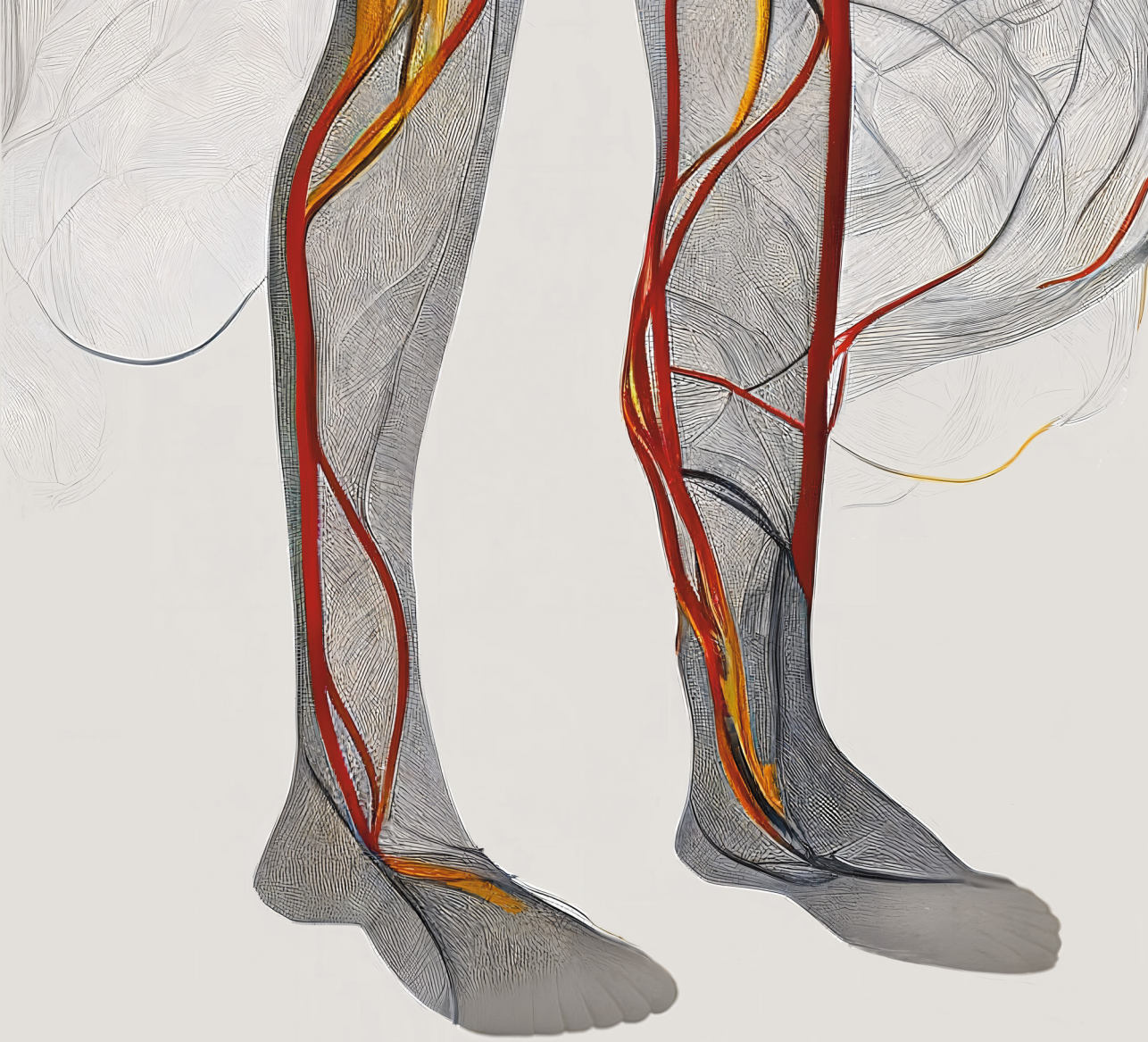

Peripheral Artery Disease

Evaluation of Novel Biomarkers and Adverse Events in Unexplored Subtypes

M.C. Verwer

Peripheral Artery Disease

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Maarten Cees Verwer

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Peripheral Artery Disease

Evaluation of Novel Biomarkers and Adverse Events in Unexplored Subtypes

Evaluatie van nieuwe biomarkers en ongewenste uitkomsten in niet eerder onderzochte subtypes van perifeer arterieel vaatlijden
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
op gezag van de
rector magnificus, prof. dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op

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door

Maarten Cees Verwer

geboren op 15 februari 1992
te Deventer

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Contents

1

General introduction and thesis outline

Maarten Verwer

Parts of this chapter have been published in the Nederlands Tijdschrift voor Geneeskunde

General introduction and thesis outline

Cardiovascular disease (CVD) is a class of non-communicable diseases that involves the heart and vasculature. With an estimated 17.8 million deaths in 2017, representing over 30% of all global deaths, it remains the most common cause of death worldwide.[1, 2] In Europe, over 60 million potential years of life are lost due to CVD annually.[3] Even though the increase in cardiovascular death is largely due to ageing and growing populations, the prevalence of CVD is on the rise as an epidemic of metabolic disease is affecting high-income and low-income countries alike.[4] The burden of CVD is not only comprised of the deaths caused by it, but chronic comorbidities of CVD affect the lives of millions of people every day as it leads to 18% and 10% of disability-adjusted life years lost for high-income and low to moderate-income countries respectively.[3]

Atherosclerosis

The majority of CVD is caused by atherosclerosis, the deposition of fatty and/or fibrous material in the innermost layer of the arteries, the intima. The term atherosclerosis is derived from the Greek word for "gruel" or "porridge', which describes the appearance of the core of a typical atherosclerotic plaque (or atheroma). With progression, the atheroma becomes more fibrous and accumulates calcium minerals. This, in turn, can result in gradual luminal loss, impeding blood flow, leading to tissue ischaemia distally from this culprit lesion. Non-flow-limiting plaque can disrupt blood flow and provoke thrombus formation, which can occlude the arterial lumen and cause acute ischemia.[5]

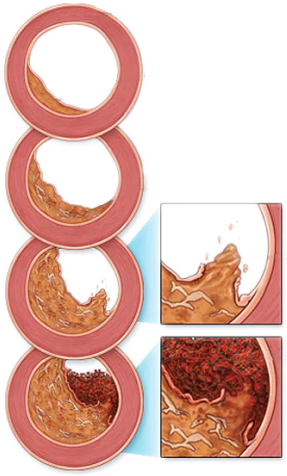

Figure 1.1: Atherosclerotic model with from top to bottom: arterial lining with plaque, disruption of arterial lining with plaque, plaque rupture, blood cloth formation limiting blood flow

The nature and severity of the symptoms caused by atherosclerosis depend on the affected vascular segment and the degree of perfusion decline. As a result, carotid plaque formation is considered the culprit for cerebrovascular diseases such as transient ischemic attack or cerebral stroke. Likewise, atherosclerosis of the coronary arteries can lead to acute coronary syndromes such as myocardial infarction or chronic conditions like stable angina pectoris. Finally, when the peripheral arteries are affected, impeding blood flow can result in exercise-induced pain or ischaemic ulcerations of the extremities.

Peripheral artery disease

Although there is much overlap between these CVD subtypes, this thesis focuses primarily on the latter, which is generally referred to as peripheral arterial/artery disease (PAD) or lower extremity arterial disease (LEAD). This atherosclerotic subtype involves the aortoiliac segments through to the pedal arteries.

Its clinical spectrum is vast, as it includes asymptomatic individuals and those with life-threatening symptoms of the legs, such as ischemic gangrene. The balance between metabolic

demand and the capacity to deliver oxygen to the tissue is critical to this difference. Unfortunately, this is also dependent on many factors. Relatively minor impairment results in characteristic exercise-induced muscle pain that is relieved upon rest: its severity is often quantified by the pain-free walking distance. Further decrease of flow is characterised by rest pain, in which basic consumption is slightly in deficit. On the most extreme end of the spectrum is the onset of ischemic wounds, when the oxygen supply does not meet the metabolic demands of tissue regeneration, compromising the viability of the limb.

General management of peripheral artery disease

The management of PAD patients, much like all other patients with CVD, hinges on preventive actions and therapeutic interventions. In general, all patients with PAD should receive preventive treatment, which is aimed at the multifactorial nature of atherosclerosis and its complications. Such risk factors include, but are not limited to, hypertension, high cholesterol, thrombotic events, metabolic disease, and tobacco use.[5] As such, recent guidelines have adopted strict LDL-c levels as benchmarks for single use or a combination of statins, ezetimibe, or PCSK9 -inhibitors.[6] Mono-antiplatelet therapies are recommended for secondary prevention, but new therapy regimes, such as dual pathway inhibition and dual platelet inhibition, a combination of anticoagulant and antiplatelet drugs, and two antiplatelets, respectively, are considered for high-risk patients.[7–9] New therapies, such as colchicine treatment, are being investigated and may be indicated for high-risk patients.[10] Although promising, these add-on medical therapies inevitably expose patients to more side effects and potential adverse events and should only be provided to those with adequate benefits.

Therapeutic interventions in PAD encompass minor or major surgical procedures and endovascular therapies. Due to their invasive nature, risk of complications, and limited patency, these are often reserved for those with severe symptoms.[11] Although an "endovascular intervention first" strategy is generally considered preferable, some arterial lesions are more amenable to a direct surgical approach. Unfortunately, this distinction is often not as straightforward. Furthermore, when the efficacy of revascularisation is doubted, more drastic measures like amputation could be considered. The paramount issue is the selection of patients who may benefit from early amputation when there is no immediate threat to survival.

Evidence-based approach to treatment

As part of an evidence-based approach, the treatment of patients with PAD hinges on three axes: Patient risk, Limb severity and ANatomical complexity (PLAN).[11] Models like the Wound, Ischemia, Foot Infection (WIfI) classification and Global Limb Anatomical Staging System (GLASS) have been proposed as instruments for the latter two.[11] However, clinicians are not yet able to effectively estimate patients' risks according to the hazard of adverse events, and it is difficult to select those who will benefit from additional preventive therapies. Thus, an objective score could substantiate surgical decisions when interventions are considered. Although risk models that use patient characteristics exist in PAD, they lack efficacy. Therefore, attempts have been made to find other factors that can guide risk stratification. These are called biomarkers. Some of these biomarkers have already become indispensable in daily practice, as inflammatory markers such as C-reactive protein (CRP) indicate (the

severity of) infection, the ankle-brachial index (ABI) is used to diagnose and stratify the severity of PAD, and ultrasound or a computed tomography angiography (CTA) is used to identify significant carotid stenoses.

Although there is a plethora of research on biomarkers in CVD, it remains challenging to distinguish patients at extremely high risk and those at high risk. Much of this research has been dedicated to coronary artery disease, so PAD is relatively underrepresented in research on prognostic biomarkers. However, we are still at the very start of world-changing research: genome sequencing is becoming increasingly affordable, new proteomic panels are being developed to measure a multitude of proteins with a few microliters of blood, and the discovery of other biomarker sources makes the possibilities seem endless.

The latter includes extracellular vesicles (EVs), small lipid-bilayer membrane particles secreted by all cell types into body fluids such as plasma, saliva, and urine.[12] Their cargo, which consists of nucleic acids, lipids, and proteins, reflect (patho)physiological processes such as apoptosis, coagulation, and inflammation of the parent cell. Consequently, EVs are considered a 'liquid biopsy' that can help determine disease progression and the future risk of adverse events in patients with CVD.

Aim of this thesis

The overall aim of this thesis is to investigate adverse events in patients with PAD. This information can be used to objectify how susceptible patients are to certain adverse events, to validate existing risk models, and to identify blood plasma, EV and plaque biomarkers that are associated with these events and can potentially be used for risk stratification.

Thesis outline

This thesis consists of three parts. **Part I** focuses on patients with severe forms of PAD, in whom revascularisation is not a viable option because their peripheral arterial pathway is too severely compromised. These patients are at the mercy of optimal conservative treatment and natural progression and are consequently expected to have an abysmal prognosis. However, the latter has not been investigated in much detail yet.

Hence, in *Chapter 2*, we investigated five-year outcomes such as survival and amputation risk in a population of non-revascularisable patients. In *Chapter 3*, these data were used to validate a new risk model for mortality in PAD (the Vascular Quality Initiative), which had not yet been validated in these so-called 'no-option' patients.

Part II of this thesis focuses on patients with Pseudoxanthoma Elasticum (PXE), a genetic disease that causes the mineralization of elastic fibres in tissues such as the skin, eyes and blood vessels. Since the pathophysiological processes in PXE are different from those in "normal" PAD, the predisposition of PAD in these patients is still unclear. Furthermore, one case series and two case reports anecdotally reported poor outcomes after vascular interventions in patients with PXE. *Chapter 4* is therefore devoted to clarifying the predisposition of PXE to PAD, and the application of vascular interventions, using the world's largest PXE cohort.

From **Part III** onwards, the focus of this thesis shifts to biomarker research. In *Chapter 5*, three biological biomarker sources are compared in patients undergoing carotid endarterectomy: plasma, EVs, and atherosclerotic plaque. So far, biomarker research has mostly been limited to a single marker within a single source. As such, it remains unknown how circula-

tory markers (plasma and EV) correlate to markers within the culprit lesion (atherosclerotic plaque) and how these biomarkers correlate to clinically relevant endpoints. In *Chapter 6*, four EV-derived proteins (Cystatin C, CD14, Serpin C1 and Serpin G1) were examined for an association with major adverse limb events (MALE) and major adverse cardiovascular events (MACE) in patients with PAD. Similarly, in *Chapter 7*, we investigated the association of plasma lipoprotein(a) with MALE and MACE in patients undergoing femoral endarterectomy.

Finally, **Part IV** provides the general discussion and a Dutch summary in *Chapter 8* and *Chapter 9*, respectively.

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I

Adverse events in patients with non-revascularisable chronic limb-threatening ischemia

2

Long term survival and limb salvage in patients with non-revascularisable chronic limb threatening ischaemia

European Journal of Vascular and Endovascular Surgery, **Volume 62**, Issue 2, p. 225 - 232

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2

Abstract

Objective: *This study aimed to provide long-term survival and limb salvage rates for patients with non-revascularisable (NR) chronic limb-threatening ischaemia (CLTI).*

Methods: *This was a retrospective review of prospectively collected data derived from a randomised controlled trial (JUVENTAS) investigating the use of regenerative cell therapy. Survival and limb salvage of the index limb in CLTI patients without viable options for revascularisation at inclusion were analysed retrospectively. The primary outcome was amputation-free survival, a composite of survival and limb salvage, at five years after inclusion in the original trial.*

Results: *In 150 patients with NR-CLTI, amputation-free survival was 43% five years after inclusion. This outcome was driven by an equal rate of all-cause mortality (35%) and amputation (33%). Amputation occurred predominantly in the first year. Furthermore, 33% of those with amputation subsequently died within the investigated period, with a median interval of 291 days.*

Conclusion: *Five years after the initial need for revascularisation, about half of the CLTI patients who were deemed non-revascularisable survived with salvage of the index limb. Although the prospects for these high-risk patients are still poor, under optimal medical care, amputation-free survival seems comparable with that of revascularisable CLTI patients, while the major amputation rate within one year, especially among NR-CLTI patients with ischaemic tissue loss, is very high.*

Introduction

Despite medical and technological treatment advances, patients with peripheral artery disease (PAD) still have a high morbidity and mortality risk compared with the general population.[1] This is particularly true for patients with chronic limb-threatening ischaemia (CLTI), with reported five year all-cause and cardiovascular mortality rates twice as high (57% and 29%) compared with patients with intermittent claudication (IC) (31% and 15%), respectively, according to a Dutch national registry study.[1] Furthermore, the amputation rate in CLTI patients of 15% – 20% at one year reflects a large impact on quality of life and healthcare costs.[2]

Alarmingly, the prevalence of PAD will probably grow as populations are ageing and the prevalence of risk factors for PAD, such as diabetes mellitus (DM), increase. Between 2017 and 2045, the prevalence of DM is expected to rise from 451 to 693 million people worldwide.[3] Already, up to 30% of all patients with IC and 50% of all patients with CLTI are diagnosed with DM, which co-prevalence is associated with lower revascularisation success rates, decreased wound healing and higher amputation and mortality rates compared with those without diabetes.[4–8] The increasing prevalence of patients with DM is expected to lead to a parallel increase in the number of patients with non-revascularisable or so-called "no option" PAD, and specifically no option or non-revascularisable CLTI (NR-CLTI).

Although the clinical prognosis of NR-CLTI patients has been reported, the evidence is limited to one-year mortality and amputation rates in non-consecutive case series and randomised controlled trials that report these outcomes as an ancillary result. Available data combined in a meta-analysis investigating the natural history of NR-CLTI reported a one-year mortality and amputation rate of 22%.[9] Within this analysis of 11 studies, only two reported a follow-up exceeding two years, but both were published more than 30 years ago (study periods were 1979–1986 and 1971–1983, respectively).[10, 11]

Hence, the current long-term prognosis of CLTI patients without revascularisation options remains unclear, while knowledge about the contemporary prognosis in this specific population is valuable for numerous reasons, for example, counselling patients and family, substantiating treatment decisions (not limited to PAD alone, as these patients often have multiple morbidities), the timing of palliative care, and optimal selection of patients for future (regenerative therapy) trials.

This study aimed to provide long-term survival and limb salvage rates for NR-CLTI patients. Five-year survival and amputation-free survival were investigated in "no option CLTI patients" who participated in a randomised controlled trial (RCT).

Methods

The details of the JUVENTAS trial design were published previously.[12] In short, in this single-centre, double-blind, placebo-controlled RCT, the clinical effects of repetitive infusion of bone marrow mononuclear cells into the common femoral artery were investigated in 160 patients. Notable inclusion criteria were the ineligibility for surgical or endovascular revascularisation (thus deemed nonrevascularisable [NR]), as defined by a multidisciplinary team of vascular surgeons and radiologists in the University Medical Centre of Utrecht, and severe PAD consisting of severe IC, persistent recurring rest pain or non-healing ulcers present for more than four weeks. Noteworthy exclusion criteria were a history of malig-

nancy within the ten years prior to inclusion and a life expectancy of less than one year.

The primary outcome of the initial study was major amputation of the index limb within six months after randomisation. All-cause mortality was a secondary outcome. Inclusion was conducted between 2006 and 2012. No effect of the trial intervention was observed.[13]

For the current study, only the NR-CLTI population included in the JUVENTAS trial was analysed. For the baseline, the original information was used without any new retrospectively reconstructed data (such as the Society for Vascular Surgery Wound, Ischaemia, and foot Infection [WIfI] classification).[1] But in addition to the original protocol, information about major amputation and all-cause mortality was successfully requested from the general practitioners more than five years after inclusion (n = 158). The patient and the referring hospital were contacted when the follow-up was unknown by the general practitioner (n = 2). The leg on which a patient was included in the original trial was defined as the index limb. Major amputation was defined as amputation through or above the ankle joint. The primary outcome of this study was ipsilateral amputation-free survival (AFS), the inverse composite of ipsilateral major amputation and all-cause mortality. The study was conducted according to the Declaration of Helsinki, the medical ethics board in the participating hospital approved the study, and all patients provided written informed consent.

Statistical analyses

Baseline characteristics, such as risk factors, medication use, wound characteristics, and the ankle-brachial index (ABI), stratified for AFS, are provided. Categorical variables were reported as numbers with percentages, non-normally distributed data were reported as median with inter-quartile ranges (IQR) and normally distributed results were given as mean with standard deviation (SD). The normality of data was analysed using the Shapiro-Wilk test. Continuous variables were analysed using the Student t-test or Mann-Whitney U test, as appropriate. Categorical variables were analysed using Fisher's exact test.

Additional analyses were performed to evaluate contributing factors for lower limb amputation and all-cause mortality. Scaling (z transformation) was performed after log10 transformation of non-normally distributed continuous variables. Univariable Cox proportional hazard regression was performed on a selection of risk factors with a plausible relationship to the outcome. Multivariable analysis was performed, including predictors with a p-value < .10 in univariable analyses using a forward stepwise approach. The proportional hazard assumption was verified by examining the Schoenfeld residuals.

The statistical analyses were performed using SPSS for Windows version 25.0 (SPSS Inc., Chicago, IL, USA) and R version 4.0.0 (R Core Team, Auckland, New Zealand).

Results

Patient characteristics

Of the original 160 included patients, eight patients had severe IC (Rutherford stage 3) and were excluded from analyses (none underwent amputation or died within five years). Two of the remaining 152 CLTI patients were lost to follow-up in an early phase. Hence five-year follow-up data were available for 150 patients, including 102 males (68%), with a median age of 67 (IQR 56, 76) years, of whom 56 (37%) patients had DM. At the time of inclusion, 51 patients had rest pain (Rutherford stage 4), 90 patients had ischaemic ulceration not exceeding the digits of the foot (Rutherford stage 5), while nine patients had severe ischaemic ulcers or gangrene (Rutherford stage 6).

Outcomes

After five years, 64 of the 150 patients (43%) survived without major amputation of the index limb. Of the other 86 patients, 53 (35% of the total) died, and 49 (33% of the total) underwent a major amputation. In 16 patients, amputation was performed prior to their death within the five-year interval. The median time between amputation and death was 291 days (IQR 35, 583). The Kaplan–Meier curves for AFS, amputation, and death, are shown in Figure 1. As seen, all-cause mortality is evenly distributed along the five-year interval, while amputation occurs predominantly within the first year. The one-year AFS was 70% (95% CI 63 – 78), attributed to 24% (95% CI 17 – 31) major amputation and 11% (95% CI 6 – 16) mortality.

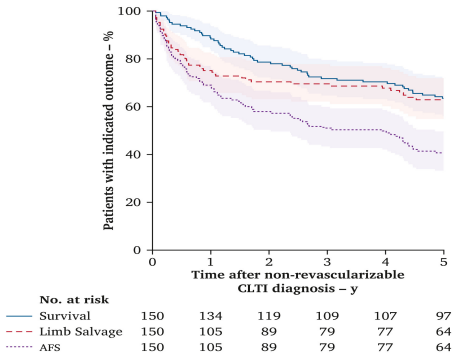


Figure 1: Cumulative Kaplan-Meier estimate of amputation-free survival (AFS), survival and limb salvage during a five-year period, with 95% confidence intervals, in patients with non-revascularisable chronic limb-threatening ischemia (CLTI)

Determinants of outcomes

Table 1 summarises the baseline characteristics of the 150 included patients, stratified by the five-year composite outcome. Male gender ($p = .033$), higher age ($p < .001$), higher Rutherford stage ($p = .004$), history of a cerebrovascular event ($p < .001$) and cardiogenic chest pain ($p = .001$), use of diuretics ($p = .031$), lower glomerular filtration rate ($p = .013$), HDL cholesterol ($p = .005$), and haemoglobin ($p = .004$) were statistically significantly more common in the group with the composite of amputation and mortality.

