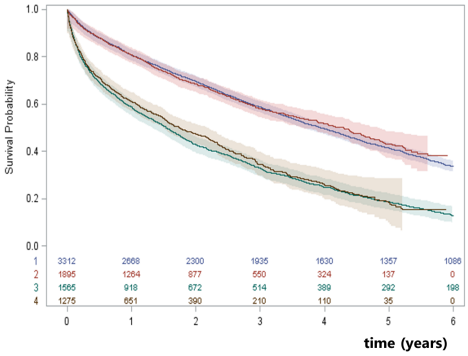
MEN AGE GROUP 45-54 YEARS



MEN AGE GROUP 55-74 YEARS

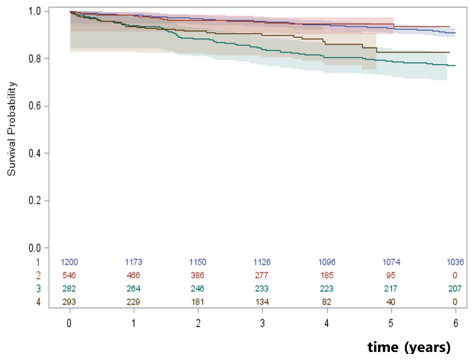


MEN AGE GROUP ≥ 75 YEARS

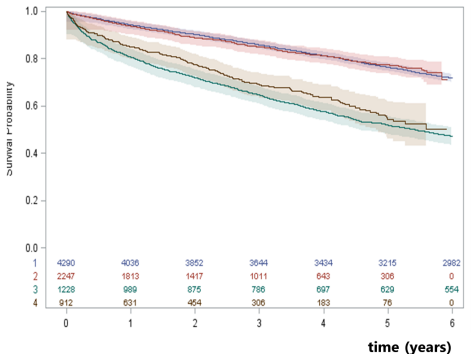


1= IC 1998-2004, 2 = IC 2005-2010, 3 = CLI 1998-2004, 4 = CLI 2005-2010,
with numbers at risk and 95% Hall-Werner bands

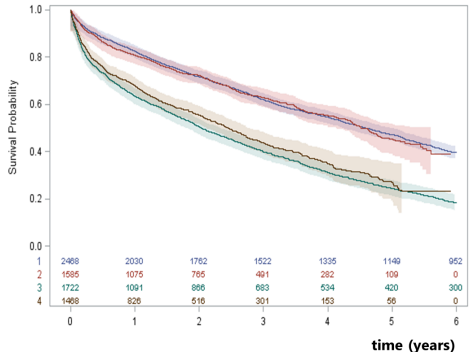
WOMEN AGE GROUP 45-54 YEARS



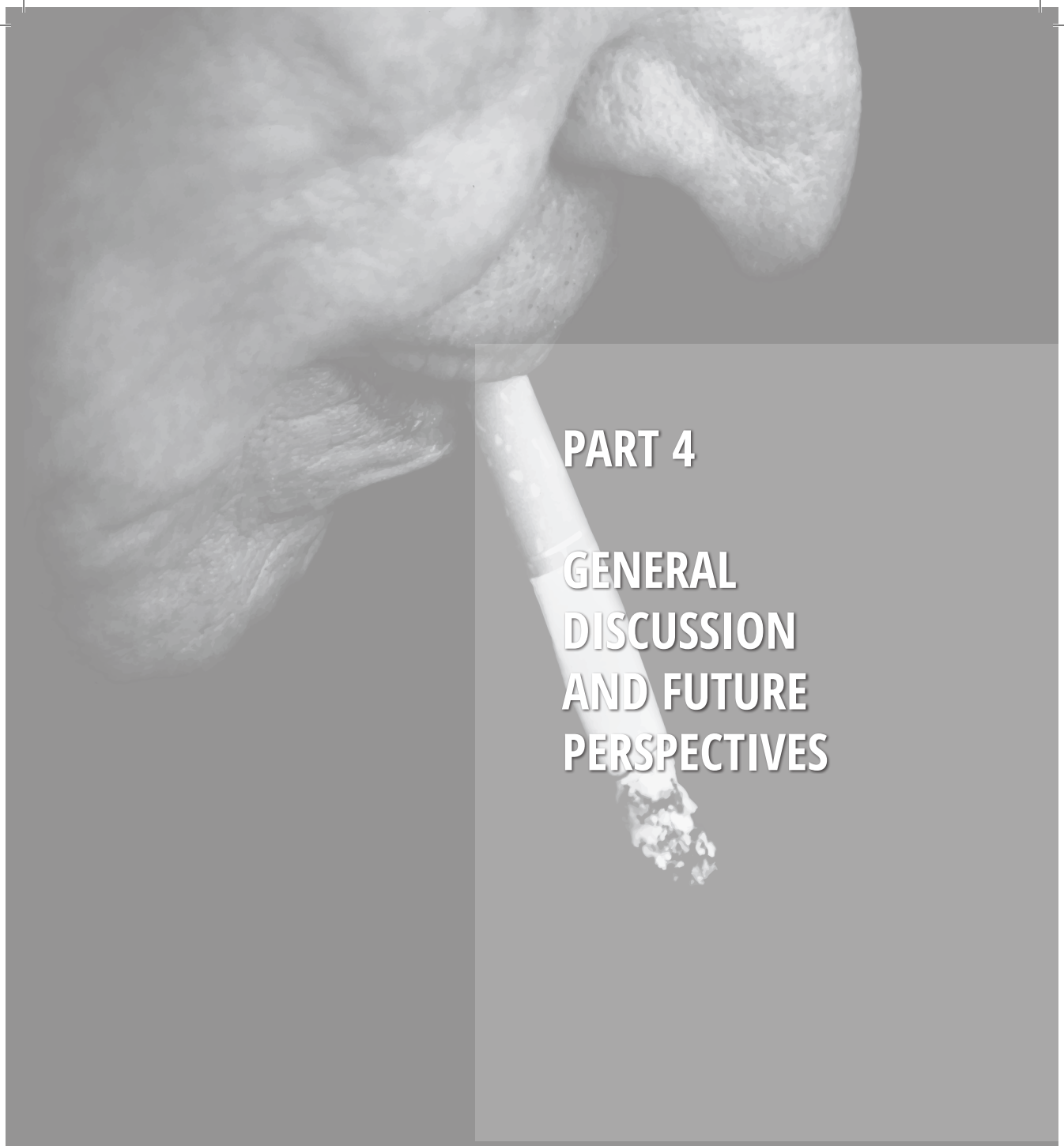
WOMEN AGE GROUP 55-74 YEARS



WOMEN AGE GROUP ≥ 75 YEARS



1= IC 1998-2004, 2 = IC 2005-2010, 3 = CLI 1998-2004, 4 = CLI 2005-2010,
with numbers at risk and 95% Hall-Werner bands



PART 4

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

CHAPTER 12

Summary, General discussion & Future perspectives

SUMMARY

Part I of this thesis summarizes the available evidence of a relatively new treatment modality for peripheral arterial disease and the evidence for a commonly used protocol to treat PAD patients after revascularization for a certain period with dual antiplatelet therapy. The conclusion both reviews share is that available evidence is scarce, and that existing evidence is often heterogeneous or derived from studies conducted in other vascular beds.

One of the issues noted in **Chapter Two** is that although multiple studies were performed with the use of bioresorbable stents, these tend to include different patients (eg claudicant patients and/or patients with critical limb ischemia) and patients with atherosclerotic lesions in different segment of the infrainguinal arteries (above the knee or below the knee). As several new stent platforms are tested, these different baseline characteristics only add to the heterogeneity of the available data.

Similarly, the use of dual antiplatelet therapy after endovascular revascularization with stent placement in PAD patients, for which available evidence was investigated in **Chapter Three**, is based on results from the Cardiology field and studies performed after stenting in Carotid arteries. The quality of evidence is variable and the results are certainly not unambiguously. The use of dual antiplatelet therapy carries a small but significant increased risk of adverse bleeding events with possible serious consequences, for example hemorraghic CVA. In coronary and carotid arteries the small increased risk of bleeding events is acceptable as compared to the serious adverse effects of stent thrombosis. However, in peripheral arteries that balance is different, with less far-reaching outcomes if stent thrombosis occurs. The widespread use of dual antiplatelet therapy in PAD patients without any evidence is remarkable and future studies into this subject are warranted.