The results of univariable and multivariable Cox proportional hazard regression analyses are detailed in Table 2 for the composite outcome and Table 3 for individual outcomes. Age (HR 1.77, 95% CI 1.35 – 2.32; $p < .001$), Rutherford 5 (HR 1.79, 95% CI 1.07 – 2.99; $p = .027$), Rutherford 6 (HR 3.48, 95% CI 1.46 – 8.27; $p = .005$), and HDL cholesterol (HR 0.68, 95% CI 0.53 – 0.88; $p = .003$) were independent predictors for the composite of amputation and death. Figure 2 presents AFS for these predictors, in which the continuous variables age

Table 1: Characteristics of 150 patients with non-revascularisable chronic limb-threatening ischemia stratified by endpoint

	Amputation-Free Survival 64	Amputation or Mortality 86	P
Gender = Female (%)	27 (42.2)	21 (24.4)	.033
Age - y	60.50 [51.50, 70.00]	71.00 [62.25, 79.00]	<.001
BMI kg/m^2	26.56 [24.53, 29.17]	25.15 [22.72, 27.77]	.055
Peripheral Artery Disease			
Rutherford Classification (%)			.004
Rutherford 4	31 (48.4)	20 (23.3)	
Rutherford 5	31 (48.4)	59 (68.6)	
Rutherford 6	2 (3.1)	7 (8.1)	
History of (%)			
Cerebrovascular Event	2 (3.1)	20 (23.3)	.001
Cardiogenic chest pain	15 (23.4)	44 (51.2)	.001
Coronary Intervention	13 (20.3)	32 (37.2)	.040
Ipsilateral Minor Amputation	5 (7.8)	10 (11.6)	.62
Ipsilateral Bypass	34 (53.1)	41 (47.7)	.62
Ipsilateral PTA or Stent	37 (57.8)	53 (61.6)	.76
Contralateral Major Amputation	2 (3.1)	8 (9.3)	.24
Contralateral Minor Amputation	3 (4.7)	5 (5.8)	1.0
Contralateral Bypass	9 (14.1)	16 (18.6)	.61
Contralateral PTA or Stent	13 (20.3)	21 (24.4)	.69
Dialysis	2 (3.1)	3 (3.5)	1.0
Hypertension	37 (59.7)	53 (63.1)	.80
Diabetes Mellitus	19 (29.7)	37 (43.0)	.13
Smoking			.12
Never	6 (9.4)	15 (17.9)	
History of Smoking	36 (56.2)	51 (60.7)	
Currently	22 (34.4)	18 (21.4)	
Use of medication			
Antiplatelets			.008
None	20 (31.2)	25 (29.1)	
Aspirin	41 (64.1)	39 (45.3)	
Clopidogrel	1 (1.6)	6 (7.0)	
Aspirin + Clopidogrel	1 (1.6)	13 (15.1)	
Persantin	1 (1.6)	3 (3.5)	
Anticoagulants			.74
None	42 (65.6)	51 (59.3)	
Acenocoumarol	19 (29.7)	29 (33.7)	
Fenprocoumon	3 (4.7)	6 (7.0)	
Lipid Lowering Drugs			.97
None	10 (15.6)	15 (17.4)	
Statin	51 (79.7)	66 (76.7)	
Ezetimibe	0 (0.0)	1 (1.2)	
Statin + Ezetimib	3 (4.7)	4 (4.7)	
ACE inhibitors	20 (31.2)	38 (44.2)	.15
Angiotensin-2 receptor blockers	13 (20.3)	18 (20.9)	1.0
Diuretics	22 (34.4)	46 (53.5)	.031
Beta-blockers	24 (37.5)	42 (48.8)	.22
Laboratory Results			
Glomerular Filtration Rate	78.36 [64.12, 86.76]	62.04 [44.34, 86.83]	.013
Total cholesterol	4.40 [3.50, 5.17]	4.20 [3.32, 4.80]	.15
Triglycerides	1.40 [0.90, 1.92]	1.45 [1.00, 2.05]	.44
HDL cholesterol	1.32 [0.96, 1.55]	1.06 [0.84, 1.30]	.005
Hemoglobin	8.40 [7.88, 8.95]	7.80 [7.12, 8.50]	.004
Thrombocytes	283 [223, 330]	279.50 [234, 343]	.92
Leucocytes	7.90 [6.83, 9.72]	8.55 [7.03, 10.15]	.17
Outcomes			
Mortality	0	53	
Amputation	0	49	

Data are presented as n (%) or median (interquartile range). PTA = percutaneous transluminal angiography; ACE = angiotensin-converting enzyme; GFR = glomerular filtration rate: HDL = high-density lipoprotein; BMI = body mass index. Parametric continuous data were tested with the Student t-test, non-parametric continuous data with the Mann-Whitney U test, and categorical data with Fisher's exact test.

and HDL cholesterol are categorised based on their median value.

Similarly, a history of a cerebrovascular event (HR 2.49, 95% CI 1.19 – 5.20; p = .015), history of contralateral amputation (HR 3.3, 95% CI 1.44 – 7.60; p = .005), higher leucocytes (HR 1.48, 95% CI 1.12–1.95; p = .006), and lower haemoglobin (HR 0.72, 95% CI 0.56 – 0.92; p = .010) were predictors for amputation, whereas age (HR 2.26, 95% CI 1.49 – 3.44; p < .001), lower glomerular filtration rate (HR 0.64, 95% CI 0.47 – 0.87; p = .004), and HDL cholesterol (HR 0.54, 95% CI 0.39 – 0.76); p < .001) were independent predictors of death. The proportional hazard assumption holds for all of these, and thus, these predictors were not time-dependent.

Discussion

The long-term prognosis in this well-defined and granulated CLTI population without options for revascularisation (NR-CLTI) was revealed to be poor, with 43% of the patients completing five years of survival without limb loss. This result was driven by an equal rate of all-cause mortality and amputation: one-third of the patients died (35%), and one-third underwent amputation of the index limb (33%). Furthermore, a third of those with limb loss after inclusion died within the five-year time interval (33%). The present data correspond with the findings of a small, long-term retrospective observational study (n = 30), the only published equivalent, reporting a five-year mortality of 30% for this NR-CLTI subgroup.[14] No registry studies have been performed; thus, prognostic information for NR-CLTI patients is very limited. As such, the present data provide the best available insight into today's perspective for these patients in terms of mortality and limb salvage. Two registry studies concerning the "real world" CLTI population reported higher all-cause mortality rates of 54% and 57% for four and five years, respectively. This may relate to the fact that the present study population was younger and had lower prevalences of history of coronary artery disease and DM.[1, 15]

In trial-selected patients treated for severe limb ischaemia, the BASIL trial reported an AFS of 38% within the completed follow-up (3 – 7 years), which was mainly driven by mortality (56%), possibly as a result of an older study population.[16] Although the overall amputation rate was not given, only 7% of the patients alive at the final follow-up underwent amputation, compared with 22% in the present study. This seems particularly high, but four-year amputation rates of CLI patients in a retrospective cohort, according to Rutherford stages 4, 5, and 6 (12%, 35%, and 67%, respectively) were more comparable with the present cohort (20%, 38%, and 56%, respectively).[17]

In the present cohort, 33% of those who underwent amputation subsequently died within the investigated period. This rate is relatively low as amputation is an established risk factor for death, and five-year mortality rates of up to 85% have been reported in elderly CLI amputees, and seven-year rates after below and above-the-knee amputations in a veteran cohort (published in 2003) were 72% and 80%, respectively.[18–20] However, subjects were much older in both studies, which troubles comparison.

More published evidence is available on the short-term outcomes of this subgroup. At one year, NR-CLTI patients in JUVENTAS were at an especially high risk of amputation (24% of total), but mortality was lower (11%). In comparison, two meta-analyses reported one-year amputation rates of 22% and 34% and mortality rates of 22% and 20%.[9, 21]

This is perhaps the result of a similar design of some of the included studies in these

Table 2: Results of Cox proportional hazard regression analysing the predictors for the composite endpoint of amputation and death in 86 patients with non-revascularisable chronic limb-threatening ischaemia

	Composite endpoint			
	Univariate analysis		Multivariate analysis *	
	HR (95% CI for HR)	P	HR (95% CI for HR)	P
Gender (Female)	0.63 (0.38-1)	.062		
Age [a]	1.73 (1.3-2.3)	<.001	1.77 (1.35-2.32)	<.001
BMI [a]	0.97 (0.92-1)	.21		
Peripheral artery disease				
Rutherford 5 [b]	2.08 (1.25-3.46)	.005	1.79 (1.07-2.99)	.027
Rutherford 6 [b]	4.05 (1.71-9.61)	.001	3.48 (1.46-8.27)	.005
History of				
Cerebrovascular Event	2.93 (1.8-4.9)	<.001		
Cardiogenic Chest Pain	2.07 (1.4-3.2)	<.001		
Coronary Intervention	1.65 (1.1-2.6)	.026		
Contralateral Major Amputation	1.99 (0.96-4.1)	.063		
Contralateral Minor Amputation	1.19 (0.48-2.9)	.70		
Ipsilateral Minor Amputation	1.15 (0.59-2.2)	.68		
Contralateral Bypass	1.29 (0.75-2.2)	.35		
Contralateral PTA or Stent	1.13 (0.69-1.8)	.63		
Ipsilateral Bypass	0.88 (0.58-1.3)	.56		
Ipsilateral PTA or Stent	1.19 (0.77-1.8)	.44		
Dialysis	0.87 (0.27-2.7)	.81		
Diabetes Mellitus	1.45 (0.94-2.2)	.090		
Use of medication				
ACE inhibitors	1.39 (0.91-2.1)	.13		
Angiotensin-2 receptor blockers	1.05 (0.62-1.8)	.87		
Diuretics	1.64 (1.1-2.5)	.023		
Beta-blockers	1.37 (0.9-2.1)	.14		
Laboratory Results				
Glomerular Filtration Rate [a]	0.81 (0.65-1)	.064		
Total cholesterol [a]	0.85 (0.69-1)	.12		
Triglycerides [a]	1.06 (0.86-1.3)	.57		
HDL cholesterol [a]	0.74 (0.59-0.92)	.008	0.68 (0.53-0.88)	.003
Hemoglobin [a]	0.73 (0.61-0.88)	<.001		
Thrombocytes [a]	1.07 (0.86-1.3)	.56		
Leucocytes [a]	1.15 (0.94-1.4)	.17		

Data are presented as n (%), median [interquartile range] or mean ± standard deviation. PTA = percutaneous transluminal angiography; ACE = angiotensin converting enzyme; HDL = high density lipoprotein; HR = hazard ratio; CI = confidence interval. Empty fields are not entered into the final model; BMI = Body Mass Index.

* Multivariable HRs were calculated with the Cox proportional hazard analysis using a forward stepwise approach (derived from factors with $p < .10$ in univariable analysis).

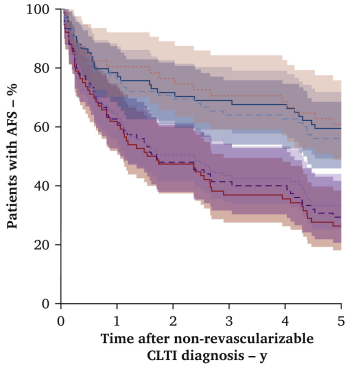
[a] Non-parametric continuous data were log-transformed and scaled to provide an HR per standard deviation increase.

[b] Rutherford 5 and 6 are compared with Rutherford 4 stage.

meta-analyses: most recent short-term prognostic data are derived from small RCTs investigating gene or cell therapy in no-option patients.[22–24] Other (older) case series included in these meta-analyses do not provide up-to-date information for the current CLTI population, especially as recent studies show a gradual reduction of amputation and mortality rates.[1, 9–11, 25, 26]

Short and long-term results considered, the present results indicate that NR status is associated with an increased early risk of major amputation, although this risk tails off in subsequent years. In contrast, mortality is fairly evenly distributed throughout follow-up. This is important for both patients and physicians and might imply that NR status is not the primary cause of death but rather a gradation of a common denominator: progressive systemic atherosclerotic disease. Direct comparison between CLTI and NR-CLTI is difficult, but outcomes are generally in the same order of magnitude. In contrast, a more benign (PAD) population with means of intervention recently revealed considerably better outcomes, as all-cause mortality and amputation rates of just 9.1% and 3.5% at three years in the placebo arm of the recent VOYAGER-trial demonstrate.[27]

As the difference in outcomes for CLTI and NR-CLTI patients is less pronounced than that of CLTI and IC patients, NR status is perhaps not a major risk factor. With regard to this concept, although the long-term prognosis is poor, the present authors believe that NR status in CLTI does not drive towards immediate amputation per se if the best medical/wound treatment can be applied, contrary to what perhaps seems the general belief of vascular specialists, and neither does this amputation always lead to premature death (compared with CLTI patients with revascularisation options). The emphasis for management of these high-risk patients should therefore lie on strategies to decrease the amputation risk in the short term and enable optimal management of comorbidities in the long term. This approach could facilitate vascular specialists in medical management and patient counselling and is otherwise crucial in the design of future regenerative trials and their selection of patients.



No. at risk						
Age < median	74	58	53	51	50	44
Age > median	76	47	36	28	27	20
HDL < median	75	47	36	31	30	22
HDL > median	75	58	53	48	47	42
Rutherford 4	51	41	39	36	35	31
Rutherford 5 & 6	99	64	50	43	42	33

Figure 2: Cumulative Kaplan-Meier estimate of amputation-free survival (AFS) during a five year period, with 95% confidence intervals, stratified by independent risk factors: Rutherford classification, median age, and median high-density lipoprotein (HDL) in patients with non-revascularisable chronic limb-threatening ischaemia (CLTI).

Putting the study outcomes into perspective is difficult because of a paucity of prognostic data for NR-CLTI and the heterogeneity of study design and populations. The disparity between the present results and some of the current literature could be attributed to a trial effect, selection bias, definition, and time. The so-called "trial effect" has been suggested to influence the outcome, although little evidence is available on this topic.[28, 29]

However, extensive care and strict surveillance, as implemented in these trials, are thought to reduce adverse outcomes in cardiovascular disease and thus hypothetically support this claim.[30–32] If these assumptions are valid, the present relatively benign results compared with the CLTI registry studies suggest that extensive care could improve the prognosis of the no-option patient significantly, even for a relatively short amount of time (as in this study),

and thus more effort is warranted to enable optimal management.

On the other hand, differences in the present outcomes, caused by a discrepancy between real-world and trial patients, are possibly the result of selection bias. Participation in a time-consuming study with potential adverse events could potentially favour a compliant patient with, ultimately, a lower a priori risk of mortality because of better adherence and disease awareness. Undoubtedly, the exclusion criteria in JUVENTAS, in combination with an average to good ambulatory state (a non-ambulatory state can be a disincentive to participation because of the frequency of follow-ups), influences both short and long-term outcomes.[33–35]

Furthermore, there is a lack of a standard definition of "no option", which could comprise patients without feasible intervention and patients whose medical condition is too frail to justify the exposure to additional intra- and postoperative risks. Patients included in JUVENTAS match the first category, as established by a multidisciplinary team of vascular surgeons and radiologists in an academic hospital. However, other mentioned studies combined these categories, which subsequently influenced these outcomes.[24–26]

Whether the no-option patient of today is comparable with the no-option patients of 10 or 20 years ago in terms of AFS is arguable, as secondary and tertiary prevention has improved, and innovations have led to improved revascularisation alternatives.[1, 36] Furthermore, a time-dependent shift in aetiology (macro to microvascular) could lead to different patient characteristics. However, the main principle of the present no-option definition remains the same: all patients are subject to inadequate perfusion, resulting in high-grade ischaemia, without any means of treatment in the foreseeable future. A uniform description should be considered for general use and research, in which it is proposed that there is an emphasis on the "no option anatomy" category, as mentioned in the Global Vascular Guidelines on the management of CLTI.[37]

A limitation of the present analysis is the extension of the original follow-up without additional contacts or visits within this interval. However, the endpoints remained the same, and almost no loss to follow-up occurred. The two patients lost to follow-up were removed from the analysis because there was a significant gap between their last confirmed medical status and five-year follow-up. Both treatment and placebo arms were included in the present analyses. The JUVENTAS trial did not find a treatment-related effect on AFS. At five years, the present study reaffirmed no difference in AFS (46 vs 40, p = .53), amputation (27 vs 22, p = .56), or mortality (29 vs 24, p = .58) for treatment vs placebo, respectively.[13] Thus, including patients from both trial arms is justified.

In conclusion, the present study provides the necessary contemporary long-term follow-up data for NR-CLTI patients. The poor amputation-free survival and general survival underscore the poor prospects for these patients. Compared with other studies, the present analysis suggests that AFS and survival in NR-CLTI are no worse than in CLTI patients with revascularisation options.

Table 3: Results of Cox proportional hazard regression analysing the predictors for amputation and death in 150 patients with non-revascularisable chronic limb-threatening ischaemia

	Amputation = 49				Mortality = 53			
	Univariate analysis		Multivariate analysis *		Univariate analysis		Multivariate analysis *	
	HR (95% CI for HR)	P	HR (95% CI for HR)	P	HR (95% CI for HR)	P	HR (95% CI for HR)	P
Female gender	0.8 (0.43–1.5)	.48			0.51 (0.26–1)	.049		
Age [a]	1.27 (0.93–1.7)	.14			2.27 (1.5–3.5)	<.001	2.26 (1.49–3.44)	<.001
BMI [a]	0.93 (0.87–1)	.061			0.97 (0.91–1)	.40		
Peripheral artery disease								
Rutherford 5 [b]	2.24 (1.11–4.55)	.025			2.26 (1.15–4.42)	.018		
Rutherford 6 [b]	5.20 (1.77–15.3)	.003			2.69 (0.86–8.46)	.09		
History of								
Cerebrovascular event	2.06 (1.02–4.2)	.044	2.49 (1.19–5.20)	.015	2.28 (1.2–4.3)	.012		
Cardiogenic chest pain	1.47 (0.84–2.6)	.18			2.54 (1.5–4.4)	<.001		
Coronary intervention	1.49 (0.83–2.7)	.18			1.58 (0.91–2.8)	.11		
Contralateral major amputation	3.16 (1.4–7)	.010	3.3 (1.44–7.60)	.005	0.75 (0.23–2.4)	.62		
Contralateral minor amputation	1.26 (0.39–4.1)	.70			1.61 (0.58–4.5)	.36		
Ipsilateral minor amputation	0.76 (0.27–2.1)	.60			1.63 (0.77–3.5)	.20		
Contralateral bypass	1.56 (0.79–3)	.20			0.89 (0.42–1.9)	.75		
Contralateral PTA or stent	1.27 (0.67–2.4)	.46			0.85 (0.44–1.7)	.64		
Ipsilateral bypass	1.02 (0.58–1.8)	.95			0.66 (0.38–1.1)	.13		
Ipsilateral PTA or stent	1.37 (0.76–2.5)	.30			0.85 (0.5–1.5)	.57		
Dialysis	1.03 (0.25–4.2)	.97			1.04 (0.25–4.3)	.96		
Diabetes mellitus	1.78 (1–3.1)	.043			0.94 (0.54–1.7)	.84		
Use of medication								
ACE inhibitors	1.41 (0.8–2.5)	.24			1.3 (0.76–2.2)	.34		
Angiotensin 2 receptor blockers	0.89 (0.43–1.8)	.76			0.78 (0.38–1.6)	.49		
Diuretics	1.3 (0.74–2.3)	.36			1.94 (1.1–3.3)	.017		
Beta blockers	0.95 (0.53–1.7)	.85			1.96 (1.1–3.4)	.016		
Laboratory Results								
Glomerular filtration rate [a]	1.08 (0.81–1.5)	.59			0.56 (0.43–0.73)	<.001	0.64 (0.47–0.87)	.004
Total cholesterol [a]	0.72 (0.55–0.95)	.020			0.87 (0.67–1.1)	.29		
Triglycerides [a]	0.93 (0.7–1.2)	.59			1.12 (0.85–1.5)	.41		
HDL cholesterol [a]	0.76 (0.57–1)	.057			0.62 (0.47–0.83)	.001	0.54 (0.39–0.76)	<.001
Haemoglobin [a]	0.69 (0.54–0.88)	.002	0.72 (0.56–0.92)	.010	0.71 (0.55–0.9)	.005		
Thrombocytes [a]	1.39 (1.1–1.8)	.019			0.83 (0.63–1.1)	.20		
Leucocytes [a]	1.3 (1–1.7)	.052	1.48 (1.12–1.95)	.006	1.05 (0.81–1.4)	.72		

Data are presented as n (%), median [interquartile range] or mean ± standard deviation. PTA = percutaneous transluminal angiography; ACE = angiotensin converting enzyme; HDL = high density lipoprotein; HR = hazard ratio; CI = confidence interval. Empty fields are not entered into the final model; BMI = Body Mass Index. * Multivariable HRs were calculated with the Cox proportional hazard analysis using a forward stepwise approach (derived from factors with p < .10 in univariable analysis).

[a] Non-parametric continuous data were log-transformed and scaled to provide an HR per standard deviation increase.

[b] Rutherford 5 and 6 are compared with Rutherford 4 stage.

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3

External validation of the vascular quality initiative prediction model for survival in no-option chronic limb-threatening ischemia patients

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Abstract

Objective: *Chronic limb-threatening ischemia (CLTI) is associated with high morbidity and mortality rates. More than 50% of all CLTI patients die within five years after presentation. Patient-specific survival prediction is critical for informing treatment strategies, even for those without a clear option for revascularisation. We validated a survival prediction model developed in a revascularised Vascular Quality Initiative (VQI) cohort in a Western European no-option CLTI cohort.*

Methods: *The VQI survival prediction model was applied to the validation cohort (N = 150) to compare estimated mortality and observed mortality two years after baseline. The performance of the VQI model was tested by evaluating discrimination using the receiver operating characteristic area under the curve and calibration using the Hosmer-Lemeshow goodness-of-fit test.*

Results: *The two-year survival rate was 79% in the validation cohort compared with 83% in the VQI cohort. Baseline characteristics were significantly different for 13 of 17 variables. The C statistic was 0.86 (95% confidence interval, 0.78-0.95), which indicates good discrimination. The Hosmer-Lemeshow goodness-of-fit test had a P value of .30, which indicates a good fit.*

Conclusion: *This is the first external validation of the VQI survival prediction model. The good model performance suggests that it can be used in different CLTI populations, including no-option CLTI, and underlines its contributory role in this challenging population.*

Introduction

Globally, >200 million people are living with peripheral artery disease (PAD), and this prevalence has increased during the last decade.[1] Although chronic limb-threatening ischemia (CLTI) represents <10% of all PAD patients, it comes with a huge burden in terms of morbidity, mortality, and socioeconomic costs. At 12 months, both the mortality and amputation rate are approximately 20%.[2] Estimates indicate that >50% of all CLTI patients die within five years after presentation.[3] A more recent Dutch study supported this five-year estimate and showed a slight decrease in all-cause mortality between 1998 and 2010.[4]

CLTI is a multifactorial disease with mainly ischemic, neuropathic, and microvascular determinants. Recognizing the multifactorial aetiology has led to improved therapeutic strategies for these patients. Yet, choices for revascularisation are mostly based on expert opinion or the personal preference of the physician treating the patient. There is no standardized therapeutic approach to the CLTI patient, and therefore the recently published Global Vascular Guidelines attempted to provide a framework for evidence-based revascularisation.[5] This framework is composed of three dimensions: (1) Patient risk; (2) Limb status; and (3) ANatomic pattern (PLAN). Staging of the limb by the Wound, Ischemia, and foot Infection (WIfI) classification and staging of the anatomic disease pattern by the Global Limb Anatomic Staging System (GLASS) are the fundaments of the second and third dimensions of PLAN. A reliable tool to assess patient risk, the first element of the PLAN framework, has yet to appear.

Multiple tools have been developed to assist clinicians with predicting all-cause mortality, major amputation, amputation-free survival, and perioperative events. The only randomized controlled trial comparing open lower extremity bypass with endovascular intervention (Bypass vs Angioplasty in Severe Ischaemia of the Leg [BASIL]) showed that open bypass is the preferred treatment in patients with survival >2 years.[6] Estimating the prognosis of a CLTI patient is, therefore, not only useful to inform the patient. It can also affect therapeutic choices by determining whether revascularisation is indicated and, if so, which approach should be preferred. A prognostic model derived from BASIL data was externally validated and showed modest performance in this heterogeneous population. Moreover, variables in BASIL are less convenient and not available in routine clinical practice.[7] Other models show similar limited predictive values.[8–10]

A prediction model was recently developed using the Vascular Quality Initiative (VQI) database, in which CLTI patients underwent either an endovascular intervention or infrainguinal bypass. The chosen covariates were easily obtainable, and internal validation showed acceptable discrimination.[11] However, external validation of the VQI-derived model has not yet been performed. The model was developed in patients who had undergone revascularisation; its performance in patients without a revascularisation option is unknown. If the VQI model proves to have good performance in a no-option CLTI population, this will underline that the model could be useful in a broader population to substantiate treatment decisions on amputation and palliative strategies and to aid in the optimal selection of patients in future regenerative therapeutic trials (e.g., cell therapy) in no-option CLTI patients.

Therefore, our goal was to investigate the applicability and performance of the VQI model in predicting survival at two years in a well-defined Western European no-option CLTI population.

Methods

Study design

We aimed to apply the VQI survival predictive model for two-year mortality on the baseline characteristics in the Rejuvenating Endothelial Progenitor Cells via Transcutaneous Intra-arterial Supplementation (JUVENTAS) cohort and to compare them with the observed mortality at two years. We analysed two-year mortality and disregarded the 30-day time point because of the clinical relevance and limited sample size. We analysed the performance of the VQI survival prediction model by evaluating discrimination and calibration.