In part II in-vivo experiments were conducted to examine the effects of a new treatment modality to inhibit intimal hyperplasia (IH) in a minimally invasive pig iliac artery damage model. In addition, in a goat venous model the effects of Mechanochemical endovenous ablation (MOCA) therapy on a histological level was researched.

Chapter Four highlights the difficulties in animal research and the pros and cons of an endovascular damage model to induce IH in pig iliac arteries. The chosen method was effective, but with extensive arterial wall damage and a difficult to standardize model due to balloon sizes and inflation variability. As a result, it is questionable whether the proposed model is a reliable method to research IH in pigs. The Synthetic Intimal Coating (SIC) as a novel treatment to inhibit IH was tested in **Chapter Five** in the earlier mentioned pig arterial damage model. The aim was to prove efficacy of a gel-coating which should be non-toxic, applicable and absorb within a certain timeframe. No toxic effects of the SIC were observed during follow-up, but we were unable to establish a circumferential placement of the SIC against the (damaged) arterial wall. In the current setting the SIC did not prevent the formation of IH, probably

due the encountered limitations, such as the placement and adhesion of the SIC on the arterial wall. Therefore, further studies are necessary to investigate adequate SIC placement and absorption on the arterial wall.
MOCA therapy is an established therapy to treat varicose veins in humans. However, the working mechanism is largely unknown and the only animal study investigating the histological effects of MOCA has been conducted by the manufacturer, and has published only partial results. MOCA therapy and its separate components were investigated in an animal goat venous model in **Chapter Six**, using the same protocol as in a clinical setting and the same goat model as previously described in the manufacturers study. Unfortunately, the application of stockings after MOCA-therapy was not allowed due to animal welfare concerns, which could have led to the lower occlusion rates observed in the goat study as compared to clinical reports and the study of the manufacturer.

In Part III plaque characteristics and patient characteristics of patients undergoing ilio-femoral endarterectomy were investigated.
In **Chapter Seven** plaque characteristics of plaques retrieved over the course of several years were compared, and it was observed that the characteristics of retrieved plaques changed over time into what we consider more stabilizing plaque characteristics. These findings supported results from the same biobank in plaques retrieved from the Carotid artery, and highlights the need to investigate contemporary patient plaques. The prevalence of secondary cardiovascular events in ilio-femoral endarterectomy patients included in the Athero-Express are described in **Chapter Eight**, and whether this changed over time. While the plaques changed over time, the prevalence of secondary cardiovascular events decreased only in the last two-year cohort.
Further research into the direct link between several plaque characteristics and the occurrence of secondary cardiovascular events in patients who underwent ilio-femoral endarterectomy was conducted in **Chapter Nine**. No direct link between the investigated plaque characteristics and secondary cardiovascular events was observed, including peripheral re-interventions. One of the limitations of current retrieved plaques in PAD patients is that these plaques are usually removed in an end-stage of the disease, after medical therapy and guided walking therapy have proven to be insufficient. This means there is a certain selection bias into the inclusion of these patients and that the plaque has possibly remodeled into a more stable plaque. We could not investigate the effects of early plaque characteristics, rate of plaque progression or acute symptomatic plaque characteristics on secondary cardiovascular events in this study. In **Chapter Ten** the plaque characteristics of patients with diabetes were investigated and compated to plaques retrieved from patients without diabetes. The prevalence of diabetes is rising and it is an important risk factor for peripheral arterial disease. It is known that these patients often have more calcified plaques in arteries below the knee, and the hypothesis was that this would also hold true in plaques retrieved from ilio-femoral arteries. Indeed, these patients more often had

moderate to severely calcified plaques. In addition, patients with diabetes developed more secondary cardiovascular events during three-year follow-up.

In **Chapter Eleven** the one and five-year mortality rates in PAD patients with claudication (IC) complaints or those presenting with critical limb ischemia (CLI) in nation-wide hospital registries were investigated. The mortality risks for both IC and CLI patients significantly declined in the Netherlands over time, in a sex-specific manner. However, absolute (cardiovascular) mortality risk remained high in both IC and CLI patients. The need for future studies remains clear, as these patients have a high risks of secondary cardiovascular events and (cardiovascular) death.

GENERAL DISCUSSION & FUTURE PERSPECTIVES

Recently it has been shown that peripheral arterial disease (PAD) patients have worse clinical prognosis when compared to patients with cardiac or carotid disease[1]. PAD patients often have chronic complaints or disabling symptoms, leading to a high disease burden for both patients and economic costs for society[1–4]. Of PAD patients, the prognosis of patients with critical limb ischemia (CLI) is even worse compared to claudicant (IC) patients[5,6]. Studies indicate that secondary prevention methods (such as medical therapy, but also guided walking therapy), is still not given to all PAD patients[7,8]. When a patient is diagnosed with PAD, clinicians need to be aware of the severity of this disease. In addition, it is essential to keep studying this vulnerable patient population - if possible in a more standardized method.

Justification of animal studies

One of the primary goals of this thesis was to investigate a novel treatment to counter restenosis due to intimal hyperplasia (IH) after endovascular treatment. Unfortunately, we could not prove efficacy of the Synthetic Intimal Layer (SIC) gel-coating as an inhibitor of intimal hyperplasia in an endovascular pig iliac arterial damage model in **Chapter Five**. This was contributed to several possible causes, but resulted from the fact that we were unable to establish a circumferential placement of the SIC against the (damaged) arterial wall. Our study highlighted another problem of in vivo animal studies, namely that no golden standard animal model exists to investigate restenosis due to IH in peripheral arteries and that inducing intimal hyperplasia is more difficult in peripheral arteries than in coronary arteries[9–11]. (Similarly, there is currently no golden standard animal model for atherosclerotic lesions, although there are promising developments[12]). In **Chapter Four** the pros and cons of the currently used animal model are described. Similarly in the goat animal study in **Chapter Six**, the animal model was not 100% in accordance with practice in human veins due to animal welfare concerns – which did not allow for stockings to be applied up to 24 hours after the procedure. This may be one of the causes of the lower occlusion rates observed in comparison with current clinical use of MOCA for venous ablation[13].

Notwithstanding these results, the use of animals in pre-clinical studies have gained medical science much knowledge and is still indispensable[10,12,14]. Our results do reflect the great care that should be taken into account to limit animal suffering and the number of animals used for these pre-clinical studies. A standard animal model to investigate restenosis due to IH or remodeling after treatment for PAD would greatly benefit standardized pre-clinical in-vivo research and limit bias due to the use of different protocols to induce IH in pig iliac arteries. This could lead to a decrease in the number of animals needed to proof feasibility of a new treatment and to increased comparability between pre-clinical animal studies. The model we have currently used is in our opinion not sufficient for this task, due to the extensive amount of arterial wall damage which was hard to control and the impossibility to standardize

thrombectomy balloon inflation and cutting balloon sizes. The use of a non-compliant balloon overstretch model is probably a better suited model to investigate IH, even though this model is not as successful in inducing IH as the currently used model. In addition, available literature is not unambiguous as to the extent of oversizing, duration of oversizing, and the number of times to repeat oversizing needed to successfully induce IH[9,15].

Plaques characteristics as markers for future secondary cardiovascular events

Several observations were made from ilio-femoral plaques included in the Athero-Express biobank. Contrary to intraplaque hemorrhage (IPH) in Carotid artery plaques[16], which are usually retrieved in a semi-acute setting if possible within 14 days of the initial cerebrovascular event[17], characteristics of ilio-femoral plaques were not associated with secondary cardiovascular events (**Chapter Nine**). Ilio-femoral plaques are mostly retrieved years after initial symptoms begin and patients often received medical therapy[7] and guided walking therapy[18] before a plaque was removed, in which the plaque could have remodeled and stabilized. In a prospective study it was observed that collagen rich plaques retrieved from the common and superficial femoral artery did increase the risk of restenosis measured with echo duplex during follow-up[19]. However, the definition of restenosis and (re-)intervention is not the same. The indication is that a collagen rich plaque, which is considered stable and/or remodeled, could predict restenosis, but not necessary indicate an increased risk of re-intervention within three years of follow-up in the Athero-Express cohort.