The Institutional Review Board of the University Medical Center Utrecht approved the study. The study was conducted according to the Declaration of Helsinki, and all patients provided written informed consent.

Initial model cohort

The specifics of the VQI model and cohort can be found in the original article.[11] In summary, the model was derived from a large eponymous cohort of CLTI patients in the United States who underwent open or endovascular revascularisation of the infrainguinal segment of the lower extremity between 2003 and 2017. The primary endpoint was survival at two years, defined as freedom from all-cause mortality. A backward stepwise selection was performed after univariable analysis, which resulted in a Cox proportional hazards model with 12 covariates: age; race; rest pain or tissue loss; smoking status; coronary artery disease; congestive heart failure (CHF); chronic obstructive pulmonary disease (COPD); chronic kidney disease stage; ambulation status; and preoperative use of beta blocker, antiplatelet, and statin (see Table for corresponding coefficients). The equation for the Cox survival model is

$$h(t, X) = h0(t)exp(\sum \beta ixi)$$

where X = (x_1, x_2,...., xp) are the covariates, and h0 (t) is the hazard function, with an empirical baseline hazard of 0.952 at two years in the VQI cohort. Internal validation demonstrated acceptable discrimination with a C statistic of 0.72 at two years. The Hosmer-Lemeshow goodness-of-fit test was $P < .05$.

Validation cohort

The JUVENTAS trial was a single-centre, double-blind, placebo-controlled, randomized controlled trial performed between 2006 and 2012 in The Netherlands.[12] It showed no difference in major amputation or all-cause mortality six months after repetitive infusion (three times at three-week intervals) of bone marrow mononuclear cells in 160 patients with severe, nonrevascularisable (no-option) PAD compared with placebo. A multidisciplinary team of experts determined no-option status because of either poor health status or technical impossibility for revascularisation.

For our analysis, two patients with missing information on outcomes were excluded from

Table 1: Baseline characteristics of Rejuvenating Endothelial Progenitor Cells via Transcutaneous Intra-arterial Supplementation (JUVENTAS) and Vascular Quality Initiative (VQI) cohorts

	Beta coefficient	JUVENTAS (N = 150)	VQI (N = 38,470)	*P*
Age, years				.036
<60	Referent	45 (30)	8400 (22)	
60-70	0.32	45 (30)	12,105 (31)	
71-80	0.68	40 (27)	10,030 (26)	
>80	1.17	20 (13)	7935 (21)	
Sex, male		102 (68)	23,533 (61)	.10
Race, nonwhite	−0.25	9 (6.0)	9376 (24)	<.001
Indication				.38
Rest pain	Referent	51 (34)	11674 (30)	
Tissue loss	0.43	99 (66)	26,796 (70)	
Smoking status				<.001
Never	Referent	21 (14)	11,368 (30)	
Prior history	0.09	89 (59)	15,621 (41)	
Current	0.11	40 (27)	11,412 (30)	
Hypertension		95 (63)	34,577 (90)	<.001
CAD				.005
None	Referent	90 (60)	26,715 (70)	
History of MI, asymptomatic or stable angina	0.18	59 (39)	10,799 (28)	
Unstable angina or MI within 6 months	0.31	1 (0.7)	916 (2.4)	
CHF	0.49	9 (6.0)	8888 (23)	<.001
Diabetes		56 (37)	24,328 (63)	<.001
COPD				.017
None	Referent	129 (86)	29,766 (77)	
Not treated or on medication	0.24	21 (14)	7670 (20)	
Home oxygen	0.52	0 (0)	1034 (2.7)	
Chronic kidney disease stage				.004
GFR >90 mL/min/1.73 m2	Referent	31 (21)	7295 (22)	
GFR >60-89 mL/min/1.73 m2	0.02	69 (46)	12,597 (37)	
GFR 30-59 mL/min/1.73 m2	0.22	34 (23)	11,506 (34)	
GFR 15-29 mL/min/1.73 m2	0.64	12 (8.0)	1949 (5.8)	
GFR <15 mL/min/1.73 m2	1.09	4 (2.7)	303 (0.9)	
Ipsilateral treatment		122 (81)	15,066 (39)	<.001
Major amputation		10 (6.7)	2799 (7.3)	.90
Ambulation status				.015
Independent	Referent	88 (59)	24,576 (64)	
With assistance	0.33	39 (26)	10,066 (26)	
Wheelchair bound	0.52	23 (15)	3266 (8.5)	
Bedbound	0.91	0	428 (1.1)	
Baseline medication				
Beta blocker	0.12	66 (44)	23,353 (61)	<.001
Antiplatelet	−0.13	105 (70)	29,029 (75)	.15
Statins	−0.19	124 (83)	25264 (66)	<.001
Endpoints				
Survival		119 (79)	31,880 (83)	.29

Data are presented as n (%), median [interquartile range] or mean ± standard deviation. CAD = Coronary artery disease; CHF = congestive heart failure; COPD = chronic obstructive pulmonary disease; GFR = glomerular filtration rate; MI = myocardial infarction. Beta coefficients are derived from the VQI model. Characteristics without a beta coefficient are not implemented in the VQI model. The numbers of patients are given with percentages in parentheses. A $\chi 2$ analysis was used for all categorical characteristics. For baseline medication, numbers correspond with patients who use medication at baseline.

the validation analyses. Another eight patients with Rutherford classification stage 3 were excluded because they did not meet the CLTI criteria; per definition, they had no rest pain or ischemic wounds.

Endpoints

The primary outcome was all-cause mortality after two years of inclusion.

Statistical analyses

A $\chi 2$ analysis was performed to compare categorical baseline characteristics of the JUVENTAS and VQI groups. A logistic regression model for mortality at two years was constructed using the coefficients for the corresponding Cox model in the original article. Three survival curves were constructed by the Kaplan-Meier method, with each curve representing one tertile of the VQI Cox survival score. Calibration refers to the agreement between observed outcomes and predictions.[13] A Hosmer-Lemeshow goodness-of-fit model was used to assess calibration. We used g = 10. A P value <.05 means the model is not a good fit; a nonsignificant P value indicates good calibration. We constructed receiver operating characteristic (ROC) curves with a calculation of the concordance (C) statistic, which is equal to the area under the curve (AUC) in binary outcomes. A C statistic is a unitless index interpreted as the probability of a random pair of patients in which the patient who meets the endpoint has a higher predicted probability compared with the patient who does not.[14] A C statistic of <0.6 was considered fail, 0.6 to 0.7 was considered poor, 0.7 to 0.8 was considered fair, 0.8 to 0.9 was considered good, and 0.9 to 1 was considered excellent in the ability to predict the all-cause mortality of the patient. SPSS 21.0 (IBM Corp, Armonk, NY) and R 3.5.1 (R Core Team, Auckland, New Zealand) were used for the execution of all statistical analyses.

Results

Patient characteristics

In the JUVENTAS cohort, for a total of 150 patients, the 2-year follow-up was completed. Baseline characteristics of the VQI and JUVENTAS cohorts are presented in the Table. Distribution of the age groups was significantly different between JUVENTAS and VQI (P = .036). The majority of the patients were male (68% vs 61%; P = .10) and white (94% vs 76%; P < .001) and had tissue loss (66% vs 70%; P = .38) in both cohorts. In JUVENTAS, 14% of the patients never smoked, 59% had only a history of smoking, and 27% were current smokers. This was significantly different in the VQI cohort, with 30%, 41%, and 30%, respectively (P < .001). Hypertension was less common in JUVENTAS (63% vs 90%; P < .001). CHF was present in only 6% compared with

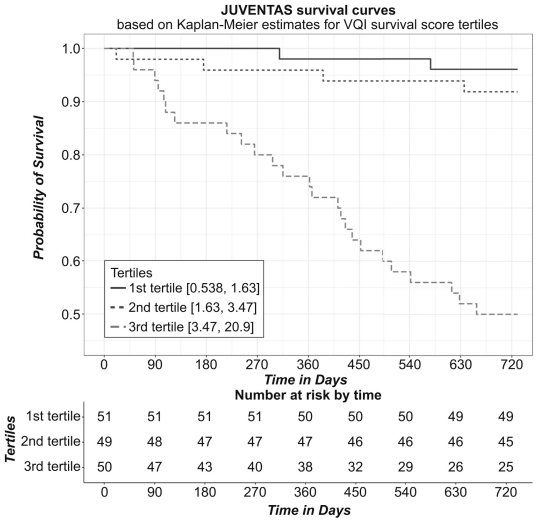

Figure 1: Kaplan-Meier curves for Vascular Quality Initiative (VQI) survival model score tertiles. Tertiles of VQI survival scores were made (ranging from [0.538-1.63], [1.63-3.47], and [3.47-20.9]) for patients in JUVENTAS. The first (and lowest) tertile indicates a lower risk of mortality, the last (and highest) tertile indicates a higher mortality risk.

23% (P < .001) and diabetes in 37% vs 63% (P < .001), and most patients had no history of COPD (86% vs 77%; P = .017). A large proportion of the patients from the JUVENTAS cohort had previous ipsilateral treatment (81% vs 39%; P < .001). Use of beta-blockers (44% vs 61%; P < .001) and statins (83% vs 66%; P < .001) was statistically different in the cohorts.

Follow up

All-cause mortality within two years after inclusion in the JUVENTAS trial was 21% (n = 31) compared with 17% (n = 6590) in the VQI population (P = .29). In Figure 1, the Kaplan-Meier curves are shown for tertiles of the VQI Cox survival score to indicate at what time a patient dies, depending on the patient's survival probability. The highest tertile (the highest risk scores derived from the VQI model) mortality is higher compared with the lower two tertiles. At two years, 50% of the patients in the highest tertile have died, compared with 8.1% and 3.6% in the middle and lowest tertiles, respectively.

Prediction model performance

In Figure 2, the ROC curve of the 2-year all-cause mortality is shown. The AUC was 0.86 (95% confidence interval, 0.78-0.95), which is generally regarded as providing good discrimination. The P value of the Hosmer-Lemeshow goodness-of-fit was .30, indicating an agreement between observed outcomes and prediction and, therefore, good model calibration.

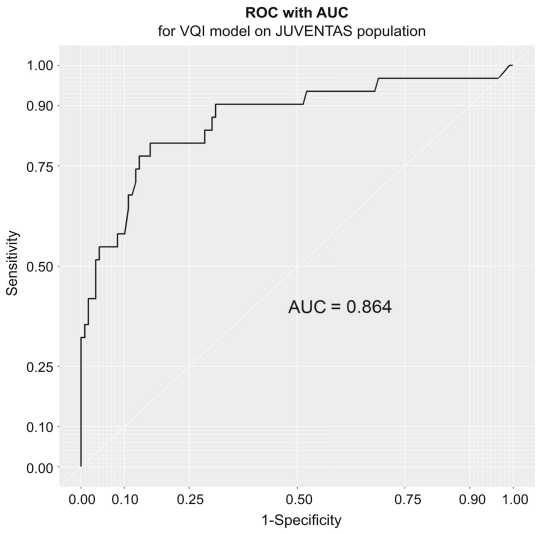

Figure 2: Discrimination of the model for overall mortality at two years. Receiver operating characteristic (ROC) curves for predicted and observed mortality at two years are obtained from all JUVENTAS patients. The area under the curve (AUC) is 0.864 (95% confidence interval, 0.781-0.948).

Figure 3 shows the calibration plot of the predicted and actual mortality probabilities. There is an underestimation of low predicted probability (0.1-0.3), indicating that patients with a predicted probability in this range have an actual mortality that is slightly higher. There is a good estimation when the predicted probability is higher (0.5-1.0). The Supplementary Figure (online only) shows a regression curve of survival based on the VQI Cox survival score; 30 is not the highest possible score, but no patient exceeded this risk score in the JUVENTAS cohort. To test whether the predictive performance was coincidentally good only at two years, we also calculated the ROC AUC for 180 and 365 days. Likewise, this shows good discrimination with a ROC AUC of 0.83 (9 events) and 0.86 (16 events), respectively.

Discussion

The initial VQI survival prediction model showed good performance in an internal validation study of patients who underwent revascularisation. Here we present the first external validation study of the VQI model in an independent well-defined no-option CLTI cohort from Western Europe. Our study showed good discrimination (C statistic = 0.86) and a nonsignificant Hosmer-Lemeshow goodness-of-fit test result (P = .30), indicating good calibration. Our findings support the VQI model as a good tool to evaluate survival estimates of no-option patients as part of the first dimension of the PLAN framework.

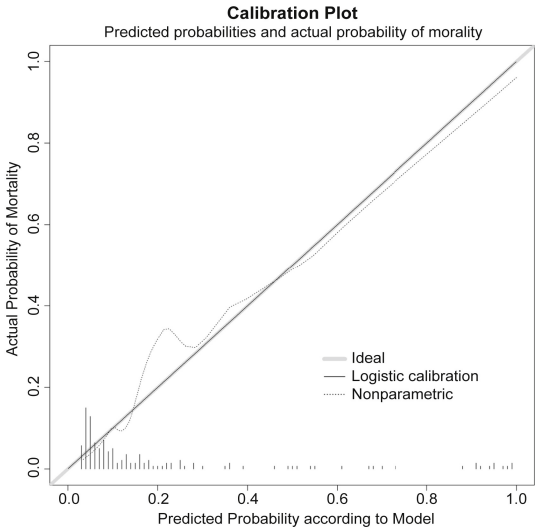

Figure 3: Calibration plot for Vascular Quality Initiative (VQI) survival model at two years. Standard calibration curve for the predicted probability of mortality and actual probability of mortality. The diagonal line indicates the ideal line (where the predicted probability matches the actual probability of mortality). On top of the x-axis, an indication of the number of patients is given in a histogram to show the distribution of patients along the predicted probability according to the VQI survival model (on the x-axis). There are relatively more patients with a lower probability of mortality (left side of the x-axis) compared with a higher probability of mortality (right side of the x-axis).

Survival predictions in no-option patients may seem less relevant as surgical revascularisation strategies are not expected to be successful or patients are deemed to be too frail to be exposed to the risks of intervention. However, a reliable estimation of the prognosis of no-option CLTI patients could be relevant, especially considering future therapeutic options. The model is useful in providing an optimal selection of no-option patients who might benefit most from novel therapeutic strategies (e.g., biologics). These trials are costly, and results are affected when there is a high loss to follow-up due to earlier (unexpected) death. Patients within the third tertile of survival scores perform much worse compared with patients in the first two tertiles. Therefore, in this high-risk group, the least advantage from other treatments is expected, and palliative care should be considered. Furthermore, patients within the lower-risk groups have better life expectancies and should be the focus of therapeutic research in the future.

In reviewing the JUVENTAS and VQI cohorts, the majority of baseline characteristics differ significantly despite the fact that both cohorts included CLTI patients, which underlines the heterogeneity of the CLTI population. For age >80 years, race, hypertension, diabetes, CHF, COPD, and use of beta-blockers, the incidences and, therefore, risk indicators were much higher in the VQI cohort, whereas for the history of smoking, myocardial infarction, ipsilateral treatment, and use of statins, the covariates in the JUVENTAS cohort were more prevalent. The difference in baseline characteristics and hence predictor values between the cohorts may be explained by demographic and genetic differences, different structures of the healthcare system, and, most important, the selection of patients. The VQI

cohort is derived from patients who were able to receive either surgical or endovascular intervention and were prospectively observed from this time point. The JUVENTAS trial, in contrast, was a study performed in patients without revascularisation options. The specific reason to be considered no-option varied between patients; most were deemed technically ineligible for revascularisation, and in other patients, the poor overall condition precluded an intervention.

Despite these differences, survival was not significantly different between groups and in line with previous literature.[2] This could partially be explained by the exclusion criteria in the JUVENTAS trial: life expectancy <1 year and no history of malignant disease in the past ten years. This may have reduced the number of patients at risk for mortality in the trial. Furthermore, it could be hypothesized that inclusion in a clinical trial is related to better outcomes, and patients who are motivated and consent to participate in a clinical trial have characteristics that put them in a prognostic better no-option subgroup; for example, their compliance could be higher, and risk factors are addressed better because of a prevalent follow-up within the trial. This selection bias complicates a direct baseline comparison between the cohorts.

Analysing the covariates independently, age, CKD stage, and ambulation were the most contributory to discrimination in our population. This is unsurprising as beta coefficients were highest in these groups, although very few patients in JUVENTAS were in the highest risk categories (i.e., >80 years old, glomerular filtration rate <15 mL/min/1.73 m2, and bedbound). In this respect, a limitation of the model might be the dichotomization of age and CKD stage. Part of the information is lost this way.

In the VQI model, coronary artery disease and preoperative ambulation violated the proportional hazards assumption. When checking the Schoenfeld residuals of the covariates independently, we found no interaction with time in the JUVENTAS data. This is probably due to a combination of the very small deviance in coefficient (ranging from -0.011 to -0.005, as reported in the original article) and the small sample size in JUVENTAS. The majority of the covariates used in the VQI survival model could be regarded as general health indicators.

In this model, only the indication for intervention can be considered a direct CLTI-dependent risk factor for mortality. By expanding the predictive model with more PAD-specific variables, performance could perhaps be improved. Unfortunately, ankle-brachial index (ABI) measurements were missing in >40% in the VQI cohort, as it would be interesting to see whether including ABI would lead to increased model performance. Other studies have shown ABI to be a good predictor for long-term survival in PAD.[15–17] However, ankle pressure was not incorporated in the BASIL predictive score because of the lack of statistical significance, and other prediction models have not incorporated ABI either.[6, 18] In the future, it could be assessed whether other factors like perfusion (e.g., toe pressure or toe-brachial index) and blood-derived parameters or biomarkers further improve the VQI model.[19]

However, the main advantage of this model is the overall easy availability of its covariates, and even without CLTI-specific covariates, the VQI survival model performs well. Moreover, in comparison with the BASIL and Edifoligide for the Prevention of Vein Graft Failure in Lower Extremity Bypass Surgery Phase III (PREVENT III) survival models, which were validated within the JUVENTAS cohort, the VQI model has better performance.[20] Therefore, the VQI model seems to serve as a good indicator for no-option patients in the first

dimension of the PLAN framework. Although the JUVENTAS cohort represents a selection of specific and high-risk patients, we were able to show that the VQI model performs well in this subgroup. In the absence of alternative prediction models, we suggest that based on our analysis, the VQI model can be applied to no-option CLTI patients to predict survival. External validation on all-comers should be performed to extend its validity and utility.

Strengths and limitations

As JUVENTAS is a clinical trial, cohort covariates were gathered prospectively, and missing data were scarce. There was a low loss of follow-up within the JUVENTAS trial, even though the original follow-up stopped at six months. Only two patients were lost at the 2-year interval and were excluded.

A limitation of this study is its small absolute number of events. The small event size in our study could lead to an overestimation of the model's performance. The relatively low number of events could be due to selection bias in the JUVENTAS trial. To have a lower loss to follow-up in the original trial and to reduce the loss of patients due to factors other than the one studied, patients with concomitant disease with an estimated life expectancy of <1 year were excluded from the trial. This could be considered another limitation of this study because the population included in the trial could be considered a relatively healthier selection than the general no-option population. However, the estimation of frailty was purely subjective, and looking at the Kaplan-Meier curves, a linear decrease in the probability of survival across the whole 2-year interval is seen. If the initial estimation were better, we would expect a more convex slope if high-risk patients were excluded, with fewer deaths in the first year and increased mortality in the following year. Consequently, this underlines the difficulty of accurately predicting mortality, which shows the importance of a prediction model, such as the VQI, for this CLTI subgroup.

In this first external validation study, the VQI survival model showed good performance. Our study indicates that the model applies not only to surgically or endovascularly treated patients in the United States but can also be used in nonrevascularisable Western European CLTI patients. Therefore, the VQI survival prediction model seems a reasonable tool to be used as part of the three-dimensional PLAN strategy proposed in the Global Vascular Guidelines for the treatment of CLTI. Whether the model can be further improved with the addition of blood-derived or CLTI-specific parameters, such as ABI, should be elucidated in future studies. Moreover, the VQI model should be validated in a large prospective all-comer CLTI population to show its usefulness in the general CLTI population.

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Supplemental Material

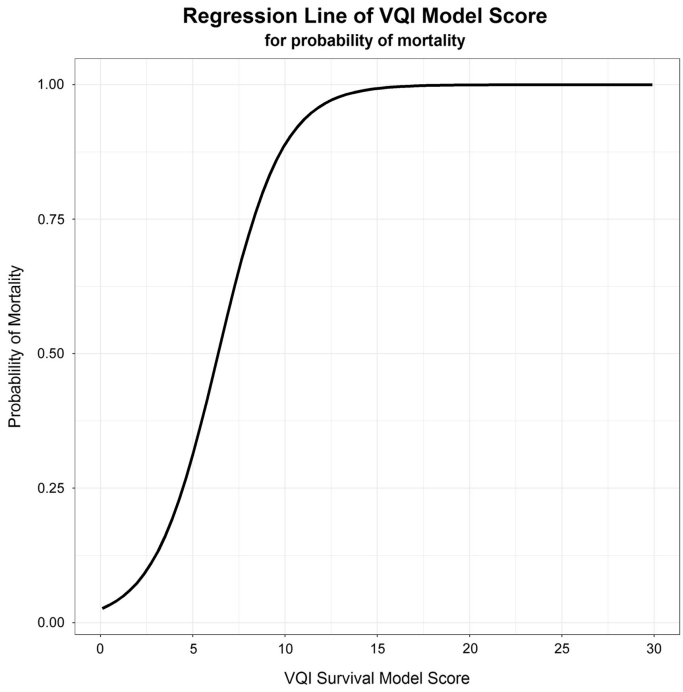

Figure S1: Regression curve of mortality probability. A regression curve shows the probability of mortality dependent on the Vascular Quality Initiative (VQI) survival model score. Patients with a score of 5 have approximately a 31% chance of mortality within two years. Patients with a score of 10 have approximately 88% chance of mortality within two years.

II

Risk and complications of peripheral artery disease in pseudoxanthoma elasticum

4

Peripheral interventions in patients with pseudoxanthoma elasticum

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Abstract

Introduction: *Pseudoxanthoma elasticum (PXE) is an autosomal recessive metabolic disorder that may be associated with a high prevalence of peripheral artery disease (PAD) and related symptoms. However, the evidence supporting this association is weak, as only small cohort studies are available. Furthermore, limited data are available on the outcome of peripheral arterial interventions (PAI) in patients with PXE.*

Methods: *From the Dutch National Expertise Centre database for PXE, we examined the clinical data of consecutive patients with a definitive diagnosis of PXE. The primary endpoint was the prevalence of PAD (defined as an ankle-brachial index of <0.9). The secondary endpoint was to report an overview of PAI and target lesion revascularisations.*

Results: *In 285 PXE patients (median age 58 years), 50.9% of patients (N = 145) met the criteria for PAD. Seventeen patients underwent a PAI, mostly for intermittent claudication, at a median age of 51 years. The incidence of PAI was 2.25 per 1,000 patient-years in patients with PAD and PXE. A total of 58 interventions were recorded, of which 35 were target lesion revascularisations in 9 patients. Twenty-one revascularisations were performed within a year following the primary intervention, in 16 cases due to an acute occlusion.*

Conclusion: *Within a well-phenotyped and large PXE cohort, the diagnosis of PAD was prevalent in one of every two patients. The observed rate of peripheral interventions was low, while the reintervention rate was unfavourable after endovascular or bypass surgical procedures, with over half of the reinterventions indicated within a year.*

Introduction

Pseudoxanthoma elasticum (PXE) is an autosomal recessive metabolic disorder characterised by an ectopic accumulation of calcium phosphate complexes, leading to pheno-typical expressions that include yellowish papules on the sides of the neck and in flexural areas. The disease is caused by mutations in the ABCC6 gene, and with an estimated prevalence of around one in every 25 000 – 50 000 people in the general population, knowledge about the disease remains limited.[1] Nevertheless, the available literature on PXE indicates that the prevalence of leg arterial calcifications (LAC) and lower extremity peripheral artery disease (PAD) are, with rates up to 80%, remarkably high in these patients.[2, 3] Furthermore, in these small cohorts, as many as 56% of these patients reported intermittent claudication, which often manifests at an early age and in the absence of other major risk factors for cardiovascular disease.[2, 3]

It is currently believed that these complications result from the mineralisation of elastic fibres in the medial arterial layer, which impairs the elasticity of the blood vessels (increased stiffness and decreased compliance) and leads to increased intima-media thickness (IMT).[4] These pathophysiological processes are different and develop at a different rate from those in PAD in patients without PXE. As a result, questions have been raised about the effectiveness of conventional preventive and interventional management of patients with PXE. Anecdotally, high failure rates for femoral angioplasty have been observed in this high-risk group, suggesting that (endo)vascular interventions are less preferable in PXE.[5] However, the true prevalence of PAD and peripheral arterial interventions (PAI) in patients with PXE remains unclear due to its rarity: current results are derived from small cohorts (n =32, 38, 53 and 71) and are thus susceptible to all sorts of bias. Furthermore, no cohort studies have investigated PAI; the only knowledge comes from one case series and two case reports.

The present study used clinical data from the Dutch Expertise Centre for PXE to primarily elucidate the diagnosis of PAD in patients with PXE. The second objective was to report PAI and target lesion revascularisations (TLR) in PXE patients.

Methods

Cohort population

Dutch PXE patients are followed and treated within the Dutch National Expertise Centre for PXE, situated in the University Medical Centre Utrecht (UMCU). The clinical history was collected retrospectively, and specifically for patients with PAI, additional procedural data were obtained on request by contacting referring hospitals and the general practitioner. The follow-up data of all PXE patients were recorded prospectively and included a protocolised diagnostic program with genetic, dermatological, ophthalmological, and standardised vascular screening. The diagnosis of PXE was established on predefined criteria.[6] Given the retrospective use of routine care data, no formal approval of this study was required, as stated by the Medical Ethics Committee of the UMC Utrecht. Participants gave written informed consent to use their medical files for research purposes. This substudy was performed in accordance with the declaration of Helsinki.

Vascular Screening

During the initial visit after referral for PXE to the UMCU and subsequent visits thereafter, systematic history taking was performed in which patient characteristics such as vascular risk factors were collected. These included a physical examination and regular diagnostic cardiovascular blood work. Ankle-brachial index (ABI) measurements before and after the treadmill test were performed by experienced technicians. Pre-test ABI was measured after 10 minutes of supine rest by measuring the systolic blood pressure of both brachial and posterior tibial and dorsal pedal arteries. Treadmill exercise was performed on a 10% slope and at a speed of 3.5 km/h for six minutes. Post-test ABI measurements were done similarly. For patients who underwent peripheral revascularisation, a pre-interventional ABI was used when these data were documented and available. Diabetes mellitus was defined as a history of diabetes mellitus in the medical files or the use of glucose-lowering agents. Hypercholesterolaemia was defined based on laboratory work or the use of cholesterol-lowering treatment. Hypertension was a composite of either elevated blood pressure on both arms (repeated measurements) or the use of antihypertensive drugs.

Peripheral artery disease definition

Lower extremity PAD was defined as a post-treadmill test ABI lower than 0.9 at the limb with the lowest measurement. Since the efficacy of the ABI as a diagnostic instrument has never been proven in PXE, the PXE group was also stratified according to an ABI below and above 0.5.

Arterial interventions and failure of intervention

Peripheral arterial interventions were defined as open surgery, endovascular interventions (including thrombolysis), or a combination (hybrid) to treat (symptoms of) lower extremity PAD. TLR was defined as a re-intervention for both clinically and radiologically driven stenoses or occlusions in the same arterial segment as a previous procedure. Acute occlusions were defined as a sudden loss of limb perfusion, unrelated to the time after the previous intervention, resulting in acute worsening of limb-related symptoms and requiring revascularisation.

Statistical analyses

Quantitative data were expressed as mean (±standard deviation (SD)) or as median (interquartile range, (IQR)) as appropriate to their distribution and were compared with the Student t-test and a Mann-Whitney U test, respectively. Discrete data were presented as frequencies and percentages and were compared using the chi-square or Fisher exact test. A comparison of baseline characteristics was performed for two groups stratified by diagnosis of PAD and history of PAI. For PAI, the incidence per 1000 person-years was calculated.

All p values were 2-tailed, with a $P < 0.05$ considered statistically significant. Statistical analyses were performed with R version 4.0.4 inside an R Studio 1.4.1103 environment.(7)

Table 1: Overall characteristics and characteristics stratified for presence or absence peripheral artery disease of lower extremities of patients with pseudoxanthoma elasticum recorded in the Dutch Expertise Centre database

	Overall 285	**PAD** 145 (50.8%)	**No PAD** 140 (49.2%)	**P**
Female	177 (62.1)	94 (64.8)	83 (59.3)	.40
Age - y	52 [44, 60]	55 [47, 63]	49 [34, 58]	<.001
Cardiovascular risk factors				
Smoking status				.71
Current, every day smoker	42 (14.8)	24 (16.7)	18 (12.9)	
Current, someday smoker	5 (1.8)	2 (1.4)	3 (2.2)	
Former smoker	123 (43.5)	64 (44.4)	59 (42.4)	
Never smoker	113 (39.9)	54 (37.5)	59 (42.4)	
CAD	16 (5.6)	11 (7.6)	5 (3.6)	.23
CVA	33 (11.6)	26 (17.9)	7 (5.0)	.001
Lower limb peripheral intervention	17 (6.0)	17 (6.0)	0	<.001
Hypertension	87 (30.5)	53 (36.6)	34 (24.3)	.034
Hypercholesterolemia	140 (49.1)	79 (54.5)	61 (43.6)	.085
DM type 1	1 (0.4)	1 (0.7)	0 (0.0)	1.00
DM type 2	10 (3.5)	8 (5.5)	2 (1.4)	.12
BMI				
Overweight	68 (23.9)	36 (24.8)	32 (22.9)	.80
Obese	38 (13.3)	19 (13.1)	19 (13.6)	1.00
Severely obese	7 (2.5)	5 (3.4)	2 (1.4)	.47
Morbidly Obese	2 (0.7)	0 (0.0)	2 (1.4)	.46
Peripheral characteristics				
ABI pre-walking test	0.98 [0.78, 1.06]	0.79 [0.66, 0.96]	1.05 [1.00, 1.10]	<.001
ABI post walking test	0.90 [0.63, 1.04]	0.63 [0.44, 0.78]	1.04 [0.98, 1.09]	<.001
Medication				
Antihypertensive medication	59 (20.7)	41 (28.3)	18 (12.9)	.002
Antithrombotic/anticoagulant medication	60 (21.1)	43 (29.7)	17 (12.1)	.001
Acetylsalicylic acid	27 (9.5)	20 (13.8)	7 (5.0)	.020
Carbasalate calcium	10 (3.5)	3 (2.1)	7 (5.0)	.341
P2Y12 inhibitor	16 (5.6)	15 (10.3)	1 (0.7)	.001
Vitamin K antagonist	5 (1.8)	4 (2.8)	1 (0.7)	.39
Direct Oral Anticoagulant	4 (1.4)	3 (2.1)	1 (0.7)	.64
Dipyridamole	8 (2.8)	6 (4.1)	2 (1.4)	.31
Glucose lowering medication	6 (2.1)	5 (3.4)	1 (0.7)	.23
Lipid lowering medication	75 (26.3)	57 (39.3)	18 (12.9)	<.001
Lab Results				
eGFR - mL/min/1.73m^2	85.24 (9.93)	84.10 (10.79)	86.51 (8.74)	.051
Calcium - mmol/L	2.37 (0.19)	2.39 (0.14)	2.34 (0.24)	.076
Triglycerides - mmol/L	1.34 (0.95)	1.39 (0.85)	1.29 (1.05)	.37
Cholesterol - mmol/L	5.04 (1.16)	5.04 (1.17)	5.05 (1.15)	.99
LDL - mmol/L	2.92 (1.01)	2.88 (1.01)	2.96 (1.00)	.51
HDL - mmol/L	1.52 (0.39)	1.53 (0.40)	1.52 (0.38)	.80
Albumin - mmol/L	42.27 (2.97)	41.95 (3.01)	42.62 (2.89)	.071

Data are presented as n (%), median [interquartile range] or mean ± standard deviation. PAD = peripheral artery disease; TIA = transient ischaemic attack; DM = diabetes mellitus; ABI = ankle-brachial index; eGFR = estimated glomerular filtration rate; LDL-c = low-density lipoprotein cholesterol; HDL-c = high-density lipoprotein cholesterol.

Results

Baseline characteristics

A total of 285 patients with a definitive diagnosis of PXE were included (Table 1). The median age at inclusion was 52 years (IQR, year, 44-60), and the median follow-up time within the PXE registry was 4.8 years (IQR, year, 2.11–6.23). This corresponds to a retrospective follow-up of 14,256 and a prospective follow-up of 1,271 patient years. The majority of patients were female (62.1%), hypercholesterolemia (49.1%) was relatively prevalent, and only 3.9% of patients had a history of diabetes mellitus (DM). A percutaneous coronary intervention (PCI) and coronary artery bypass graft surgery (CABG) were performed in 16 patients (5.6%), whereas 33 patients (11.6%) had a history of a cerebrovascular accident (CVA).

Peripheral artery disease and ABI measurements

According to ABI measurements, 50.8% of the total PXE cohort met the criteria for PAD (Table 1). ABI levels were lower in higher age groups (Figure 1), which is also reflected by the significantly higher age of patients with PAD vs those without (55 vs 49 years, p <.001) and the proportions of PAD per age category (Figure 2). Hypertension was more prevalent (36.6% vs 24.3%, p = .034) compared to patients without PAD. Cardiac interventions such as coronary artery bypass graft surgery and percutaneous coronary intervention were equally prevalent in both groups, but a history of CVA or TIA was reported significantly more by patients with PAD than those without PAD (17.9% vs 5.0%, p =.001). Patients with PAD were significantly more likely to receive antihypertensive, antithrombotic and lipid-lowering drugs. A total of 52 patients had a recorded ABI of < 0.5 (Supplementary Table S1). In patients with a lower ABI, virtually all major risk factors for cardiovascular disease were significantly more prevalent. Atherosclerotic complications such as cardiac interventions (13.5% vs 3.4%, p = .010) and CVA or TIA (32.7% vs 6.9%, p <.001) were over three times more prevalent in the lower ABI group compared with PXE patients with an ABI >0.5.

Peripheral interventions

Of the 285 consecutive patients, 17 (6%) reported a medical history of PAI before inclusion (Table 2). Their first PAI was performed at a median age of 51 years (IQR, year, 45, 62). After inclusion, there were no new patients who underwent PAI. Thus, the incidence of PAI was 1.20 per 1 000 person-years in the total cohort and 2.25 per 1 000 person-years in patients with PXE and PAD. The ABI values of all patients with PAI were low, both pre- (0.65 [IQR 0.50, 0.81]) and post-walking test (0.43 [IQR 0.16, 0.61]) and none of these patients had ABI measurements above 0.9. For the primary intervention, 13 patients had disabling intermittent claudication as an indication for intervention, one had rest pain, and three had ischaemic ulcers. These symptoms were caused by occlusion (n = 5, of which none were acute) or stenosis (n = 8) in the target lesion; in four cases, the (older) reports were ambiguous. Two patients' symptoms worsened from intermittent claudication to rest pain after an intervention. Ulcer healing was reported in the three patients with ischaemic wounds, and no patients required lower limb amputation or any other ipsilateral amputation. Seven patients underwent a single PAI, and 51 interventions were performed in the remaining ten patients. Of these, nine patients underwent at least one PAI in both limbs. In summary: endovascular interventions (n = 28), bypass surgery (n = 12), endarterectomy (n = 1), throm-

Table 2: Characteristics of patients with pseudoxanthoma elasticum with or without a history of peripheral intervention recorded in the Dutch Expertise Centre database

	Peripheral intervention 17	No peripheral intervention 268	P
Female	11 (64.7)	166 (61.9)	1.00
Age - y	59 [56, 68]	51 [42, 60]	.001
Cardiovascular risk factors			
Smoking status			.21
Current, every day smoker	3 (17.6)	39 (14.7)	
Current, someday smoker	0 (0.0)	5 (1.9)	
Former smoker	11 (64.7)	112 (42.1)	
Never smoker	3 (17.6)	110 (41.4)	
CAD	4 (23.5)	12 (4.5)	.006
CVA	4 (23.5)	29 (10.8)	.23
Hypertension	9 (52.9)	78 (29.1)	.072
Hypercholesterolemia	12 (70.6)	128 (47.8)	.12
DM type 1	0 (0.0)	1 (0.4)	1.00
DM type 2	2 (11.8)	8 (3.0)	.22
BMI			
Overweight	6 (35.3)	62 (23.1)	.40
Obese	3 (17.6)	35 (13.1)	.86
Severely obese	0 (0.0)	7 (2.6)	1.00
Morbidly Obese	0 (0.0)	2 (0.7)	1.00
Peripheral characteristics			
Highest grade			
Rest pain	3 (17.6)	0	<.001
Ischemic Wounds	3 (17.6)	0	<.001
ABI pre-walking test	0.65 [0.50, 0.81]	0.99 [0.82, 1.06]	<.001
ABI post walking test	0.43 [0.16, 0.61]	0.93 [0.70, 1.04]	<.001
Medication			
Antihypertensive medication	11 (64.7)	48 (17.9)	<.001
Antithrombotic/anticoagulant medication	15 (88.2)	45 (16.8)	<.001
Acetylsalicylic acid	8 (47.1)	19 (7.1)	<.001
Carbasalate calcium	0 (0.0)	10 (3.7)	.90
P2Y12 inhibitor	5 (29.4)	11 (4.1)	<.001
Vitamin K antagonist	4 (23.5)	1 (0.4)	<.001
Direct Oral Anticoagulant	0 (0.0)	4 (1.5)	1.00
Dipyridamole	2 (11.8)	6 (2.2)	.12
Glucose lowering medication	1 (5.9)	5 (1.9)	.80
Lipid lowering medication	15 (88.2)	60 (22.4)	<.001
Lab Results			
eGFR - mL/min/1.73m^2	84.62 (14.00)	85.28 (9.64)	.80
Calcium - mmol/L	2.40 (0.08)	2.37 (0.20)	.49
Triglycerides - mmol/L	1.48 (0.66)	1.33 (0.97)	.55
Cholesterol - mmol/L	4.63 (0.95)	5.07 (1.17)	.13
LDL - mmol/L	2.55 (0.78)	2.94 (1.01)	.13
HDL - mmol/L	1.42 (0.37)	1.53 (0.39)	.28
Albumin - mmol/L	41.71 (2.63)	42.30 (2.99)	.46

Data are presented as n (%), median [interquartile range] or mean ± standard deviation. PAD = peripheral artery disease; TIA = transient ischaemic attack; DM = diabetes mellitus; ABI = ankle-brachial index; eGFR = estimated glomerular filtration rate; LDL-c = low-density lipoprotein cholesterol; HDL-c = high-density lipoprotein cholesterol.

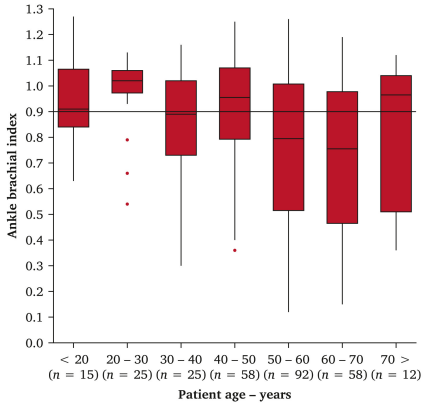

Figure 1: Lowest ankle-brachial index (ABI) measurements stratified by age categories in a cohort of 285 patients with pseudoxanthoma elasticum recorded in the Dutch Expertise Centre database. Boxplots demonstrate median and interquartile ranges, whiskers depict the highest or lowest ABI within 1.5 times the interquartile range. Outliers (values >1.5 times the interquartile range beyond either end of the box) are represented by single points.

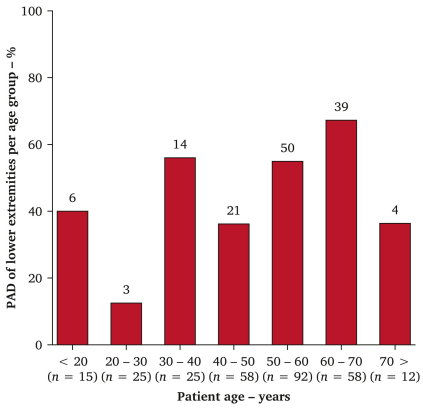

Figure 2: The relative frequency of peripheral artery disease (PAD) of the lower extremities with ankle-brachial index < 0.9 across different age groups in a cohort of 285 patients with pseudoxanthoma elasticum recorded in the Dutch Expertise Centre database. Counts of patients with PAD are given above each bar.

bolysis (n = 5), embolectomy (n = 10), and hybrid (n = 2) interventions were most commonly performed in the femoropopliteal segment (n = 47), followed by iliac arteries (n = 8), and below the knee (n = 3) lesions.

Target lesion revascularisations

A total of 35 TLRs were performed on nine patients. The numbers of successive TLR were scored as follows: three patients with one and three re-interventions, one with six, seven, and ten re-interventions (Figure 3, Supplementary Fig. S1). Seven patients reported TLR within half a year, equating to a six-month TLR rate of 41% (7/17). The other two patients had an interval of over ten years between interventions. Twenty-one (60%) of these re-interventions were performed within one year of the initial PAI in that segment (overall median time was 183 days [IQR 32, 1 425]). Seventeen TLRs were performed after bypass surgery and 18 following endovascular interventions due to 21 occlusions and 10 re-stenoses (four indications were undefined) (Table 3). Sixteen of these occlusions were defined as acute, but this was disproportionate as these occurred in only five patients (i.e., one patient with nine acute occlusions, another with four).

Discussion

To date, this is the largest study addressing PAD in patients with PXE. In a cohort of 285 Dutch PXE patients, half met the criteria for PAD, which is consistent with previous small cohort studies.[2, 7, 8] This prevalence is remarkably high compared to the prevalence of

PAD in the general population of high-income countries (7.37%) and cannot be attributed to the skewed age distribution of our cohort.[9] Even across lower age groups, PAD was frequently seen and was nearly 20 times more common than in the general population (at age 30 - 40: 2.19 - 2.79% in the general population versus 60% in our PXE cohort).[9]

It remains debatable whether the utility of the ABI, with a cutoff point of 0.9, provides the best diagnostic efficacy in patients with PXE. Analogous to diabetes mellitus, in which ABI measurements have a lower sensitivity for the diagnosis of PAD, PXE has been thought to be associated with elevated ABIs that hinder these diagnostic capabilities.[10] However, the present study demonstrated that only two patients exhibited noncompressible arteries, and no other patients had an ABI of > 1.3. This aligns with another study (n = 53) that reported only a single PXE patient with an ABI >1.4.[2] The present results are surprising given the general concept that medial arterial calcifications, as seen in PXE in up to 80% of patients, are thought to be responsible for increased ABI.[3, 11] However, a recent study disputing the association between medial arterial calcification and elevated ABI supports the present findings.[12]

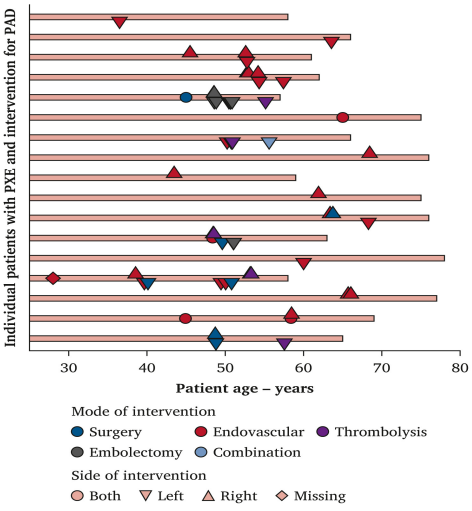

Figure 3: Peripheral artery interventions performed in 17 patients among the cohort of 285 patients with pseudoxanthoma elasticum (PXE) recorded in the Dutch Expertise Centre database with peripheral artery disease (PAD) of lower extremities plotted against age.

Fortunately for patients with PXE, disease progression to critical limb ischaemia seems rare, as the present cohort included only three patients with (a history of) rest pain and three patients with (a history of) ischaemic wounds. Furthermore, rest pain was described as a result of acute post-interventional occlusions in two of these patients, and it remains unknown whether the natural history of PXE would have led to symptom progression in these patients. The available literature includes only one case study that reported a manifestation of ischaemic wounds, but whether this is genetically confirmed or PXE-like disease remains unknown.[13] Other PXE cohorts with a particular interest in PAD neither confirmed nor denied the presence of ischaemic ulceration in any of the patients. A natural history of rest pain has only been documented in one case report.[14]

Only a minority of patients in the present cohort recorded a history of a PAI for arterial stenotic or occlusive disease. These interventions were mostly performed to alleviate intermittent claudication due to either stenoses or chronic occlusive disease. The latter is in line with an observational study that found a high prevalence of chronic occlusion in one or more peripheral vessels in

patients with PXE.[7] Most of the present patients underwent multiple revascularisations, almost always bilaterally, and TLR within a year of the initial PAI was frequently observed. The rate of acute occlusion was high after both intervention types but especially after bypass surgery, although this is biased by a high rate of recurrent acute occlusion in only a few patients. It is, therefore, difficult to determine whether this may be more related to the characteristics of these patients or local therapeutic management or whether it can be attributed entirely to PXE. To the present authors' knowledge, the (pathophysiological) association of occlusive disease and PXE has hardly been investigated, and this should perhaps be an area for future research.

Comparable data on PAI and PXE are sparse and limited to case reports and case series.[5, 15, 16] In summary, two interventions with good outcomes (no re-stenosis or occlusion within one year) and four patients with interventions that failed in both the short and long term provided the best evidence for the efficacy of PAI in PXE thus far. In the patients of these studies, the segment of intervention was most often the superficial femoral artery followed by the iliac arteries. The present data confirm this distribution pattern. To the present authors' knowledge, no reports have been made of below-the-knee interventions in PXE patients, and so this is the first study to report such cases.

Finally, and especially as the cohort may be considered small when compared with more common diseases, it is important to see the present results in the context of the characteristics of the cohort. The present data support the female predominance of PXE.[17] One in three patients had a history of hypertension, which is on par with equivalent studies.[18, 19] In contrast, hypercholesterolaemia was reported often (49%) in our study population, and its prevalence is higher than that of other PXE cohorts (27% and 19.4%).[8, 19] However, our reported prevalence might not be unreasonably high as a Belgian study found hypercholesterolaemia in over 75% of carriers of ABCC6 mutations.[20] Other characteristics or laboratory values are as expected, and thus it is believed that the prevalence of PAD and the number of PAI are not influenced by a disproportionate cohort.

Strict recommendations cannot be made based on the present and other available data, as the number of PXE patients with PAI is low. In summary, a PAI has a reasonable effect in some patients but has major implications in those with interventions that fail (prematurely). Although in PXE, the number of patients with PAD is exceptionally high, the natural history seems less malign, with rare reports of rest pain or ischaemic ulceration. As a consequence, the risk of postoperative adverse events seems to outbalance the potential short-term benefit in patients with mild complaints (i.e., intermittent claudication), and other means of treatment should be pursued first.

Strengths and limitations

The greatest strengths of this study are its sample size and the prospective well-phenotyped registration; data are provided from one of the largest cohorts of PXE worldwide. These patients completed systematic vascular screening, enabling a detailed description of the PAD phenotype. The vast majority of patients were referred to the present authors' centre of expertise because of symptoms of PXE and only a few because of a family screening. Although most patients are under surveillance in the UMC Utrecht, some chose to attend their local physician and hospital or were treated there before referral. Consequently, the selection and implementation of PAI are not centralised and may therefore vary from one institution to

another. Moreover, PXE was sometimes not diagnosed until after these intervention(s), and hypothetically, a different strategy would have been chosen had this condition been known.

The retrospective nature of this study leads to recall bias but is insurmountable as PXE expertise centres have only been started within the last decade. Furthermore, reports on interventions that were carried out a long time ago were not as detailed as present reports. Cardiovascular risk management has evolved in recent years, and thus the efficacy of interventions undertaken a long time ago perhaps does not reflect the current risks and advantages of PAI. Heterogeneity of post-procedural follow-up in the multiple hospitals (and across time) impaired the investigation into (radiological) patency, and thus TLR was chosen as a fitting outcome after PAI.

In conclusion, within a well-phenotyped and large PXE cohort, the diagnosis of PAD was prevalent in one in two patients. The observed rate of peripheral interventions was low, while the re-intervention rate was unfavourable after endovascular or bypass surgical procedures, with over half of the re-interventions indicated within a year.