The absence of an association between the investigated plaque characteristics and the cardiovascular endpoint indicates that future research should focus on other plaque characteristics, for example plaque erosions on the surface of the plaque[20]. Obtaining computerized continuous data regarding plaque characteristics could enhance the accuracy with which to identify plaque characteristics with possible prognostic values. If certain plaque characteristics are identified as predictors for secondary CVE, radiological studies could benefit from this knowledge in a clinical setting or in future research[21–23]. In addition, to be able to stratify patients into risk groups at the hand of plaque markers from retrieved plaques could help indicate which patients would benefit from stricter secondary preventive care control and follow-up[7,24].

Changing plaques… and patients

Another observation in ilio-femoral plaques within the Athero-Express Biobank, which was also observed in Carotid plaques[25], is that plaque characteristics alter over time towards more stable plaques in PAD patients (**Chapter Seven**). In both CEA patients and IFE patients these changed plaque characteristics did not coincide with reduced secondary cardiovascular events in these patient populations, except for a lower rate of re-interventions in the years 2011-2012 in ilio-femoral endarterectomy patients (**Chapter Eight**). Additionally, a recent population study observed changing patterns in population risk factors[26]. Indeed, in the Athero-Express several patient characteristics

changed over time, with an increase in hypertension, an increased statin use and a (subsequent) lowering of lipid levels and cholesterol. When studying plaque and patient characteristics for possible prognostic markers, one should keep in mind that this might be best served with the use of a contemporary plaque and patient data. The combination of data from multiple (multinational) databases and biobanks may be necessary to gain sufficient statistical power to properly investigate these plaques and patients within a shorter timeframe.

Quality of evidence in peripheral arterial disease

As observed in the review and meta-analysis of this thesis (**Chapter Two** & **Chapter Three**), the indication for treatment in PAD patients is sometimes based on thin evidence, and sometimes extrapolated from study results in the Cardiology field. It remains questionable whether this extrapolation is justified, as complications such as stent thrombosis, stent fracture or in-stent restenosis have a much worse clinical outcome in cardiac patients than in PAD patients[27]. The patient numbers reached in Cardiac trials will probably never be reached in PAD patient trials. Yet, with a multicenter and if possible a multinational approach, reaching sufficient power in PAD trials should be a possibility. In- and exclusion criteria should adequately reflect the average vascular patient population, as not to hamper inclusion into clinical trials. In addition, strict instructions for use (IFU) of some of the new devices, and vague contra-indications for use, could reduce practicability of these devices in clinical trials.

Many PAD trials include a wide-spread range of patients, from IC patients with Fontaine 2b (or Rutherford 2-3) to patients with severe CLI (Fontaine 4 or Rutherford 5-6). The atherosclerotic disease burden in severely affected CLI patients is probably much higher as compared to IC patients. Also, the prognosis of IC and CLI patients are not comparable as observed in **Chapter Eleven**. Yet, narrowing the inclusion criteria to a certain atherosclerotic substrate or too specific patients characteristics could hamper inclusion/participation rates of PAD patients. Studies should focus on using clear inclusion criteria, comparability with current practice and the patient population should reflect every-day practice, with a separation of IC and CLI patients. A new way to classify atherosclerotic lesions and patient wounds in CLI patients would enable more standardized research and give physicians a better indicator for the (technical) success of a (new) treatment.

The focus of research into new treatment modalities

The main reason for decreased patency rates is (in-stent) restenosis after initial (endovascular) treatment of an atherosclerotic lesion[28–30]. Several new treatment modalities have been developed to counter restenosis due to IH or negative remodeling, such as: a drug-coated balloon, drug-eluting stent or a (drug-eluting) bioresorbable stent[31,32]. Each option has its up- and downsides, but a particular concern is that long term effects of intimal formation inhibiting drugs (such as everolimus, paclitaxel[33]) are currently unknown. In addition, some of these new techniques show less restenosis

and improved primary and secondary patency rates, but in contrast scarce evidence on clinically important outcomes for the patient is available (such as walking distance, wound healing, and pain)[34–36]. Future studies into new treatment modalities need to focus on clinically important results for the patient. In addition, histological animal studies towards the (local) effects of a new treatment on the arterial wall should be thoroughly conducted in order to get a clear idea what the long term effects of a new treatment could be. Our own animal studies towards a new possible treatment to inhibit the formation of intimal hyperplasia unfortunately did not yield positive results. However, we hope our findings could be used to base further research upon.