Table 3: Target lesion revascularisation counts after type of surgery because of stenosis or occlusion in patients with pseudoxanthoma elasticum and peripheral artery disease of lower extremities recorded in the Dutch Expertise Centre database

Indication for revascularisation	**Prior intervention performed**		
	Bypass	Endovascular	Total
Occlusion	15	6	21
Stenosis	1	9	10
Missing	1	3	4
Total	17	18	35

Data are presented as n.

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Supplemental Material

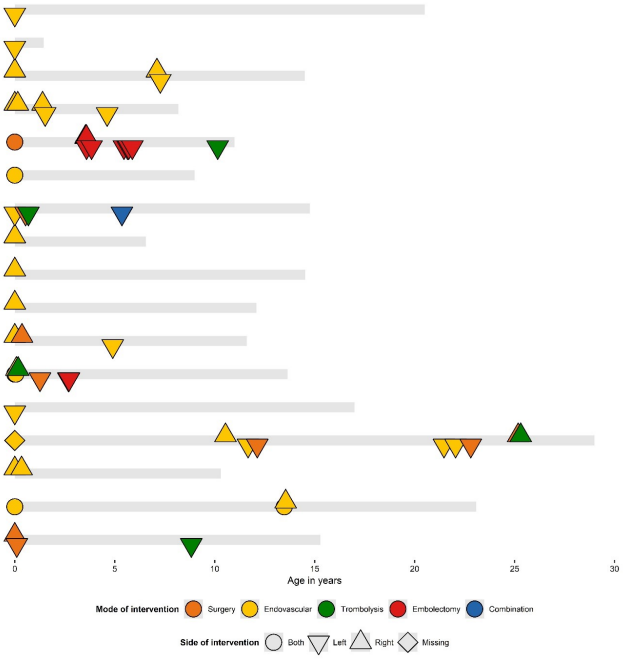

Figure S1: For each patient that underwent a peripheral intervention for lower limb claudication, a line is drawn from the first intervention until the latest follow-up, reflecting the follow-up time. The type of intervention is depicted in different colours. Symbols indicate which side is operated on: both sides (circle), left (downward triangle) and right (upward triangle).

Table S1: Characteristics of patients stratified by ABI values below or above 0.5

	ABI <0.5 52	**ABI >0.5** 232	**P**
Female (%)	29 (55.8)	148 (63.8)	.36
Age in years (median [IQR])	63 [57, 70]	56.00 [44, 66]	<.001
Cardiovascular risk factors			
Smoking status (%)			.014
Current, every day smoker	15 (28.8)	27 (11.7)	
Current, someday smoker	0 (0.0)	5 (2.2)	
Former smoker	20 (38.5)	102 (44.3)	
Never smoker	17 (32.7)	96 (41.7)	
CAD	7 (13.5)	8 (3.4)	.010
CVA	17 (32.7)	16 (6.9)	<.001
Hypertension (%)	22 (42.3)	64 (27.6)	.055
Hypercholesterolemia (%)	35 (67.3)	104 (44.8)	.005
DM type 1 (%)	0 (0.0)	1 (0.4)	1.00
DM type 2 (%)	5 (9.6)	5 (2.2)	.026
BMI			
Overweight (%)	15 (28.8)	53 (22.8)	.46
Obese (%)	10 (19.2)	27 (11.6)	.21
Severely obese (%)	1 (1.9)	6 (2.6)	1.00
Morbidly Obese (%)	0 (0.0)	2 (0.9)	1.00
Peripheral characteristics			
Highest grade			
Rest pain (%)	3 (5.8)	0	<.032
Ischemic Wounds (%)	2 (3.8)	0	<.032
ABI pre-walking test (median [IQR])	0.65 [0.56, 0.74]	1.02 [0.92, 1.07]	<.001
ABI post walking test (median [IQR])	0.37 [0.26, 0.44]	0.96 [0.79, 1.05]	<.001
Medication			
Antihypertensive medication (%)	18 (34.6)	40 (17.2)	.009
Antithrombotic/anticoagulant medication (%)	25 (48.1)	34 (14.7)	<.001
Acetylsalicylic acid (%)	13 (25.0)	13 (5.6)	<.001
Carbasalate calcium (%)	1 (1.9)	9 (3.9)	.783
P2Y12 inhibitor (%)	8 (15.4)	8 (3.4)	.002
Vitamin K antagonist (%)	3 (5.8)	2 (0.9)	.065
Direct Oral Anticoagulant (%)	2 (3.8)	2 (0.9)	.32
Dipyridamole (%)	4 (7.7)	4 (1.7)	.059
Glucose lowering medication (%)	3 (5.8)	3 (1.3)	.14
Lipid lowering medication (%)	29 (55.8)	45 (19.4)	<.001
Lab Results			
eGFR - mL/min/1.73m²	81.67 (11.92)	86.23 (9.08)	.003
Calcium - mmol/L	2.40 (0.09)	2.36 (0.21)	.17
Triglycerides - mmol/L	1.48 (1.02)	1.31 (0.93)	.25
Cholesterol - mmol/L	4.98 (1.17)	5.06 (1.16)	.64
LDL - mmol/L	2.87 (1.01)	2.94 (1.00)	.62
HDL - mmol/L	1.44 (0.38)	1.54 (0.39)	.091
Albumin - mmol/L	41.44 (2.86)	42.48 (2.97)	.026

PAD = peripheral artery disease; IQR = interquartile range; CAD = coronary artery disease; CVA = cerebrovascular accident; DM = diabetes mellitus; BMI = body mass index; ABI = ankle-brachial-index; eGFR = estimated glomerular filtration rate; LDL = low-density lipoprotein; HDL = high-density lipoprotein.

III

Biomarker discovery in subtypes of peripheral artery disease

5

Comparison of cardiovascular biomarker expression in extracellular vesicles, plasma and carotid plaque for the prediction of mace in cea patients

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Abstract

Introduction: *Extracellular vesicles (EV) are a novel biomarker source for the diagnosis and prognosis of cardiovascular disease. A protein comparison of plasma EVs in relation to blood plasma and atherosclerotic plaque has yet to be performed but would provide insight into the origin and content of biomarker sources and their association with atherosclerotic progression.*

Methods: *Using samples of 88 carotid endarterectomy patients in the Athero-Express, 92 proteins (Olink Cardiovascular III panel) were measured in citrate plasma, plasma-derived LDL-EVs and atherosclerotic plaque. Proteins were correlated between sources and were related to preoperative stroke and 3-year major adverse cardiovascular events (MACE).*

Results: *Plasma and EV proteins correlated moderately on average but with substantial variability. Both showed little correlation with plaque, suggesting that these circulating biomarkers may not originate from the latter. Plaque (n = 17) contained the most differentially-expressed proteins in patients with stroke, as opposed to EVs (n = 6) and plasma (n = 5). In contrast, EVs contained the most differentially-expressed proteins for MACE (n = 21) compared to plasma (n = 9) and plaque (n = 1).*

Conclusion: *EVs appear to provide additional information about the severity and progression of systemic atherosclerosis than can be obtained from plasma or atherosclerotic plaque.*

Introduction

Extracellular vesicles (EVs) are a heterogeneous group of bilayer membrane particles that have emerged as a potential novel biomarker source. These secreted encapsulated carriers of biological material act as intercellular messengers of different cell types.[1] Based on their cargo, which reflects the origin cell and comprises of proteins, nucleic acids, lipids and metabolites, EVs have been implicated in cell processes such as inflammation, coagulation, stem cell expansion, neuronal communication and carcinogenesis.[2] As such, the content and composition of EVs can function as a liquid biopsy of cellular processes.

In cardiovascular disease (CVD), EVs have been shown to be associated with traditional risk factors such as smoking, metabolic diseases and hypertension.[3–5] Furthermore, studies demonstrated that EVs could also accumulate in atherosclerotic plaque, where they stimulate foam cell formation, influence smooth muscle cell proliferation and promote endothelial dysfunction, all crucial contributors to atherosclerotic plaque progression.[6] As such, biomarkers in different EV-subpopulations are elevated in patients with myocardial infarction, ischemic stroke, and cardiovascular patients with future major adverse cardiovascular events (MACE).[7–12] Albeit a plethora of research on potential pathophysiologic mechanisms and content of EVs are available, a study comparing the protein content of EVs in relation to plasma and atherosclerotic plaque is lacking. These comparisons would elucidate how EV and plasma proteins are expressed in relation to one another. A high correlation may consequently reflect a similar (patho-)physiological state or origin. In contrast, when the expression of the proteins in these sources is not uniform, the biomarkers may indicate different cellular processes and/or origins. Furthermore, by reviewing the correlation of proteins in plasma and EVs with proteins isolated from atherosclerotic plaque, the extent to which local disease is reflected by circulating biomarkers could be elucidated. This information could guide further biomarker research and increase our understanding of plasma, EV and plaque content in relation to atherosclerotic disease.

Patients undergoing carotid endarterectomy (CEA) serve as excellent biomaterial donors for this investigation because carotid artery surgery offers the opportunity to procure atherosclerotic plaque tissue as well as plasma and plasma EVs. Although this procedure is performed to reduce the risk of (recurrent) ischaemic cerebral or ocular events in patients with significant carotid stenosis, these patients still have a 13% residual risk of MACE within three years. Biomarker analysis at the time of carotid surgery provides both a representation of disease severity at surgery and a clinically relevant starting point for future events in the three years after surgery.[13]

Hence, we used citrate plasma and carotid plaque tissue from 88 matched CEA patients that were included in the Athero-Express biobank. For our first objective, we compared protein levels across three biomarker sources: plasma, plasma EVs and atherosclerotic plaque. The secondary objective was to find a differentiation of protein levels in the three biomarker sources between patients with and without stroke and those with and without the three-year MACE.

Methods

Study population and design

The Athero-Express Biobank Study (AE) is an ongoing prospective vascular biobank with a collection of biological materials such as blood and atherosclerotic plaque, in addition to baseline characteristics and three-year follow-up data.

Patients undergoing CEA or femoral endarterectomy in two hospitals in the Netherlands (UMC Utrecht and St. Antonius Hospital, Nieuwegein) are eligible for inclusion in the AE. The study design has been described in more detail.[14] The study has been approved by the Institutional Review boards of both hospitals and written informed consent was obtained from all patients. The study is conducted in accordance with the declaration of Helsinki.[15]

All patients included in the AE and underwent CEA, with complete three-year follow-up, were eligible for inclusion in this sub-study. Exclusion criteria were lack of citrate plasma, plaque, or follow-up data. Patients were matched for the presence of MACE and no MACE based on gender, age, history of coronary artery disease and the presence of peripheral artery disease. A MACE/no MACE ratio of 1:3 was chosen because it would facilitate statistical testing with a smaller sample size, even though it is slightly higher than the overall prevalence of MACE in the Athero-Express (13%).

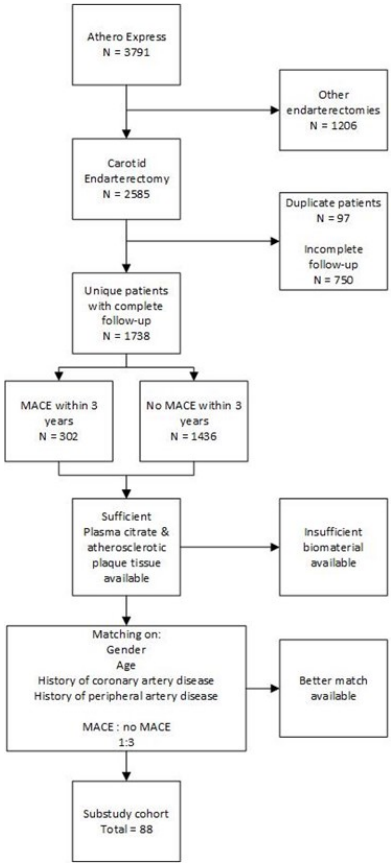

Figure 1: A flow chart of the matched patient's selection.

Blood collection, tissue collection and plaque processing

Venous blood was collected in citrate tubes the day before surgery. Citrate tubes were centrifuged (10 min, 1850xg @ room temperature (RT)) within 30 minutes after collection. Plasma was aliquoted and directly stored at -80°C. Freshly dissected carotid plaques were divided into 0.5 cm segments and processed following standard procedure.[14] Plaques were ground in liquid nitrogen, and approximately 500 μl of residue was used for the extraction of proteins with 500 μl 40 mM Tris buffer (pH 7.5) and an EDTA-free proteinase inhibitor cocktail (Roche) and 20 seconds of iced sonification. After centrifugation (10 min, 13 krpm @ 4 °C), the supernatant was stored at -80 °C as the Tris fraction.

Isolation of extracellular vesicle plasma subfractions

LDL subfraction was obtained from citrate plasma using Dextrane Sulphate (DS) of 0.05% (end concentration) and MnCl2 of 0.05M (end-concentration). For this, DS and MnCl2 were added to phosphate-buffered saline (PBS) (Gibco), giving a volume of 95 μl. Next 5µL magnetic beads (Nanomag®-D plain, 130mm (1:25) (Micromod)) were added, followed by the addition of 25 μl citrate plasma and mixed. The mixture was incubated for 5 minutes at room temperature (RT). Subsequently, the samples were

placed on a bio-plex handheld magnet (Bio-Rad) and incubated for 15 minutes at RT. After removal of the supernatant, pellets were lysed with 125μL Roche complete lysis-M with protease inhibitors (Roche). To remove magnetic beads and other debris, samples were centrifuged (10 min, 3200xg @ RT).

Characterisation of plasma extracellular vesicles subfractions

EV characterisation in the plasma EV subfractions has been reported previously.[16–18] For this study, we performed additional experiments in order to confirm the presence of proteins (Urokinase receptor (UPAR), NT-proBNP, CD31 and Cathepsin D (CTSD)) in EVs with an electrochemiluminescence immunoassay (Quickplex SQ120, Meso Scale). Density gradient centrifugation of the LDL EV subfraction resulted in 12 density gradient fractions.

CD9 (Santa Cruz Biotechnology #SC13118, primary antibody) and Syntenin-1 (Novusbio, #nb100-53807 as primary antibody) western blot analysis showed that vesicles markers are present in fractions 7-10 with densities between 1.07 and 1.13. Our Mesoscale measurements of UPAR, NT-proBNP, CD31 and CTSD proteins show that these protein concentrations are primarily in density gradient fractions 5-10, which largely overlaps with the EV markers (Figure 2).

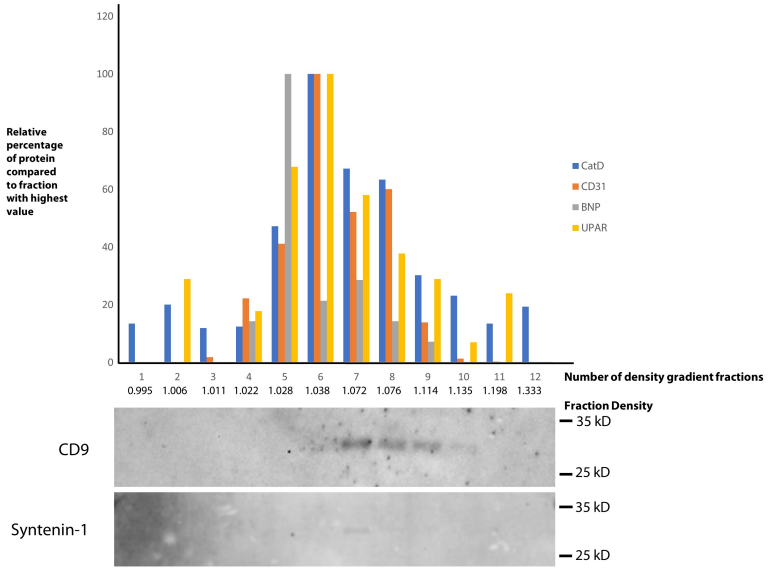

Figure 2: The bar plot at the top shows density gradients for 12 fractions of CTSD, CD31, BNP and UPAR. Below is the western blot analysis in which CD9 vesicle markers are present in fractions 7-10.

Blood collection and protein measurements

The multiplex OLINK® proteomics immunoassays Cardiovascular III panel (OLINK® Proteomics, Uppsala, Sweden) was used to determine 92 cardiovascular disease-related biomarker proteins simultaneously. A proprietary Proximity Extension Assay (PEA) technology was used to achieve high-level

multiplexing, which transforms the protein values into Normalised Protein eXpression (NPX), a relative unit on a log2 scale. These NPX cannot be converted into absolute protein concentrations. All detection limits, assay performance and validation information are available on the manufacturer's website (www.olink.com). Olink measurements from each protein source were done on a separate Olink plate.

For this analysis, we excluded all protein values that exceeded three standard deviations (SD). Our primary analyses have included proteins, regardless of their LoD. This is further covered in the strengths and limitations section of the discussion.

Follow-up and clinical outcome

After the initial CEA, patients included in the AE underwent three-year follow-ups through annual questionnaires. When patients did not respond to the questionnaire, their general practitioner was contacted. An outcome event committee performed verification of the outcome event. The primary endpoint was MACE, a composite of nonfatal myocardial infarction and stroke, and cardiovascular death. Cardiovascular death was defined as one of the following: fatal myocardial infarction, fatal stroke (either haemorrhagic or ischemic), fatal ruptured abdominal aneurysm, fatal heart failure or sudden death. Only the first manifestation of a cardiovascular event was used for analysis.

Statistical analyses

Descriptive statistics of baseline characteristics between groups were compared using a Student t-test, Mann-Whitney U test, Chi-squared test or Fisher's exact test according to variable type and their respective distribution. The correlations of proteins between the different biomarker sources were analysed by calculating Spearman's rho (rs) correlation coefficients. These were interpreted as follows: 0.0-0.29 negligible, 0.3-0.49 weak, 0.50-0.69 moderate, 0.7 – 0.9 good, and > 0.9 excellent correlation. For the secondary objective, a Cox proportional hazard (PH) regression model was used for endpoint analysis. Multiple univariable regression was performed to find potential confounders.

All data were analysed with R (R Core Team (2017) version 3.6.2, Vienna, Austria). A two-sided P-value of <0.05 was considered significant. No multiple testing correction has been performed.

Table 1: Table of baseline characteristics, overall and stratified for MACE

	Overall N = 88	No MACE N = 66	MACE N = 22	P
Age (mean (SD))	71.3 (7.5)	71.3 (7.3)	71.4 (7.9)	.95
Male (%)	68 (77.3)	51 (77.3)	17 (77.3)	1.00
BMI (mean (SD))	26.2 (3.4)	25.8 (3.4)	27.3 (3.2)	.11
Smoking (%)	36 (41.4)	26 (39.4)	10 (47.6)	.68
Preoperative symptoms				.53
Asymptomatic stenosis	19 (21.6)	15 (22.7)	4 (18.2)	
Ocular	15 (17.0)	9 (13.6)	6 (27.3)	
TIA	33 (37.5)	26 (39.4)	7 (31.8)	
Stroke	21 (23.9)	16 (24.2)	5 (22.7)	
History of				
Peripheral Intervention (%)	19 (21.6)	13 (19.7)	6 (27.3)	.65
Coronary Artery Disease (%)	35 (39.8)	25 (37.9)	10 (45.5)	.71
Stroke (%)	26 (29.5)	20 (30.3)	6 (27.3)	1.00
Hypertension (%)	67 (76.1)	46 (69.7)	21 (95.5)	.030
Diabetes Mellitus (%)	19 (21.6)	12 (18.2)	7 (31.8)	.30
Medication use of				
Insulin (%)	4 (4.5)	2 (3.0)	2 (9.1)	.26
Glucose inhibitors (%)	18 (20.5)	11 (16.7)	7 (31.8)	.22
Anticoagulants (%)	13 (14.8)	10 (15.2)	3 (13.6)	1.00
Antiplatelets (%)	77 (87.5)	58 (87.9)	19 (86.4)	1.00
Lipid Lowering Drugs (%)	62 (70.5)	48 (72.7)	14 (63.6)	.59
Laboratory Results				
eGFR (mean (SD))	70.6 (20.1)	72.8 (21.0)	64.3 (15.7)	.084
Triglycerides (median [IQR])	1.40 [1.04, 1.96]	1.41 [1.02, 1.92]	1.57 [1.09, 2.06]	.46
LDL (mean (SD))	2.35 (0.95)	2.31 (0.889)	2.46 (1.11)	.51
HDL (mean (SD))	1.09 (0.34)	1.11 (0.338)	1.03 (0.35)	.35
Cholesterol (mean (SD))	4.20 (1.16)	4.17 (1.142)	4.27 (1.22)	.72
Plaque Features				
Lipid core >40%	25 (28.4)	18 (27.3)	7 (31.8)	.89
Lipid core >10%	62 (70.5)	46 (69.7)	16 (72.7)	1.00
Collagen	72 (82.8)	54 (81.8)	18 (85.7)	.94
Smooth muscle cell	58 (66.7)	43 (66.2)	15 (68.2)	1.00
Intraplaque hemorrhage	56 (63.6)	42 (63.6)	14 (63.6)	1.00
Macrophages	49 (57.0)	42 (64.6)	7 (33.3)	.024
MAC mean (mean (SD))	0.855 (1.203)	0.892 (1.161)	0.746 (1.343)	.63
SMC mean (mean ((SD))	2.013 (1.881)	2.084 (2.001)	1.814 (1.516)	.57

Data are presented as n (%), median [interquartile range] or mean ± standard deviation. BMI = body mass index; TIA = transient ischemic attack; eGFR = estimated glomerular filtration rate; LDL = low-density lipoprotein; HDL = high-density lipoprotein; MAC = macrophages count; SMC = smooth muscle cell; SD = standard deviation; MACE = major adverse cardiovascular events.

Results

Cohort selection

Out of the 3791 patients within the Athero-Express, 88 were matched and selected (Figure 1). Baseline characteristics show that the selected CEA patients represent an average CEA population with relatively high age (mean age 71 years old) and male predominance (77%) (Table I). The prevalence of CAD, diabetes mellitus and history of peripheral interventions is 39.8%, 21.6% and 21.6%, respectively. Adherence to cardiovascular risk management at baseline is high according to the medication prescriptions. In 21 patients, a recent stroke was the indication for surgical intervention, as opposed to 33 patients with TIA, 15 patients with ocular symptoms and 19 asymptomatic patients. Three years after baseline, 22 patients had a MACE with a median time of 1.47 years (IQR 0.63-2.0). Plasma, EV and plaque protein profiles

Of the potential 24.288 protein measurements done with the Olink Cardiovascular III panel in these 88 patients, we identified and removed 385 (0.9%) outliers (> 3SD), with a maximum of 4 outliers per protein.

The correlation between plasma and EV proteins was statistically significant in 83 proteins, with a distribution of 9, 23, 36 and 15 proteins showing negligible, weak, moderate and good correlation, respectively (Figure 3). Correlations of plasma and EV proteins with plaque proteins were hardly significant and often had a much lower rs, although significantly more plaque-derived proteins were weakly or moderately (R> .30) correlated with EV proteins (15 proteins) than with plasma proteins (4 proteins).

Differentially-expressed proteins for preoperative stroke

Protein levels of patients with and without recent preoperative stroke were compared in plasma, EV and plaque to identify differentially-expressed proteins (DEPs) (Supplemental Tables 1A-B). Plaque (N = 17) contained the most DEPs as opposed to plasma (N = 5) and EVs (N = 6) (Figure 3). The levels of DEPs in plaque and EV were higher (except for PAI) in patients with preoperative stroke. In contrast, plasma fraction protein levels were lower in patients with preoperative stroke than those without preoperative stroke.

Differentially-expressed proteins for MACE

With regards to 3-year MACE following surgery, the EV-fraction (N = 21) had more DEPs compared to the plasma (N = 9) and plaque fractions (N = 1) (Figure 4; Supplementary tables 2A-B). Fifteen proteins were differentially expressed in the EV fraction only, whereas six DEPs were found in both the EV and plasma fractions. Of these six corresponding proteins, the correlation between EV and plasma was weak (N = 2), moderate (N = 2) and good (N = 2). In all sources, these DEPs were higher in patients with MACE compared to patients without.

Association of plaque proteins with plaque characteristics

The seventeen DEPs for preoperative stroke were compared with plaque characteristics. Ten out of these 17 proteins were higher in plaque with a lipid core compared to plaque without a lipid core. Four proteins were higher when stratified for the presence of smooth muscle cells (IL18BP, GDF15, TR, MCP1), and one was higher in collagen-rich plaque (MCP1) and plaque with more macrophages (CASP3). The single DEP from plaque, which was elevated in patients with MACE (PON3), was not associated with semi-quantitative plaque features.

Discussion

This study shows that, within a small matched cohort of patients undergoing CEA, the majority of Olink cardiovascular panel III proteins measured in plasma and EVs show moderate to good correlations, although the range of correlations is wide. Proteins from plaque have little correlation with either EV or plasma fraction but are most frequently differentially expressed when stratified for preoperative-stroke. In contrast, stratifying protein levels for MACE within three years after surgery, EVs contain the most differentially-expressed proteins.

It has already been mentioned that our understanding of biomarkers in different sources could be much better. Our data suggest that there are both similarities and dissimilarities of (cardiovascular) protein markers in plasma compared to EVs. Consequently, the expression of well-correlated proteins may be the result of similar cellular processes or a resemblance of cell-specific origin. The latter was expected in some plasma and EV proteins, as EVs in this study were isolated from plasma.

On the other hand, some of these proteins might reflect different pathophysiological processes or origins and/or targets when comparing plasma to EVs as sources. The lower degree of correlation between some plasma and EV proteins and their dissimilar association with MACE could indicate that the cell-specific origin of some EV proteins may be different from that of their equivalent plasma proteins. This needs to be evaluated in studies and should be considered an objective for future research.[19] In effect, when a protein is identified as a potential biomarker, multiple sources should be explored to rule out its discriminatory effect.

An equally wide range of correlations between EVs and plasma has been reported in another study that used the Olink platform when they compared protein expression in patients (N = 82) with myocardial infarction to controls.[8] Tumor necrosis factor ligand superfamily member 13B (TNFSF13B) was also measured in that study and showed a similar correlation coefficient (0.71 according to our data, 0.75-0.8 according to theirs), underlining that the correlations we observed are not coincidental. Unfortunately, that protein was our study's only overlapping marker, so general conclusions cannot be deduced from these data. Other studies that looked at specific EV biomarkers rather than a large selection of biomarkers likewise showed that plasma-derived and EV-derived proteins show varying correlations.[20–22] However, these specific proteins were not used in our analyses, and thus the correlations cannot be compared with our data.

As for plaque, the correlation with either plasma or EV is limited, rendering it a unique source. In our analyses, EV proteins correlated better with plaque than plasma proteins. Since EVs have also been proven to emanate from atherosclerotic plaque, it could be hypothesised that EV proteins originate from plaque in a greater extent compared to plasma, and consequently reflect its pathophysiological state better.[3, 23] Taking an EV liquid biopsy of systemic plaque proteins can therefore provide an accurate impression of the plaque content.

However, since atherosclerotic plaques are not easily available as a regular biomarker source, a direct comparison of blood biomarkers with plaque has rarely been performed, and thus this remains ambiguous. Equivalent research is restricted to one study (N = 574), published by our research group, which determined the correlation coefficient of osteopontin (OPN) in plasma and plaque (rs = 0.15). Coincidentally, OPN was also measured with the current Olink CVD panel III, and the correlation between sources appears to be almost identical (rs = 0.16).([24]

Our secondary objective was to determine whether a disparity in protein content across these sources would result in a difference in discriminatory power for a diagnostic criterion (preoperative stroke) and future outcomes (MACE within three years following surgery). Concerning preoperative stroke, our results indicate that plasma and EVs contained limited DEP. Statistically significant proteins in EVs were higher in stroke patients, but conversely, proteins in plasma were significantly lower in the affected group than those without stroke. Our study design is not suitable to elucidate this inverse relationship specifically, however, it has described that Kallikrein Related Peptidase 6 (KLK6) and Plasminogen activator inhibitor (PAI) are decreased during subarachnoid bleeding and one week

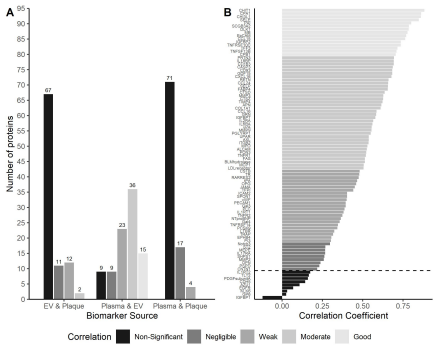

Figure 3: (A) Correlations were categorised according to statistical significance and coefficient (negligible, weak, moderate, good and excellent). This was done for all protein correlations between each protein source (EV correlation with plaque, plasma with EV and plasma with plaque). The counts of these categories are given in a bar plot. (B) The Spearman's rho of correlation of plasma and EV is given for all proteins, with their respective categories (negligible, weak, moderate, good and excellent) indicated in greyscale. Proteins under the dashed line are non-significant.

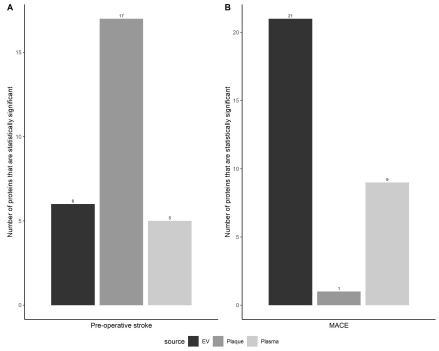

Figure 4: The bars represent the number of differentially-expressed proteins stratified for pre-operative stroke (A) and 3-year MACE (B). This is done per biomarker source (EV, plaque and plasma) out of the total 94 proteins measured.

after stroke, respectively.[25, 26] Furthermore, our results are perhaps influenced by the timing of blood sampling, as this did not take place during the stroke but in the subsequent period after initial diagnosis and treatment.

A significantly larger number of plaque proteins are differentially expressed in patients with pre-operative stroke compared to plasma and EV. Since the harvested atherosclerotic plaque is often considered the culprit lesion for cerebrovascular events, this comes as little surprise.[27] Although this has not been tested in other research, higher plaque protein levels are related to characteristics of plaque instability in several studies, which are, in turn, associated with stroke.[28–31] In addition, we found that most of these differentially-expressed plaque proteins are also statistically higher in plaques with a prominent lipid profile, underlining that plaque histology is reflected by protein expression.

With regards to the discriminatory power of future events, only one plaque-derived protein was differentially expressed for three-year MACE. This might indicate that the ongoing systemic processes associated with these events are not properly represented by the disease state of the carotid plaque alone but might be captured more accurately with systemic (EV) biomarkers. This is demonstrated with our data, as there are twenty-one differentially-expressed EV-proteins, compared to nine differentially-expressed proteins for plasma. Again, a comparative analysis of multiple biomarker sources has yet to be performed, let alone demonstrate the predictive properties of these biomarkers with respect to any long-term outcome.

Strengths and limitations

The Athero-Express biobank, with well-defined baseline descriptions and the possibility of using both blood and atherosclerotic plaque, makes it feasible to create a matched cohort of 88 patients and enough biomaterial for multiple analyses. Obviously, a larger selection of patients would have been preferred, although equivalent studies used less patients.[8] Furthermore, the insight we provide is limited to

only 92 selected cardiovascular selected proteins, which amounts to only a fraction of the entire EV, plasma and plaque proteomes. The EV precipitation method is an enrichment for EV proteins, and as such, it yields not only pure EV proteins but also proteins in EVs, on EV membranes, in EV corona and plasma.[32] For this EV isolation, the LDL fraction was preferred, as it is our experience that this fraction produces the most protein. As such, EVs in other fractions might show different correlations with plasma or plaque or different expressions when stratified for preoperative stroke and outcome.

Olink is optimised for plasma; thus, measurements for EV and plaque proteins might lead to more measurements below the limit of detection (LoD), employed by Olink. While these LoDs seem very specific and critical, their recommended utility is more nuanced, as the manufacturer suggests that proteins should be considered for exclusion when less than 25-50% of the proteins are above the LoD. Furthermore, as stated by Olink, the inclusion of data under LoD does not commonly increase false positives.[33] We have addressed this in the supplementary files and thus demonstrate that analysis of the data above the LoD does not change our general conclusions. Furthermore, although measurements of Cathepsin D are entirely below the LoD, it correlates well with MSD immunoassay measurements of the same samples, substantiating our hypothesis that the complete data can be used.

Applying these strict boundaries would lead to less viable measurements of EV and plaque proteins. However, in the supplemental material, a brief overview of these remaining data shows that our general conclusions still stand.

Since plasma samples are centrifugated only once at low speed, this might lead to platelet contaminations. This potential platelet contamination, however, is relatively equal in plasma, EV isolations and plaque extracts, and hence this could mask the association of protein level with MACE and presurgical stroke in all three protein sources.

Regarding preoperative stroke and 3-year MACE analyses, our protein-specific results should be interpreted with caution. Selection bias (a matched cohort) and limited power impair direct conclusions about the efficacy of these potential biomarkers. Moreover, this article aims to examine how cardiovascular proteins from different protein sources in one individual are associated with MACE and presurgical stroke. Although potential proteins will indeed yield predictive performance in this matched cohort, these analyses should be performed in a large cohort of patients to obtain enough patient numbers and thus statistical power and to enable multivariate regression analysis and multiple testing correction.

Although the correlation between plasma and EV proteins is moderate overall, there are protein-specific differences, which may reflect that both sources yield proteins from different cellular processes or origins. In contrast, very little correlation of either circulating source was seen with plaque, although the atherosclerotic plaque, often seen as the culprit for stroke, contained more proteins differentially expressed for stroke. EVs contain the most DEPs for three-year MACE, indicating that EVs are indeed a relatively unexplored source of systemic protein biomarkers that, compared to plasma, provide additional information on cardiovascular disease severity.

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Supplemental Material

Availability of data and materials

The datasets generated and/or analysed during the current study are available in the DataverseNL repository upon request, https://doi.org/10.34894/W8YBH6.

Considering the limit of detection (lod) in relation to our measurements

For our main paper, the LoD was ignored as these values were based on the plasma matrix. Furthermore, Olink indicated that exclusion could be considered but is not mandatory when there are less than 25-50% proteins above the LoD. Olink proposes several ways to deal with results below the LoD. These include using the actual data below LoD, replacing these values with a pre-determined value and imputing these data. In our main paper, we opted for the first recommendation to ensure the robustness of our results, and we checked what impact LoD would have on our data. As such, we excluded all measurements below the LoD and only included proteins with more than 75% (N = 66) measurements above the LoD to assure some statistical power.

As expected, removing proteins below the LoD had the least influence on plasma, as these absolute values were highest compared to EV and plaque. For plasma, extracellular vesicles (EVs) and plaque 69, 29 and 37 proteins could be analysed, respectively. These data were used to statistically test the significant association with regard to major adverse cardiovascular events (MACE) and preoperative stroke, which was part of our manuscript. For preoperative stroke, the number of EV-proteins was downgraded (6 out of 92 (6%) in the main analyses, vs 0 out of 29 with (0%) >75% above LoD). The relative number of statistically significant proteins remained unchanged for plasma (5 out of 92 (2%) in the main analyses, vs 2 out of 69 (3%) with >75% above LoD) and plaque (17 out of 92 (18%) in the main analyses, vs 8 out of 37 (21%) with >75% above LoD). This again shows (as can be expected since the carotid plaque is often the underlying cause of stroke) that more plaque proteins are associated with symptomatic stroke compared to the same proteins in plasma and EVs.

The relative number of statistically significant EV proteins for MACE remains similar (21 out of 92 (23%) in the main analyses, vs 7 out of 29 (24%) with >75% above LoD). For plasma proteins, this relative number was raised marginally (9 out of 92 (10%) in the main analyses, vs 11 out of 69 (15%) with >75% above LoD). Plaque had a limited number of statistically significant proteins for MACE in our main analyses, and this was not different when considering the LoD (1 out of 92 (1%) in the main analyses, vs 0 out of 37 (0%) with >75% above LoD). This again shows that more EV proteins are associated with MACE than plasma and plaque.

Considering the lod and cross-platform validity

The mesoscale discovery (MSD) was used to compare Cathepsin D (CTSD) with the Olink CTSD data, as all 88 samples were below the LoD in both EV and plasma. This cross-platform analysis shows a significant correlation coefficient of 0.602 and 0.705 for EV and plasma, respectively, which substantiates the hypothesis that the measurements below LoD are valid.

Table S1: EV and plasma proteins are stratified for preoperative stroke

	EV			Plasma		
	No Stroke N = 67	Stroke N =21	P	No Stroke N = 67	Stroke N = 21	P
TNFRSF14	1.61 [1.34, 1.86]	1.77 [1.48, 1.90]	.13	17.90 [15.42, 22.71]	18.81 [15.12, 24.76]	.86
LDLreceptor	0.41 [0.35, 0.51]	0.43 [0.39, 0.49]	.57	8.44 [6.02, 11.20]	7.50 [5.70, 8.89]	.28
ITGB2	0.99 [0.79, 1.14]	1.01 [0.87, 1.12]	.66	15.63 [12.43, 20.23]	13.88 [12.57, 15.57]	.25
IL17RA	0.97 [0.77, 1.09]	1.04 [0.91, 1.11]	.20	8.12 [6.52, 11.10]	8.84 [6.04, 10.64]	.87
TNFR2	2.40 [1.87, 2.76]	2.31 [1.99, 3.05]	.55	26.28 [21.97, 37.02]	27.22 [21.68, 46.02]	.34
MMP9	0.70 [0.62, 0.84]	0.82 [0.65, 0.88]	.30	10.68 [6.43, 13.90]	10.20 [7.62, 17.29]	.82
EPHB4	1.85 [1.60, 2.02]	1.85 [1.44, 2.32]	.98	26.61 [22.77, 31.60]	27.22 [21.04, 35.74]	.72
IL2RA	0.70 [0.63, 0.80]	0.76 [0.67, 0.85]	.39	10.16 [7.60, 12.96]	10.29 [7.12, 12.52]	.94
OPG	0.80 [0.69, 0.95]	0.83 [0.72, 1.00]	.51	12.26 [9.39, 14.11]	13.06 [9.85, 15.95]	.51
ALCAM	3.28 [2.73, 3.68]	3.15 [2.40, 3.35]	.27	110.26 [89.75, 129.82]	105.76 [85.85, 127.13]	.87
TFF3	1.45 [1.19, 1.70]	1.45 [1.13, 1.59]	.59	24.99 [19.24, 30.98]	23.43 [16.72, 27.22]	.55
SELP	13.91 [10.31, 18.53]	11.94 [10.39, 17.83]	.58	556.18 [465.84, 721.11]	533.12 [332.10, 769.62]	.70
CSTB	0.54 [0.48, 0.64]	0.51 [0.45, 0.60]	.29	9.82 [7.56, 13.54]	9.54 [6.73, 13.49]	.91
MCP1	0.97 [0.75, 1.12]	1.02 [0.88, 1.18]	.15	14.01 [10.36, 16.87]	14.41 [10.62, 19.52]	.84
CD163	3.64 [2.94, 4.70]	4.00 [3.20, 4.38]	.85	113.65 [97.93, 151.52]	120.27 [98.36, 146.18]	.97
Gal3	0.23 [0.20, 0.26]	0.22 [0.19, 0.23]	.33	6.92 [5.62, 8.64]	6.58 [6.02, 8.94]	.78
GRN	1.29 [1.18, 1.43]	1.31 [1.15, 1.40]	.92	29.86 [24.32, 33.52]	27.99 [20.75, 33.01]	.60
NTproBNP	2.17 [1.78, 2.44]	2.50 [2.05, 2.75]	.018	13.39 [6.58, 21.75]	14.11 [9.38, 46.00]	.36
BLMhydrolase	0.20 [0.18, 0.22]	0.19 [0.16, 0.24]	.39	2.84 [2.41, 3.40]	2.80 [2.33, 3.24]	.38
PLC	4.26 [3.64, 6.37]	4.42 [3.70, 5.08]	.90	218.23 [181.74, 281.26]	187.01 [155.02, 290.23]	.31
LTBR	0.57 [0.46, 0.67]	0.59 [0.50, 0.63]	.62	7.79 [6.30, 9.46]	8.15 [6.16, 11.03]	.83
Notch3	1.06 [0.92, 1.20]	1.02 [0.92, 1.26]	.88	22.32 [16.28, 30.03]	20.00 [17.87, 29.41]	.68
TIMP4	0.53 [0.46, 0.63]	0.63 [0.51, 0.74]	.015	9.74 [7.43, 11.69]	9.71 [8.22, 13.10]	.48
CNTN1	0.40 [0.35, 0.47]	0.39 [0.35, 0.43]	.45	9.27 [7.63, 11.40]	9.64 [7.29, 11.22]	.78
CDH5	1.46 [1.22, 1.68]	1.49 [1.33, 1.70]	.67	11.88 [9.56, 14.51]	12.26 [8.06, 15.90]	.97
TLT2	2.06 [1.64, 2.56]	2.27 [1.76, 2.69]	.26	18.24 [14.29, 22.49]	16.69 [12.87, 21.02]	.39
FABP4	1.14 [1.03, 1.58]	1.12 [0.91, 1.56]	.57	36.56 [27.14, 50.30]	32.86 [25.95, 60.92]	.99
TFPI	10.03 [7.66, 11.27]	9.37 [7.58, 11.85]	.95	388.41 [294.84, 467.99]	324.74 [263.78, 404.16]	.075
PAI	0.64 [0.54, 0.78]	0.56 [0.48, 0.62]	.017	15.97 [11.63, 26.24]	11.42 [8.79, 16.20]	.047
CCL24	0.68 [0.50, 0.82]	0.76 [0.68, 0.81]	.058	19.97 [13.19, 32.18]	20.98 [15.95, 33.39]	.41
TR	1.42 [1.24, 1.71]	1.51 [1.10, 1.75]	.99	14.48 [10.87, 20.02]	14.33 [11.11, 21.60]	.98
TNFRSF10C	3.23 [2.62, 4.19]	2.89 [2.45, 3.98]	.56	57.13 [44.75, 70.14]	59.07 [40.46, 72.30]	.86
GDF15	1.20 [0.99, 1.51]	1.35 [1.00, 1.92]	.31	49.64 [37.70, 68.02]	51.84 [33.29, 85.35]	.78
SELE	79.01 [57.79, 105.80]	66.43 [48.50, 84.85]	.22	2397.13 [1847.53, 3343.31]	2372.53 [1654.03, 2733.01]	.29
AZU1	0.28 [0.24, 0.31]	0.29 [0.23, 0.35]	.44	4.65 [3.27, 8.04]	3.99 [2.96, 7.15]	.33
DLK1	2.00 [1.62, 2.51]	1.98 [1.52, 2.45]	.59	46.38 [29.84, 61.37]	35.74 [26.25, 46.85]	.23
SPON1	1.51 [1.30, 1.74]	1.65 [1.39, 1.78]	.23	2.35 [2.04, 2.81]	2.19 [1.92, 2.51]	.13
MPO	0.59 [0.53, 0.65]	0.65 [0.56, 0.69]	.10	7.30 [5.32, 9.31]	7.93 [5.52, 9.80]	.77
CXCL16	1.52 [1.25, 1.85]	1.65 [1.38, 1.99]	.38	28.01 [22.09, 35.56]	33.51 [19.42, 35.45]	.80
IL6RA	49.27 [39.50, 55.65]	45.65 [30.41, 65.49]	.45	3060.01 [2353.02, 3954.50]	2963.45 [2257.26, 3380.08]	.44
RETN	0.98 [0.81, 1.31]	1.12 [0.85, 1.44]	.53	64.45 [46.89, 78.82]	64.83 [43.97, 80.57]	.96
IGFBP1	1.13 [0.91, 1.30]	1.21 [0.95, 1.56]	.23	17.54 [10.27, 31.26]	17.36 [10.22, 61.08]	.75
CHIT1	1.39 [1.16, 1.74]	1.56 [1.12, 1.80]	.73	31.39 [19.44, 39.72]	31.68 [19.72, 43.18]	.60
TRAP	0.32 [0.28, 0.37]	0.33 [0.29, 0.37]	.50	7.50 [5.68, 8.93]	7.63 [5.81, 9.21]	.71
GP6	0.44 [0.40, 0.53]	0.48 [0.41, 0.54]	.28	1.70 [1.43, 2.24]	1.60 [1.29, 1.89]	.24
PSPD	0.59 [0.49, 0.71]	0.60 [0.47, 0.71]	.88	4.29 [2.53, 6.66]	2.78 [1.99, 3.89]	.023
PI3	0.33 [0.30, 0.39]	0.34 [0.29, 0.35]	.39	2.75 [2.10, 3.96]	2.19 [1.86, 3.18]	.14
EpCAM	1.26 [0.99, 1.73]	1.16 [0.91, 1.60]	.27	24.64 [14.01, 48.78]	20.84 [10.91, 26.90]	.12
APN	1.05 [0.91, 1.18]	0.97 [0.82, 1.23]	.46	20.92 [16.27, 26.53]	20.99 [17.08, 23.91]	.61
AXL	7.61 [6.33, 8.58]	8.07 [6.26, 9.09]	.83	213.03 [177.89, 269.13]	209.03 [184.25, 235.79]	.59
IL1RT1	1.77 [1.51, 2.09]	1.81 [1.42, 1.90]	.51	46.55 [38.52, 54.57]	45.41 [37.02, 52.80]	.68
MMP2	0.61 [0.55, 0.65]	0.60 [0.57, 0.67]	.47	7.24 [5.21, 8.52]	6.21 [5.64, 7.90]	.48
FAS	0.86 [0.73, 0.98]	0.87 [0.75, 1.02]	.64	30.03 [23.68, 34.97]	25.30 [21.86, 36.42]	.33
MB	3.11 [2.59, 4.70]	3.42 [2.48, 3.96]	.97	113.23 [80.85, 163.44]	104.42 [77.61, 163.73]	.79
TNFSF13B	3.08 [2.57, 3.78]	2.76 [2.36, 3.82]	.20	92.46 [72.30, 126.05]	79.41 [59.70, 93.64]	.094
PRTN3	0.42 [0.36, 0.51]	0.53 [0.42, 0.59]	.009	8.00 [5.65, 10.97]	8.42 [6.78, 16.51]	.058
PCSK9	0.82 [0.71, 0.99]	0.88 [0.75, 0.98]	.26	4.59 [3.93, 5.91]	4.72 [3.58, 5.90]	.74
UPAR	1.72 [1.48, 2.01]	1.70 [1.51, 1.93]	.80	29.26 [21.33, 37.00]	27.67 [22.80, 35.86]	.89
OPN	0.71 [0.62, 0.83]	0.81 [0.68, 0.94]	.080	97.71 [74.65, 137.10]	90.72 [80.88, 162.00]	.67
CTSD	0.25 [0.22, 0.28]	0.25 [0.22, 0.32]	.74	5.61 [4.08, 6.64]	5.55 [3.78, 6.77]	.85
PGLYRP1	2.14 [1.61, 2.51]	2.29 [2.02, 2.67]	.19	116.53 [86.64, 152.67]	104.58 [85.65, 153.64]	.57
CPA1	1.06 [0.81, 1.43]	0.94 [0.81, 1.17]	.22	36.47 [24.73, 50.22]	28.46 [17.19, 40.45]	.087
JAMA	0.46 [0.38, 0.61]	0.45 [0.32, 0.53]	.11	11.19 [9.10, 14.00]	8.35 [7.44, 13.25]	.051