Homogenous research in peripheral arterial disease

As mentioned earlier, the field of PAD research is small, yet heterogeneous, and sometimes clear evidence is even absent due to extrapolated results from other cardiovascular fields[27]. Especially in CLI patients and treatment of atherosclerotic lesions below the knee evidence is thin. To counter these limitations in the future, a multicenter, or even a multinational approach with specialized vascular surgery hospitals is in my opinion the way to move forward with new techniques and treatment options. A clear histological basis for the use of novel techniques is necessary to "understand what you're doing"[37] in the artery – and thus the search for a proper animal model. New devices should have clear and 'clinically workable' instructions for use (IFU). Including patients should be done rationally, with a separation of IC patients and CLI patients due to a different prognosis, but possibly also different technical success of a treatment and treatment outcomes. The focus on the extent of atherosclerotic lesions in a study should be based on workable clinical setting – as not to exclude too many patients due to advanced atherosclerotic disease. Negative results should be published to limit publication bias and give a clear overview to what is being investigated in the field. Lastly and perhaps most importantly, clinical trials should focus their endpoints on clinically important outcomes for the patient.

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12

CHAPTER 13

Dutch summary
Review committee
List of publications
Acknowledgements
Curriculum Vitae

DUTCH SUMMARY

Deel I van dit proefschrift omvat een samenvatting van het beschikbare bewijs voor een relatief nieuwe behandeling voor perifeer vaatlijden en het bewijs voor een veelgebruikt protocol om patiënten met perifeer vaatlijden na revascularisatie met duaal anti-bloedplaatjes therapie te behandelen. De conclusie die beide reviews delen is, dat het beschikbare bewijs vaak schaars is, heterogeen en/of overgenomen vanuit studies die in andere vaatbedden zijn uitgevoerd.
Eén van de zaken die in **Hoofdstuk Twee** wordt aangestipt is, dat hoewel er meerdere studies zijn uitgevoerd met oplosbare stents, deze studies vaak verschillende typen patiënten includeren (zoals patiënten met claudicatio intermittens: etalagebenen of patiënten met kritieke ischemie aan de benen). Deze patiënten hebben atherosclerotische laesies (vaatverkalking) in verschillende segmenten van de arteriën van de lies tot aan de voet, namelijk in het bovenbeen of in het onderbeen, waaraan ze worden behandeld. Daarnaast wordt een aantal nieuwe stents met verschillende eigenschappen onderzocht wat verder bijdraagt aan de heterogeniteit van de beschikbare data.
Het gebruik van duaal anti-bloedplaatjes therapie na endovasculaire revascularisatie met stentplaatsing in patiënten met perifeer vaatlijden, waarvoor bewijs was onderzocht in **Hoofdstuk Drie**, bleek gebaseerd op resultaten verkregen uit cardiologische onderzoeken en studies waarbij onderzoek was gedaan na het stenten van halsslagaderen. De kwaliteit van de beschikbare onderzoeken was wisselend en de resultaten waren niet unaniem in het voordeel van deze therapie. Het gebruik van duaal anti-bloedplaatjes therapie heeft een klein, maar niet verwaarloosbaar, risico op het veroorzaken van ongewilde bloedingen met mogelijk ernstige gevolgen zoals bijvoorbeeld een hersenbloeding. Bij het stenten in kransslagaderen en halsslagaderen is het kleine extra risico op bloedingen te accepteren omdat de gevolgen van een verstopte stent veel groter zijn. Echter, het is de vraag of deze balans ook opgaat voor slagaderen van de benen, aangezien het effect van een verstopte stent minder ernstig is. Het is opvallend dat deze duaal anti-plaatjes therapie veel wordt gebruikt, zonder dat er veel beschikbaar bewijs voor is. In de toekomst zou dit beter onderzocht moeten worden.