Gal4	0.82 [0.72, 1.01]	0.85 [0.80, 0.96]	.72	9.44 [7.57, 14.60]	8.89 [6.66, 10.95]	.27
IL1RT2	1.01 [0.85, 1.25]	0.93 [0.88, 1.12]	.34	23.28 [19.32, 26.73]	20.94 [18.39, 27.30]	.58
SHPS1	0.86 [0.78, 0.98]	0.86 [0.80, 0.97]	.85	7.48 [5.63, 9.04]	6.55 [6.07, 8.21]	.27
CCL15	2.01 [1.58, 2.46]	1.96 [1.67, 2.21]	.88	112.02 [86.78, 166.77]	109.79 [79.92, 141.05]	.32
CASP3	0.95 [0.70, 1.36]	0.88 [0.69, 1.23]	.63	34.38 [20.34, 56.22]	23.54 [14.59, 30.73]	.046
uPA	0.62 [0.51, 0.71]	0.65 [0.55, 0.74]	.60	16.94 [13.11, 20.91]	15.54 [11.30, 19.29]	.34
CPB1	0.88 [0.74, 1.01]	0.83 [0.71, 0.98]	.43	29.72 [24.29, 45.96]	22.81 [15.58, 36.05]	.11
CHI3L1	0.31 [0.22, 0.46]	0.40 [0.29, 0.84]	.045	12.68 [6.76, 19.65]	12.49 [8.29, 25.35]	.32
ST2	1.61 [1.22, 1.91]	1.86 [1.31, 2.27]	.15	13.40 [10.99, 16.70]	16.35 [10.23, 20.51]	.49
tPA	1.53 [1.25, 1.89]	1.52 [1.32, 1.73]	.66	142.58 [104.11, 187.77]	138.73 [102.07, 165.64]	.39
SCGB3A2	0.80 [0.66, 0.92]	0.74 [0.68, 0.86]	.53	3.05 [2.01, 5.45]	2.52 [1.93, 3.58]	.34
EGFR	0.78 [0.72, 0.83]	0.76 [0.71, 0.86]	1.0	4.38 [3.71, 5.03]	4.23 [3.40, 4.92]	.57
IGFBP7	2.49 [2.19, 3.24]	2.53 [2.25, 3.51]	.39	132.87 [106.54, 165.98]	115.58 [88.68, 194.35]	.53
CD93	34.01 [27.25, 45.50]	32.71 [30.17, 47.99]	.52	1524.89 [1147.99, 1866.83]	1275.94 [972.37, 2030.87]	.65
IL18BP	1.92 [1.67, 2.26]	1.95 [1.53, 2.34]	.81	51.01 [42.57, 63.22]	48.74 [37.15, 63.24]	.87
COL1A1	0.75 [0.72, 0.78]	0.75 [0.73, 0.78]	.68	4.38 [3.78, 5.59]	4.23 [3.56, 6.08]	.97
PON3	1.53 [1.33, 1.89]	1.67 [1.33, 1.85]	.79	28.78 [21.58, 38.58]	29.03 [19.79, 41.74]	.97
CTSZ	1.13 [1.04, 1.27]	1.09 [1.01, 1.38]	.94	29.92 [23.31, 36.19]	25.63 [22.00, 37.81]	.43
MMP3	1.87 [1.28, 2.47]	2.03 [1.86, 2.16]	.48	119.31 [86.99, 202.71]	127.78 [91.02, 150.15]	.73
RARRES2	7.81 [6.30, 9.70]	8.43 [6.91, 10.33]	.19	1858.91 [1564.04, 2216.59]	1836.87 [1460.44, 2108.66]	.80
ICAM2	2.30 [1.90, 2.67]	2.35 [2.09, 2.66]	.65	23.83 [19.51, 31.87]	24.41 [19.35, 32.65]	.89
KLK6	0.23 [0.19, 0.31]	0.22 [0.19, 0.25]	.47	3.63 [2.96, 4.24]	3.27 [2.53, 3.58]	.038
PDGFsubunitA	1.02 [0.97, 1.11]	1.06 [1.01, 1.12]	.13	3.14 [2.48, 3.81]	2.43 [2.16, 3.11]	.035
TNFR1	3.31 [2.90, 3.85]	3.48 [2.83, 4.92]	.43	67.62 [52.24, 83.26]	70.14 [49.68, 110.51]	.61
IGFBP2	3.45 [2.68, 4.63]	4.64 [2.57, 5.69]	.21	207.49 [141.64, 291.93]	184.08 [148.90, 265.82]	.62
vWF	2.96 [2.48, 4.02]	3.38 [2.45, 4.41]	.67	135.04 [102.86, 244.11]	175.68 [125.45, 217.37]	.81
PECAM1	0.72 [0.62, 0.82]	0.75 [0.66, 0.80]	.58	13.88 [12.00, 17.59]	13.21 [10.76, 18.52]	.54
MEPE	1.03 [0.81, 1.20]	1.25 [1.00, 1.42]	.004	21.60 [18.01, 27.63]	19.99 [15.79, 27.33]	.53
CCL16	1.37 [1.15, 1.75]	1.31 [1.07, 1.60]	.57	66.24 [53.72, 78.74]	55.34 [42.99, 73.38]	.06

Table S2: Plaque proteins are stratified for preoperative stroke

	Plaque		
	No Stroke N = 67	Stroke N =21	P
TNFRSF14	3.41 [2.99, 4.28]	3.82 [3.16, 4.43]	.51
LDLreceptor	0.54 [0.49, 0.59]	0.57 [0.50, 0.62]	.27
ITGB2	3.32 [1.99, 5.29]	5.49 [3.90, 9.87]	.002
IL17RA	1.31 [1.11, 1.59]	1.47 [1.20, 1.64]	.40
TNFR2	3.28 [2.63, 4.22]	3.98 [3.15, 4.21]	.12
MMP9	1.29 [1.03, 1.96]	3.39 [1.92, 5.04]	<.001
EPHB4	1.75 [1.48, 2.14]	1.86 [1.67, 2.02]	.81
IL2RA	1.05 [0.94, 1.21]	1.07 [0.95, 1.23]	.98
OPG	15.99 [9.35, 28.71]	20.38 [10.95, 25.44]	.40
ALCAM	4.07 [2.83, 6.25]	4.04 [3.44, 7.84]	.58
TFF3	1.19 [1.03, 1.43]	1.16 [1.05, 1.23]	.60
SELP	7.73 [5.91, 11.52]	7.16 [6.43, 8.50]	.51
CSTB	110.50 [56.35, 167.74]	181.78 [86.77, 271.58]	.070
MCP1	4.90 [2.71, 8.67]	7.31 [5.40, 12.76]	.032
CD163	8.32 [4.76, 15.73]	15.73 [8.67, 24.22]	.015
Gal3	1.12 [0.58, 1.89]	1.42 [0.94, 1.90]	.39
GRN	1.96 [1.36, 2.58]	2.76 [1.95, 3.66]	.010
NTproBNP	2.80 [2.58, 3.15]	2.68 [2.46, 2.81]	.062
BLMhydrolase	1.21 [0.77, 1.99]	1.50 [1.07, 1.70]	.34
PLC	11.88 [5.47, 23.67]	15.82 [7.12, 19.06]	.71
LTBR	1.16 [0.91, 1.36]	1.06 [0.85, 1.33]	.89
Notch3	1.42 [1.18, 1.64]	1.28 [1.24, 1.68]	.93
TIMP4	0.70 [0.63, 0.81]	0.70 [0.62, 0.76]	.66
CNTN1	0.58 [0.53, 0.64]	0.56 [0.53, 0.59]	.30
CDH5	1.98 [1.72, 2.34]	1.89 [1.66, 2.14]	.67
TLT2	2.63 [2.19, 3.32]	2.53 [2.03, 2.90]	.12
FABP4	11.33 [4.55, 20.74]	24.32 [6.15, 50.33]	.10
TFPI	4.23 [3.31, 6.14]	4.71 [4.26, 5.25]	.49
PAI	2.85 [1.97, 5.07]	3.91 [2.95, 5.08]	.16
CCL24	0.75 [0.59, 0.95]	0.80 [0.69, 0.92]	.61
TR	0.87 [0.75, 1.12]	1.05 [0.86, 1.34]	.018
TNFRSF10C	2.89 [2.35, 3.56]	2.90 [2.32, 4.64]	.75
GDF15	2.92 [2.16, 4.37]	3.63 [3.25, 9.53]	.003
SELE	12.70 [8.95, 18.22]	14.02 [10.35, 18.24]	.46
AZU1	2.18 [1.30, 4.13]	4.26 [2.11, 6.62]	.024
DLK1	1.49 [1.35, 1.82]	1.43 [1.25, 1.64]	.18
SPON1	2.20 [1.98, 2.62]	2.15 [1.96, 2.49]	.33
MPO	1.44 [1.07, 3.10]	3.71 [1.70, 6.56]	.012
CXCL16	2.09 [1.64, 2.95]	2.53 [2.19, 3.30]	.011
IL6RA	13.51 [9.80, 17.29]	15.67 [11.97, 21.21]	.29
RETN	2.98 [2.16, 5.50]	4.60 [2.91, 7.50]	.099
IGFBP1	1.90 [1.67, 2.32]	1.97 [1.76, 2.19]	.84
CHIT1	1.62 [1.25, 2.68]	1.97 [1.45, 3.06]	.30
TRAP	1.05 [0.51, 1.49]	1.69 [0.97, 2.41]	.022
GP6	0.73 [0.68, 0.83]	0.71 [0.63, 0.86]	.48
PSPD	0.79 [0.69, 1.04]	0.75 [0.64, 1.08]	.71
PI3	0.26 [0.22, 0.32]	0.24 [0.22, 0.29]	.52
EpCAM	1.17 [0.90, 1.53]	1.22 [0.91, 1.51]	.85
APN	1.00 [0.89, 1.21]	1.14 [0.97, 1.30]	.065
AXL	5.44 [4.32, 6.68]	5.65 [4.14, 6.38]	.83
IL1RT1	1.77 [1.48, 2.20]	1.83 [1.49, 2.06]	1.0
MMP2	0.79 [0.74, 0.84]	0.79 [0.75, 0.82]	.77
FAS	1.46 [1.02, 2.23]	1.29 [1.14, 1.71]	.42
MB	9.70 [4.13, 19.73]	3.91 [2.62, 16.39]	.12
TNFSF13B	3.10 [2.34, 4.15]	3.62 [2.73, 4.40]	.22
PRTN3	1.17 [0.86, 1.56]	1.47 [1.18, 3.00]	.024
PCSK9	1.12 [0.97, 1.28]	1.10 [0.97, 1.17]	.41
UPAR	10.64 [5.23, 23.46]	25.08 [13.03, 48.99]	.001
OPN	20.78 [9.19, 54.89]	52.36 [15.42, 116.12]	.090
CTSD	5.45 [1.37, 10.69]	8.09 [1.97, 15.60]	.30
PGLYRP1	2.49 [1.87, 4.07]	3.63 [2.33, 5.44]	.085
CPA1	0.97 [0.81, 1.16]	0.88 [0.78, 1.03]	.32
JAMA	0.65 [0.54, 0.97]	0.83 [0.63, 0.98]	.29

Gal4	1.05 [0.91, 1.27]	1.04 [0.92, 1.15]	.59
IL1RT2	0.99 [0.85, 1.20]	1.04 [0.91, 1.10]	.74
SHPS1	1.68 [1.42, 1.94]	1.65 [1.43, 2.09]	.77
CCL15	1.61 [1.42, 1.88]	1.38 [1.26, 1.77]	.17
CASP3	27.80 [9.73, 44.27]	41.84 [24.93, 59.60]	.016
uPA	1.72 [1.12, 2.80]	3.10 [2.16, 4.19]	.003
CPB1	0.87 [0.79, 1.00]	0.90 [0.84, 0.99]	.65
CHI3L1	0.60 [0.36, 1.08]	1.31 [0.68, 1.98]	.014
ST2	2.29 [1.87, 2.62]	2.12 [1.83, 2.40]	.40
tPA	2.41 [1.94, 3.32]	2.48 [2.01, 3.06]	.83
SCGB3A2	0.70 [0.62, 0.77]	0.68 [0.64, 0.74]	.97
EGFR	0.96 [0.90, 1.00]	0.94 [0.92, 0.97]	.69
IGFBP7	50.73 [19.42, 105.78]	44.35 [30.02, 64.23]	.75
CD93	16.49 [10.36, 23.76]	19.32 [14.47, 25.45]	.41
IL18BP	1.63 [1.37, 2.22]	2.25 [1.82, 2.62]	.002
COL1A1	1.05 [0.94, 1.26]	1.10 [1.03, 1.60]	.25
PON3	1.25 [1.12, 1.40]	1.19 [1.07, 1.32]	.18
CTSZ	8.13 [5.15, 13.41]	13.72 [6.80, 17.96]	.049
MMP3	2.13 [1.82, 2.45]	2.02 [1.40, 3.02]	.64
RARRES2	5.20 [2.71, 10.24]	3.88 [2.41, 4.44]	.067
ICAM2	2.44 [2.18, 2.80]	2.31 [2.13, 2.54]	.22
KLK6	0.29 [0.22, 0.37]	0.29 [0.23, 0.37]	.97
PDGFsubunitA	1.48 [1.36, 1.58]	1.44 [1.33, 1.51]	.21
TNFR1	6.98 [5.78, 8.51]	7.76 [6.84, 10.67]	.12
IGFBP2	9.40 [5.73, 15.76]	8.71 [6.90, 12.40]	.85
vWF	2.00 [1.57, 2.46]	2.17 [1.85, 2.64]	.15
PECAM1	1.09 [0.89, 1.27]	1.07 [0.95, 1.20]	.98
MEPE	1.37 [1.22, 1.64]	1.36 [1.23, 1.54]	.77
CCL16	0.83 [0.72, 1.07]	0.77 [0.71, 0.92]	.49

Table S3: EV and plasma proteins are stratified for MACE

	EV			Plasma		
	No MACE N = 66	MACE N = 22	P	No MACE N = 66	MACE N = 22	P
TNFRSF14	1.56 [1.33, 1.85]	1.81 [1.64, 2.10]	.013	17.65 [14.47, 21.82]	22.49 [16.42, 25.65]	.031
LDLreceptor	0.42 [0.34, 0.50]	0.41 [0.38, 0.52]	.77	7.53 [5.76, 10.65]	8.34 [6.73, 11.26]	.68
ITGB2	0.94 [0.77, 1.14]	1.03 [0.93, 1.10]	.410	15.05 [12.29, 19.61]	15.12 [13.36, 19.12]	.58
IL17RA	0.97 [0.76, 1.08]	1.02 [0.92, 1.18]	.086	7.81 [6.30, 10.64]	9.84 [8.24, 11.99]	.072
TNFR2	2.31 [1.85, 2.75]	2.57 [2.16, 3.14]	.066	25.70 [21.97, 33.80]	32.76 [22.12, 47.98]	.080
MMP9	0.70 [0.62, 0.84]	0.78 [0.69, 0.96]	.036	10.17 [6.45, 14.30]	11.17 [6.64, 17.25]	.546
EPHB4	1.82 [1.53, 2.03]	1.94 [1.66, 2.27]	.244	25.95 [22.05, 30.87]	29.09 [24.61, 40.79]	.052
IL2RA	0.70 [0.63, 0.78]	0.70 [0.66, 0.86]	.254	10.09 [7.49, 12.18]	10.37 [8.38, 14.58]	.47
OPG	0.80 [0.71, 0.92]	0.91 [0.73, 1.00]	.103	11.98 [9.49, 14.32]	13.20 [9.90, 15.75]	.17
ALCAM	3.15 [2.62, 3.63]	3.37 [2.97, 3.89]	.108	108.37 [85.79, 129.28]	110.68 [99.62, 127.13]	.57
TFF3	1.40 [1.14, 1.60]	1.60 [1.41, 1.93]	.029	22.88 [17.04, 27.36]	26.48 [23.66, 35.32]	.021
SELP	12.65 [9.89, 17.78]	15.69 [11.79, 20.02]	.032	513.28 [410.79, 729.79]	619.12 [533.12, 736.13]	.090
CSTB	0.53 [0.46, 0.61]	0.57 [0.49, 0.64]	.230	9.54 [7.56, 12.45]	11.72 [6.95, 15.39]	.15
MCP1	0.98 [0.76, 1.07]	1.01 [0.83, 1.22]	.234	14.06 [10.33, 17.27]	14.31 [10.73, 19.32]	.37
CD163	3.72 [2.94, 4.71]	3.76 [3.28, 4.36]	.996	114.25 [97.65, 157.43]	107.24 [101.02, 130.00]	.62
Gal3	0.23 [0.20, 0.26]	0.22 [0.20, 0.24]	.670	6.62 [5.43, 8.51]	7.23 [6.34, 8.70]	.31
GRN	1.27 [1.15, 1.36]	1.38 [1.25, 1.48]	.049	29.37 [23.82, 32.80]	30.84 [25.27, 34.46]	.35
NTproBNP	2.16 [1.77, 2.47]	2.37 [2.11, 2.71]	.052	12.46 [5.56, 19.56]	20.84 [12.50, 29.66]	.004
BLMhydrolase	0.20 [0.18, 0.22]	0.20 [0.17, 0.22]	.417	2.87 [2.58, 3.38]	2.52 [2.21, 3.40]	.25
PLC	4.26 [3.53, 5.53]	5.02 [3.97, 7.01]	.077	203.15 [160.22, 270.72]	221.68 [199.28, 299.30]	.070
LTBR	0.55 [0.45, 0.62]	0.65 [0.57, 0.69]	.005	7.54 [6.07, 9.32]	8.65 [6.98, 12.06]	.070
Notch3	1.04 [0.86, 1.16]	1.10 [0.99, 1.36]	.091	21.34 [16.28, 29.42]	22.33 [17.74, 27.60]	.71
TIMP4	0.53 [0.47, 0.64]	0.59 [0.52, 0.67]	.130	9.40 [7.60, 11.31]	10.12 [7.49, 15.12]	.22
CNTN1	0.40 [0.36, 0.46]	0.40 [0.35, 0.47]	.747	9.27 [7.55, 11.39]	10.45 [6.87, 11.22]	.98
CDH5	1.46 [1.24, 1.68]	1.51 [1.37, 1.73]	.379	11.77 [9.34, 14.37]	12.51 [9.65, 15.33]	.34
TLT2	2.07 [1.65, 2.71]	2.18 [1.83, 2.50]	.992	17.76 [13.88, 21.59]	20.97 [16.23, 22.60]	.23
FABP4	1.11 [0.97, 1.47]	1.48 [1.04, 1.87]	.099	36.24 [26.98, 53.19]	32.03 [26.69, 69.36]	.75
TFPI	9.89 [7.48, 11.33]	9.58 [7.97, 11.87]	.699	385.09 [276.67, 446.25]	371.54 [294.84, 443.15]	.77
PAI	0.58 [0.51, 0.73]	0.62 [0.57, 0.75]	.383	14.45 [10.55, 24.17]	16.48 [10.92, 19.90]	.78
CCL24	0.68 [0.52, 0.81]	0.75 [0.56, 0.93]	.180	19.81 [13.86, 32.18]	20.98 [15.19, 34.44]	.50
TR	1.45 [1.21, 1.69]	1.43 [1.30, 1.87]	.605	13.65 [10.79, 19.39]	16.83 [11.78, 22.94]	.22
TNFRSF10C	2.96 [2.59, 4.04]	3.43 [2.65, 4.24]	.261	55.75 [41.30, 70.48]	60.03 [50.97, 81.79]	.27
GDF15	1.16 [0.98, 1.50]	1.49 [1.18, 2.15]	.011	46.43 [33.84, 61.59]	68.99 [47.95, 100.28]	.005
SELE	73.66 [52.00, 91.60]	91.86 [59.87, 119.25]	.204	2358.77 [1724.27, 3077.42]	2496.39 [2143.09, 3517.78]	.23
AZU1	0.28 [0.24, 0.33]	0.26 [0.24, 0.30]	.570	4.65 [3.07, 7.73]	4.56 [3.26, 7.94]	.95
DLK1	1.89 [1.56, 2.43]	2.35 [1.84, 2.54]	.044	39.38 [27.82, 59.59]	52.42 [34.42, 63.95]	.18
SPON1	1.50 [1.30, 1.76]	1.64 [1.47, 1.72]	.123	2.29 [1.97, 2.66]	2.48 [2.27, 2.92]	.075
MPO	0.60 [0.52, 0.65]	0.62 [0.56, 0.69]	.184	7.24 [5.28, 9.70]	7.76 [6.31, 9.32]	.42
CXCL16	1.51 [1.25, 1.78]	1.84 [1.31, 2.08]	.066	27.20 [20.76, 35.14]	31.17 [25.03, 35.96]	.21
IL6RA	48.14 [36.58, 63.37]	50.62 [43.49, 52.70]	.945	2984.55 [2265.78, 3573.98]	3267.84 [2426.32, 4320.49]	.19
RETN	0.97 [0.80, 1.26]	1.21 [0.92, 1.47]	.058	61.15 [43.97, 74.97]	69.50 [56.77, 84.00]	.072
IGFBP1	1.11 [0.90, 1.28]	1.38 [0.97, 1.44]	.061	18.51 [11.68, 35.26]	14.48 [8.55, 32.20]	.49
CHIT1	1.39 [1.13, 1.68]	1.66 [1.29, 1.98]	.083	27.80 [18.47, 38.98]	33.85 [24.25, 54.16]	.076
TRAP	0.33 [0.28, 0.37]	0.31 [0.28, 0.35]	.847	7.52 [5.81, 9.06]	7.19 [5.59, 8.91]	.81
GP6	0.44 [0.40, 0.52]	0.49 [0.44, 0.57]	.056	1.60 [1.38, 2.04]	1.91 [1.60, 2.52]	.091
PSPD	0.58 [0.48, 0.66]	0.67 [0.54, 0.81]	.069	3.77 [2.32, 6.27]	5.00 [2.79, 6.26]	.37
PI3	0.33 [0.30, 0.39]	0.34 [0.30, 0.38]	.682	2.38 [1.93, 3.50]	3.34 [2.62, 4.53]	.014
EpCAM	1.21 [0.99, 1.68]	1.22 [0.91, 2.05]	.751	20.84 [13.80, 42.99]	26.62 [12.44, 45.22]	.65
APN	1.01 [0.89, 1.18]	1.13 [0.95, 1.18]	.283	20.81 [15.73, 25.77]	21.81 [17.96, 24.23]	.52
AXL	7.65 [6.25, 8.89]	7.63 [6.61, 8.02]	.743	214.74 [182.35, 266.95]	206.28 [183.47, 254.94]	.59
IL1RT1	1.81 [1.47, 2.06]	1.59 [1.51, 1.96]	.531	46.27 [38.51, 52.80]	46.44 [37.40, 55.22]	.82
MMP2	0.60 [0.56, 0.65]	0.63 [0.58, 0.71]	.072	6.87 [5.21, 8.36]	7.32 [5.77, 8.34]	.64
FAS	0.84 [0.72, 0.95]	0.94 [0.83, 1.08]	.057	28.60 [21.47, 35.32]	32.02 [26.92, 36.56]	.13
MB	3.31 [2.58, 4.53]	3.41 [2.86, 5.26]	.419	112.28 [80.04, 163.66]	99.27 [79.32, 167.30]	.96
TNFSF13B	2.97 [2.44, 3.79]	3.11 [2.62, 3.58]	.782	86.85 [64.05, 127.22]	88.53 [74.52, 100.28]	.48
PRTN3	0.43 [0.36, 0.53]	0.43 [0.37, 0.53]	.767	7.98 [5.62, 11.32]	8.41 [6.35, 11.62]	.58
PCSK9	0.79 [0.70, 0.97]	0.94 [0.81, 1.06]	.022	4.58 [3.58, 5.84]	4.72 [4.19, 6.00]	.34
UPAR	1.68 [1.35, 1.94]	1.90 [1.67, 2.13]	.010	28.25 [20.96, 34.33]	32.03 [28.39, 38.78]	.045
OPN	0.69 [0.60, 0.82]	0.83 [0.73, 0.98]	.004	92.72 [76.81, 130.45]	127.78 [82.73, 210.96]	.054
CTSD	0.25 [0.22, 0.28]	0.25 [0.22, 0.32]	.566	5.59 [3.99, 6.41]	5.92 [4.20, 7.85]	.30
PGLYRP1	2.18 [1.62, 2.49]	2.28 [1.76, 3.02]	.172	106.78 [85.89, 148.83]	127.88 [97.21, 171.84]	.16
CPA1	0.98 [0.77, 1.23]	1.18 [0.94, 1.53]	.053	33.30 [21.81, 47.96]	36.33 [28.01, 53.27]	.30
JAMA	0.44 [0.36, 0.55]	0.53 [0.43, 0.62]	.024	10.33 [7.96, 13.05]	13.50 [10.61, 14.81]	.010