In Deel II zijn in-vivo experimenten uitgevoerd om de effecten van een nieuwe behandeling ter remming van intima hyperplasie na vaatchirurgische behandelingen te onderzoeken. Dit gebeurde in een minimaal invasief varkensmodel, door middel van een ballon in de heupslagader. Daarnaast zijn de effecten van een behandeling tegen spataderen (mechanische-chemische endoveneuze ablatie (MOCA-) therapie) op celniveau onderzocht in de venen van geiten.
Hoofdstuk Vier geeft de moeilijkheden van dierexperimenteel onderzoek weer en beschrijft de voor-en nadelen van het gebruikte endovasculaire vaatschade model om intima hyperplasie te veroorzaken in varkens heupslagaderen. Het gekozen model was effectief, maar met ernstige schade aan de vaatwand en het bleek moeilijk om het

model te standaardiseren door de beschikbare afmetingen van de ballon en de lastig te reproduceren mate van opblazen van de ballon. Het gevolg hiervan is, dat men zich af kan vragen of het voorgestelde model wel een betrouwbaar model is om intima hyperplasie te onderzoeken in varkens.
Een nieuwe methode om intima hyperplasie na vaatchirurgische behandelingen te remmen is onderzocht in **Hoofdstuk Vijf**. De Synthetische Intima Laag (SIC) werd getest in het eerder genoemde varkensmodel. Daarbij werd één beschadigde zijde behandeld met de SIC en één beschadigde zijde niet (links of rechts was om het even en voor de operatie bepaald). Het doel was de effectiviteit van een gel-laag te onderzoeken in het remmen van intima hyperplasie en daarnaast vast te stellen dat de SIC niet toxisch was, plaatsbaar was en binnen afzienbare tijd zou oplossen in de vaatwand. Na follow-up werden er geen toxische effecten van de SIC waargenomen. Echter, we konden ook geen circumferentiele plaatsing van de gel tegen de (beschadigde) vaatwand bewijzen. In de huidige setting remde de SIC de vorming van intima hyperplasie niet, waarschijnlijk door onvoldoende plaatsing en/of adhesie van de SIC aan de vaatwand. Nader onderzoek is nodig om adequate plaatsing, adhesie en absorptie van de SIC op de vaatwand vast te stellen.
MOCA therapie is een bekende behandelmogelijkheid tegen spataderen bij mensen. Echter, het werkingsmechanisme van deze behandeling is grotendeels onbekend. De enige dierstudie die histologische effecten van MOCA therapie heeft onderzocht, is uitgevoerd door de fabrikant van deze therapie en daarvan zijn alleen partiele resultaten gepubliceerd. In **Hoofdstuk Zes** zijn MOCA therapie en de verschillende losse componenten onderzocht in een geit venen model. Hierbij is hetzelfde protocol gebruikt als in mensen wordt toegepast en zoals eerder beschreven in de dierstudie van de fabrikant. Helaas mochten steunkousen na de behandeling niet worden aangebracht bij de geiten vanwege zorgen om dier-welzijn. Dit kan de oorzaak zijn geweest waardoor we lagere occlusiegraden zagen in deze geitenstudie dan in mensen is gerapporteerd en dan de fabrikant rapporteerde.

In Deel III van deze thesis zijn verschillende plaque en patiënt karakteristieken onderzocht van patiënten die een ilio-femorale endarterectomie ondergingen – het verwijderen van een atherosclerotische plaque direct uit de slagader van de lies. Voor onderzoek zijn deze plaques en patiënt karakteristieken opgeslagen in de Athero-Express biobank, met goedkeuring van de patiënt.
In **Hoofdstuk Zeven** zijn de plaque karakteristieken van plaques onderzocht die over het verloop van verscheidene jaren zijn verwijderd. In deze studie werd opgemerkt dat de onderzochte plaque karakteristieken in de loop van de tijd veranderden – in wat beschouwd wordt als stabielere plaque karakteristieken. Deze bevindingen ondersteunen de resultaten uit dezelfde biobank maar dan onderzocht in plaques die uit de halsslagader waren verwijderd. Dit benadrukt dat wanneer onderzoek naar plaque karakteristieken wordt gedaan, deze met recent verkregen plaques moet worden uitgevoerd.

De mate waarin secundaire cardiovasculaire events optreden tijdens de follow-up bij patiënten in de Athero-Express waarbij de plaque uit de lies of halsslagader werd verwijderd, is onderzocht in **Hoofdstuk Acht**. Hierbij is tevens onderzocht of dit veranderde in de loop van de tijd. Opvallend is, dat terwijl de plaques veranderden, de prevalentie van secundaire cardiovasculaire events alleen afnam in het laatste 2-jaar cohort van patiënten waarbij de plaque uit de lies is verwijderd.

In **Hoofdstuk Negen** is verder onderzoek gedaan naar de directe link tussen plaque karakteristieken en het optreden van secundaire cardiovasculaire events tijdens de follow-up van patiënten bij wie de plaque uit de liesslagader was verwijderd. Hierbij werd geen directe associatie tussen de onderzochte plaque karakteristieken en het optreden van deze events gevonden, ook niet wanneer we apart keken naar re-interventies aan de benen. Een limitatie van dit onderzoek was dat de huidige verkregen plaques vaak pas verwijderd worden aan het eind van een langdurig ziekteproces, na looptherapie en medische behandelingen. Hierdoor krijgt de plaque als het ware de tijd om zichzelf verder te ontwikkelen in een meer stabielere plaque. We konden niet de effecten van vroeger-stadium plaques onderzoeken, noch de mate van plaque progressie noch van acute symptomatische plaque karakteristieken op het optreden van secundaire cardiovasculaire events in deze studie.

In **Hoofdstuk Tien** zijn de plaque karakteristieken van patiënten met diabetes onderzocht en vergeleken met plaques van patiënten zonder diabetes. Diabetes komt steeds vaker voor en is een belangrijke risicofactor voor het ontwikkelen van perifeer vaatlijden. Het is bekend dat patiënten met diabetes vaker kalk in hun plaques hebben in slagaderen van het onderbeen (vaatverkalking), en onze hypothese was dat dit ook het geval zou zijn in slagaderen van het bovenbeen en de heupslagader. Het bleek inderdaad zo te zijn dat patiënten met diabetes vaker ernstig verkalkte plaques hadden in het bovenbeen. Daarnaast hadden patiënten met diabetes vaker een secundair cardiovasculair event binnen 3 jaar follow-up.

In **Hoofdstuk Elf** zijn de 1- en 5-jaars sterfte percentages van patiënten met perifeer vaatlijden onderzocht, die zijn opgevraagd uit landelijke ziekenhuisregisters. De patiënten zijn hierbij opgedeeld in patiënten met claudicatio intermittens en patiënten met kritieke ischemie aan de benen. De sterfte risico's van patiënten met claudiatio intermittens en kritieke ischemie daalden significant in Nederland in de loop van de tijd, met hierbij een verschil tussen mannen en vrouwen. Echter de absolute (cardiovasculaire) sterfte risico's blijven hoog voor deze patiënten. Het blijft nodig deze kwetsbare patiëntengroepen te onderzoeken, aangezien deze patiënten een hoog risico hebben op het ontwikkelen van cardiovasculaire events en een hoog risico hebben om te overlijden tijdens de follow-up.

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CURRICULUM VITAE

Steven Theo Willem van Haelst was born on the 11th of September 1988 in Kockengen, The Netherlands. He is the oldest of three siblings of Fons van Haelst and Bep Kuijf. As he tried to evade village life, he went to high school in Utrecht. After graduating at the Utrecht Stedelijk Gymnasium in 2006, he started studying at the medical school of the University of Amsterdam. During his study he was an active member of the Amsterdam Studenten Corps, dispuut M.A.R.N.I.X., and co-initiator of the MCSKD. Before the start of his internships, he decided to travel the world and gain knowledge of foreign cultures during a 6-month trip in Central-America.

In September 2013 Steven graduated from medical school and started working at the Flevoziekenhuis as a surgical resident not in training under the supervision of dr. P.C.M. Verbeek. There, he found renewed interest for science and decided to apply for a phd-thesis in Vascular Surgery under the supervision of prof. dr. G.J. de Borst, prof. dr. F.L. Moll, and prof. dr. G. Pasterkamp, July 2014. He has presented this work at several (inter)national conferences and the results are published in peer-reviewed international scientific journals.

Currently, Steven is building a house in Amsterdam with his girlfriend Maxime Burgers, which he hopes will be completed some day. In addition, during the time of this writing he is residing in San Francisco for a research project at the University of California San Francisco under supervision of prof. dr. M.S. Conte, with the aim to improve standardized research in peripheral arterial disease and to help establish a long-term collaboration between the UCSF and UMC Utrecht on patients with critical limb ischemia.