Gal4	0.81 [0.69, 0.90]	0.93 [0.82, 1.16]	.006	9.13 [7.23, 12.76]	10.64 [8.56, 15.87]	.12
IL1RT2	0.99 [0.80, 1.20]	1.08 [0.92, 1.28]	.114	21.75 [18.54, 27.04]	23.37 [20.95, 26.58]	.36
SHPS1	0.86 [0.77, 0.93]	0.96 [0.86, 1.03]	.011	7.13 [5.79, 8.65]	7.12 [4.98, 10.96]	.77
CCL15	2.00 [1.56, 2.19]	2.12 [1.73, 3.03]	.036	108.60 [81.89, 128.65]	126.34 [103.50, 179.44]	.013
CASP3	0.89 [0.68, 1.31]	1.13 [0.81, 1.36]	.210	30.05 [20.22, 40.04]	29.34 [18.82, 71.72]	.52
uPA	0.59 [0.51, 0.69]	0.66 [0.58, 0.75]	.061	16.34 [12.39, 20.77]	16.82 [14.82, 22.03]	.23
CPB1	0.83 [0.73, 0.96]	0.97 [0.76, 1.09]	.069	28.78 [18.60, 44.49]	32.25 [23.83, 58.77]	.20
CHI3L1	0.32 [0.22, 0.56]	0.35 [0.28, 0.54]	.487	10.92 [6.86, 19.91]	14.03 [10.42, 20.05]	.16
ST2	1.60 [1.21, 1.96]	1.71 [1.36, 2.16]	.166	12.85 [10.57, 16.62]	16.62 [13.65, 22.49]	.018
tPA	1.51 [1.24, 1.90]	1.55 [1.44, 1.80]	.292	132.62 [100.50, 178.26]	157.83 [121.82, 198.72]	.14
SCGB3A2	0.77 [0.65, 0.92]	0.74 [0.70, 0.86]	.744	3.05 [1.59, 5.01]	2.64 [2.14, 5.32]	.82
EGFR	0.78 [0.71, 0.84]	0.79 [0.74, 0.87]	.187	4.41 [3.60, 5.00]	4.17 [3.79, 5.16]	.89
IGFBP7	2.48 [2.19, 3.06]	2.82 [2.37, 3.60]	.042	128.19 [94.28, 169.28]	138.67 [107.96, 170.89]	.28
CD93	32.51 [27.37, 45.42]	40.12 [33.82, 50.94]	.055	1455.41 [1054.18, 1841.00]	1645.75 [1289.52, 2170.66]	.11
IL18BP	1.83 [1.60, 2.21]	2.23 [1.87, 2.44]	.033	48.04 [38.36, 61.66]	52.35 [48.23, 72.92]	.17
COL1A1	0.75 [0.72, 0.78]	0.75 [0.72, 0.81]	.374	4.23 [3.64, 5.59]	4.67 [3.73, 5.90]	.53
PON3	1.53 [1.33, 1.84]	1.63 [1.41, 1.97]	.571	27.98 [21.03, 38.80]	30.14 [24.22, 41.71]	.50
CTSZ	1.11 [1.01, 1.27]	1.17 [1.07, 1.39]	.214	29.61 [22.88, 36.66]	27.43 [23.66, 41.08]	.70
MMP3	1.87 [1.27, 2.40]	2.18 [1.62, 2.45]	.257	110.24 [89.90, 187.68]	132.02 [88.45, 195.15]	.48
RARRES2	7.76 [6.31, 9.33]	8.81 [7.63, 13.16]	.036	1832.44 [1456.76, 2132.89]	1964.53 [1727.83, 2359.37]	.092
ICAM2	2.30 [1.88, 2.61]	2.33 [2.08, 2.92]	.177	23.67 [19.36, 31.05]	26.81 [19.84, 33.45]	.41
KLK6	0.23 [0.19, 0.31]	0.22 [0.19, 0.26]	.236	3.47 [2.75, 4.09]	3.56 [3.01, 4.20]	.50
PDGFsubunitA	1.02 [0.97, 1.11]	1.05 [0.99, 1.17]	.340	2.84 [2.37, 3.80]	3.12 [2.44, 3.72]	.56
TNFR1	3.16 [2.78, 3.81]	3.94 [3.07, 4.99]	.011	67.44 [50.88, 81.78]	78.76 [58.50, 111.35]	.077
IGFBP2	3.45 [2.59, 4.54]	4.85 [3.03, 5.85]	.049	193.02 [134.34, 268.79]	228.95 [159.01, 296.82]	.30
vWF	2.83 [2.43, 4.09]	3.31 [2.76, 4.39]	.157	135.49 [106.28, 213.69]	198.74 [98.11, 250.20]	.34
PECAM1	0.71 [0.63, 0.79]	0.75 [0.67, 0.84]	.254	13.44 [11.32, 17.27]	15.19 [13.70, 20.13]	.058
MEPE	1.03 [0.83, 1.20]	1.19 [0.96, 1.38]	.130	20.37 [16.51, 27.70]	23.48 [19.53, 26.93]	.25
CCL16	1.35 [1.10, 1.66]	1.48 [1.27, 1.97]	.047	62.51 [47.10, 75.85]	62.90 [54.95, 92.32]	.26

Table S4: Plaque proteins are stratified for MACE

	No MACE N = 66	Plaque MACE N = 22	P
TNFRSF14	3.45 [2.99, 4.29]	3.48 [3.03, 4.15]	.84
LDLreceptor	0.55 [0.49, 0.60]	0.53 [0.49, 0.59]	.54
ITGB2	3.84 [2.26, 6.84]	3.38 [1.74, 5.75]	.27
IL17RA	1.34 [1.13, 1.59]	1.33 [1.17, 1.57]	.77
TNFR2	3.56 [2.62, 4.27]	3.36 [3.04, 4.14]	.93
MMP9	1.46 [1.09, 2.78]	1.62 [1.03, 2.51]	.99
EPHB4	1.79 [1.49, 2.08]	1.92 [1.66, 2.18]	.26
IL2RA	1.05 [0.94, 1.22]	1.07 [0.97, 1.19]	.79
OPG	19.43 [9.57, 27.15]	13.21 [10.70, 29.40]	.42
ALCAM	4.07 [2.95, 6.93]	3.35 [2.65, 5.67]	.27
TFF3	1.16 [1.03, 1.30]	1.21 [1.04, 1.50]	.37
SELP	7.65 [6.08, 10.29]	7.23 [5.65, 11.73]	.89
CSTB	114.71 [67.94, 204.86]	103.73 [48.86, 181.30]	.32
MCP1	5.55 [2.87, 9.67]	4.88 [2.88, 10.05]	.71
CD163	10.23 [6.26, 18.84]	7.47 [4.86, 15.53]	.23
Gal3	1.23 [0.63, 1.90]	1.18 [0.58, 1.89]	.60
GRN	2.11 [1.63, 2.90]	1.69 [1.28, 2.78]	.14
NTproBNP	2.78 [2.58, 3.12]	2.78 [2.57, 2.95]	.66
BLMhydrolase	1.37 [0.91, 1.93]	0.99 [0.78, 1.71]	.30
PLC	13.39 [7.44, 22.72]	12.14 [5.16, 21.38]	.44
LTBR	1.15 [0.93, 1.34]	0.98 [0.84, 1.42]	.58
Notch3	1.34 [1.18, 1.54]	1.49 [1.27, 1.73]	.11
TIMP4	0.71 [0.63, 0.81]	0.70 [0.65, 0.74]	.63
CNTN1	0.57 [0.53, 0.62]	0.56 [0.51, 0.66]	1.0
CDH5	1.97 [1.72, 2.26]	1.98 [1.68, 2.21]	.82
TLT2	2.59 [2.15, 3.30]	2.80 [2.17, 3.00]	.94
FABP4	10.85 [4.89, 27.58]	12.85 [5.72, 25.32]	.48
TFPI	4.44 [3.52, 6.15]	4.17 [3.22, 5.40]	.32
PAI	3.42 [2.13, 5.44]	2.97 [2.02, 4.11]	.34
CCL24	0.72 [0.59, 0.94]	0.80 [0.70, 0.96]	.19
TR	0.92 [0.78, 1.22]	0.88 [0.82, 1.14]	.87
TNFRSF10C	2.89 [2.34, 3.83]	2.94 [2.07, 3.68]	.82
GDF15	3.10 [2.22, 5.09]	3.20 [2.43, 4.57]	.93
SELE	12.70 [9.82, 17.95]	14.16 [9.38, 18.02]	.95
AZU1	2.67 [1.20, 5.00]	2.20 [1.75, 3.61]	.92
DLK1	1.45 [1.32, 1.79]	1.45 [1.40, 1.64]	.60
SPON1	2.19 [1.96, 2.55]	2.33 [2.07, 2.54]	.51
MPO	1.66 [1.07, 4.09]	1.87 [1.20, 3.46]	.91
CXCL16	2.27 [1.72, 3.17]	2.22 [2.03, 2.69]	.98
IL6RA	13.76 [9.00, 18.18]	14.04 [11.27, 19.47]	.61
RETN	3.44 [2.38, 5.86]	2.97 [2.20, 5.39]	.70
IGFBP1	1.90 [1.56, 2.30]	1.97 [1.76, 2.26]	.39
CHIT1	1.89 [1.28, 3.02]	1.69 [1.20, 2.61]	.48
TRAP	1.17 [0.56, 1.85]	1.12 [0.42, 1.78]	.59
GP6	0.72 [0.63, 0.83]	0.75 [0.70, 0.91]	.079
PSPD	0.75 [0.62, 1.04]	0.84 [0.72, 1.20]	.15
PI3	0.26 [0.22, 0.30]	0.26 [0.22, 0.33]	.79
EpCAM	1.15 [0.90, 1.51]	1.27 [1.03, 1.58]	.23
APN	1.01 [0.89, 1.28]	1.02 [0.91, 1.13]	.85
AXL	5.28 [4.24, 6.60]	6.08 [4.73, 6.99]	.36
IL1RT1	1.82 [1.43, 2.21]	1.67 [1.52, 2.15]	.70
MMP2	0.78 [0.73, 0.82]	0.81 [0.76, 0.85]	.097
FAS	1.50 [1.14, 2.06]	1.24 [1.02, 1.94]	.25
MB	8.21 [3.60, 20.01]	6.48 [2.90, 15.38]	.36
TNFSF13B	3.37 [2.48, 4.32]	3.09 [2.04, 4.07]	.21
PRTN3	1.19 [0.92, 1.81]	1.33 [0.96, 1.76]	.73
PCSK9	1.09 [0.97, 1.20]	1.19 [0.96, 1.35]	.14
UPAR	15.26 [6.40, 28.52]	8.72 [6.10, 20.86]	.32
OPN	23.25 [10.05, 80.42]	23.14 [11.47, 71.69]	.97
CTSD	6.05 [1.63, 12.50]	6.01 [1.44, 9.24]	.46
PGLYRP1	2.62 [1.94, 4.55]	2.60 [2.00, 3.62]	.88
CPA1	0.91 [0.78, 1.07]	1.00 [0.89, 1.21]	.054

JAMA	0.71 [0.55, 1.01]	0.62 [0.51, 0.81]	.22
Gal4	1.05 [0.92, 1.22]	1.10 [0.93, 1.35]	.46
IL1RT2	1.00 [0.85, 1.12]	1.00 [0.90, 1.29]	.74
SHPS1	1.75 [1.45, 2.01]	1.55 [1.41, 1.83]	.27
CCL15	1.59 [1.34, 1.82]	1.68 [1.43, 1.98]	.16
CASP3	29.94 [10.76, 48.61]	29.77 [19.33, 46.17]	.80
uPA	2.01 [1.34, 3.28]	1.74 [1.10, 3.15]	.40
CPB1	0.87 [0.79, 1.00]	0.93 [0.82, 1.00]	.32
CHI3L1	0.66 [0.40, 1.21]	0.70 [0.34, 1.66]	.90
ST2	2.16 [1.82, 2.54]	2.33 [1.99, 2.77]	.18
tPA	2.59 [1.96, 3.15]	2.16 [1.92, 3.13]	.24
SCGB3A2	0.67 [0.61, 0.76]	0.73 [0.70, 0.80]	.078
EGFR	0.95 [0.91, 0.99]	0.97 [0.88, 1.00]	.99
IGFBP7	48.69 [24.62, 100.28]	48.20 [22.36, 81.88]	.82
CD93	17.92 [11.16, 25.09]	15.64 [10.70, 22.99]	.59
IL18BP	1.72 [1.39, 2.36]	1.80 [1.56, 2.20]	.91
COL1A1	1.09 [0.96, 1.46]	1.02 [0.96, 1.10]	.054
PON3	1.20 [1.09, 1.35]	1.31 [1.21, 1.58]	.032
CTSZ	9.76 [5.81, 17.02]	8.98 [4.13, 12.20]	.31
MMP3	2.04 [1.58, 2.54]	2.18 [1.79, 2.33]	.60
RARRES2	4.35 [2.66, 8.49]	4.98 [2.49, 9.61]	.98
ICAM2	2.33 [2.15, 2.76]	2.49 [2.26, 2.69]	.42
KLK6	0.29 [0.21, 0.35]	0.28 [0.24, 0.40]	.61
PDGFsubunitA	1.46 [1.37, 1.57]	1.51 [1.33, 1.55]	.90
TNFR1	7.20 [6.18, 9.18]	6.95 [5.36, 8.11]	.30
IGFBP2	9.55 [5.87, 14.66]	8.27 [4.98, 11.58]	.41
vWF	2.00 [1.57, 2.52]	2.12 [1.92, 2.40]	.25
PECAM1	1.07 [0.90, 1.24]	1.08 [0.91, 1.28]	.74
MEPE	1.36 [1.19, 1.59]	1.39 [1.24, 1.69]	.61
CCL16	0.82 [0.72, 0.98]	0.85 [0.71, 1.07]	.79

6

Plasma extracellular vesicle serpinG1 and CD14 levels are associated with mace and male in patients undergoing femoral endarterectomy

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Abstract

Objectives and design: *Plasma extracellular vesicles (EV) are an emerging source of biomarkers for the diagnosis and prognosis of cardiovascular disease (CVD). Risk stratification for common adverse events such as Major Adverse Limb Event (MALE) and Major Adverse Cardiovascular Events (MACE) by an EV blood sample could improve healthcare management by individualising drug therapy or by improving informed decision-making regarding revascularisations in patients with PAD. As such, we investigated the associations of plasma EV proteins with prospectively registered MALE and MACE in consecutive patients undergoing femoral endarterectomy.*

Methods: *Using the Athero-Express biobank study, we measured four EV proteins (Cystatin C, CD14, Serpin C1 and Serpin G1) in the HDL subfraction isolated from plasma of 317 PAD patients undergoing arterial revascularisation. Multivariable Cox proportional hazard regression was used to investigate the association between plasma EV-protein levels with MACE and MALE in the three-year postoperative period.*

Results: *Most patients were treated for claudication (Fontaine II, 52.8%), although rest pain (Fontaine III, 30.1%) and ischemic wounds (Fontaine IV, 17.1%) were common in this cohort. Within three years, 51 patients died, of which 25 were due to CVD, 39 patients experienced a MACE, and 125 patients experienced a MALE. Multivariable regression models, based on statistically proven covariables and literature, showed a significant association of Serpin G1 (HR 1.49 (95% CI 1.08 – 2.06) P = .016) and CD14 (HR 1.40 (1.03-1.90) P = .029) with MACE, and of Serpin G1 (HR 1.29 (1.07 – 1.57) P = .009) with MALE.*

Conclusion: *Serpin G1 and CD14 plasma EV protein levels are associated with future MACE and MALE in patients with severe PAD.*

Introduction

Peripheral artery disease (PAD) is considered one of the most prevalent vascular conditions, affecting over 202 million people worldwide in 2010.[1] Despite the best pharmacological control of risk factors, PAD is still associated with a high incidence of cardiovascular adverse events (CVE), such as major adverse limb events (MALE) and major adverse cardiovascular events (MACE). Of patients that underwent a peripheral arterial intervention, up to 42% will have a MALE and 13% a MACE in the following three years.[2] Consequently, patients with a high risk for CVE might benefit from add-on therapies such as dual antiplatelet therapies, dual pathway inhibition, PCSK9-inhibition or colchicine treatment.[3–6] In addition, improved risk stratification could support the decision-making of interventions when the effectiveness of limb salvage is disputed or when additional arguments are warranted to select the mode of intervention.

Early identification of PAD patients with a higher risk of complications is still lacking, and consequently, prediction models that use clinical risk factors are not widely used in PAD. Biological biomarkers associated with relevant adverse events are crucial to enhance these models.

Extracellular vesicles (EVs) are a heterogeneous group of small bilayer membrane particles that act as intercellular messengers. They are secreted by a wide variety of cells and can transfer their cargo, which consists of proteins, nucleic acids, lipids and metabolites, to areas distant from their origin, where they contribute to the preservation of vascular homeostasis by influencing processes like inflammation, coagulation and stem cell expansion.[7] For this reason, EVs are considered a "liquid biopsy" of numerous diseases.[7, 8]

Levels of EV subgroups are increased in patients with acute coronary syndromes or ischemic stroke and in response to risk factors for cardiovascular diseases, such as smoking, metabolic disease and hypertension.[9–12] A study using Framingham Heart Study data showed that EV levels were associated with hypertension, dyslipidaemia and metabolic syndrome.[13] Higher levels of EV proteins in patients with PAD relate to PAD severity, although evidence is limited due to a small number of studies and a low number of patients.[14] There is only one study focusing on the association of an EV protein (calprotectin) with future events (amputation) in a PAD population.[15] As such, the prognostic properties of these novel biomarkers are relatively unexplored territory. Recently, research in patients undergoing carotid endarterectomy showed that EV proteins (Cystatin C, CD14, Serpin C1 and Serpin F2) are associated with future MACE.[16] Serpin G1 was associated with heart failure in patients with breathlessness.[17] Furthermore, Serpin C1, G1 and F2 and Cystatin C were associated with stress-induced myocardial ischemia in women.[18] We hypothesised that these EV proteins might also be associated with future CVE in PAD patients.

Hence, we investigated whether preoperative levels of four EV-proteins (Cystatin C, CD14, Serpin C1 and Serpin G1) were associated with MACE and MALE in patients after femoral endarterectomy. Histological plaque characteristics were related to EV protein levels to explore potential pathophysiological mechanisms.

Methods

Study population and design

Patients undergoing endarterectomy of the femoral artery in two hospitals in the Netherlands (UMC Utrecht and St. Antonius Hospital, Nieuwegein) are eligible for inclusion in the Athero-Express (AE). This biobank was established to collect important biological material, such as atherosclerotic plaque and preoperative blood, which can be used for research into pathophysiology, predictive biomarkers, and other applications. The AE has therefore been used extensively and has been described in greater detail previously.[19] The study has been approved by the Institutional Review boards of both hospitals, and written informed consent was obtained from all patients. The study is conducted in accordance with the declaration of Helsinki.[20]

For this research, we selected PAD patients who underwent femoral endarterectomy with complete data regarding three years of follow-up. Of these, 218 patients underwent thromboendoarterectomy (TEA), 69 underwent endarterectomy with a ring strip cutter (RSC), and 30 underwent bypass surgery after endarterectomy. Two patients underwent RSC in addition to the TEA, eleven underwent stenting in addition to TEA, and five underwent RSC and stenting in addition to TEA. Patients without sufficient biomarker material (citrate plasma) and patients who died during surgery or underwent lower limb amputation were excluded from our analysis.

Blood collection and processing

In the 24 hours before surgery, venous blood was collected in citrate tubes. These were centrifuged (10 min, 1850xg @ room temperature [RT]) within 30 minutes after collection. Plasma was aliquoted and directly stored at -80 °C.

Isolation of extracellular vesicle plasma subfractions and mesoscale immuno assay

The HDL subset of plasma EVs, which co-precipitates with HDL particles, was isolated according to previously published protocols.[18] This subset was chosen since this subfraction contained the most differentially expressed proteins for MACE in patients undergoing carotid endarterectomy.[16] In short, solutions of dextran sulphate (MP Biomedicals, Santa Ana, CA, USA) and manganese (II) chloride (MnCl2; Sigma-Aldrich, St Louis, MO, USA) were used to precipitate the HDL-EV subset. Cystatin C (CysC), CD14, Serpin C1 (SC1) and Serpin G1 (SG1) were quantified in this subset using an electrochemiluminescence immunoassay (Quickplex SQ120; Meso Scale, Rockville, MD, USA). SerpinF2 was not investigated in this cohort as it showed very low levels in a large percentage of PAD patients in a test run.

Characterisation of extracellular vesicles

Both the modified protocol which was used as well as extracellular vesicle characterisation are described in detail in two previously published papers (especially in the supplemental materials of Zhang et al.)[17, 21] In short, we used density gradient centrifugation of the HDL plasma subfractions, with density gradient fractions characterised by CD9 western blot analysis as EV specific antibody. The proteins studied in this manuscript (SC1, CD14, SG1, and CC) were shown in the density gradient fractions that were shown with CD9 western blotting and EM and absent in the density gradient fractions with lipid particles. To get easy access to these data, an EV-track ID was created: EV200044, in which the data is structured in a uniform way.

Table 1: Baseline characteristics for AE femoral endarterectomy patients.

	Overall 317
Age [a]	68.5 (9.0)
Male	229 (72.2)
BMI [a]	26 (4.1)
Smoking	124 (39.7)
Fontaine Stage	
II	142 (52.8)
III	81 (30.1)
IV	46 (17.1)
History of	
Coronary Artery Disease	135 (42.7)
Stroke	54 (18.4)
Hypertension	276 (87.1)
Diabetes Mellitus	91 (28.7)
Medication use of	
Insulin	28 (8.8)
Glucose inhibitors	72 (22.7)
Anticoagulants	46 (14.5)
Antiplatelets	273 (86.4)
Lipid Lowering Drugs	238 (75.1)
Laboratory Results	
GFR-MDRD [a]	79.7 (26.4)
LDL [a]	2.41 (0.89)
HDL [a]	1.11 (0.37)
Triglycerides [a]	2.08 (1.56)
Total Cholesterol [a]	4.38 (1.19)
Plaque characteristics	
Fat >40%	16 (6.2)
Fat >10%	73 (28.2)
Intraplaque Haemorrhage	114 (47.3)
Smooth Muscle Cell	174 (73.1)
Calcification	151 (58.5)
Collagen	192 (81.0)

BMI = Body Mass Index; ABI = Ankle-Brachial-Index; GFR-MDRD = Glomerular Filtration Rate - Modification of Diet in Renal Disease; LDL = Low-density lipoprotein; HDL = High-density lipoprotein; EVs = Extracellular Vesicles; SD = standard deviation.

Histological atherosclerotic plaque examination

The atherosclerotic plaque was processed and immunohistochemically analysed for the number of macrophages (CD68 stain), smooth muscle cells (SMCs)(alpha-actin stain), microvessel (CD34, endothelial stain), amount of collagen (picro-sirius), intraplaque haemorrhage (IPH)(Haematoxylin Eosin stain and Elastic Van Gieson), lipid core (picro-sirius and Haematoxyin Eosin stain), and calcifica-

tions by the standardised AE protocol.[19] Two experienced observers examined plaque and scored macrophage, SMC, calcifications and collagen content as no/ minor staining or moderate/heavy staining. The lipid core was estimated and categorised as <10%, >10% and <40%, >40% of the total plaque area.

Follow-up and clinical outcomes measures

Following surgery, patients included in the AE underwent a three-year follow-up, which consisted of an annual questionnaire. Important outcomes described in the original AE were validated, which required official letters describing these events.[19] For this study, two important endpoints (MACE and MALE) were used. Major Adverse Cardiovascular Events (MACE) is a composite of nonfatal myocardial infarction or stroke and death contributed to cardiovascular disease. The latter was defined as fatal myocardial infarction, fatal stroke (both haemorrhagic or ischemic), fatal ruptured abdominal aneurysm, fatal heart failure and sudden cardiac death. Major Adverse Limb Event (MALE) is defined as all vascular interventions of either lower limb, including bypass or endarterectomy surgery, endovascular therapies with or without stenting, (catheter-directed) thrombolysis, and above-the-ankle amputation (major amputation). Only the first events were used in the analyses.

Statistical analyses

Protein measurements were log10 transformed for normalisation. The distribution of these proteins was analysed by reviewing protein levels across the different study numbers and creating density plots.

Descriptive statistics of baseline data were compared using the Student t-test, Mann-Whitney U test, Chi-square or Fisher's exact test, depending on the type of variable and their respective distribution.

Cox Proportional Hazard regression analysis was used to investigate the association of proteins with either major outcome. A multivariable model was created by implementing important risk factors. These were derived from our own statistical analysis, which consisted of a univariable Cox proportional hazard regression analysis of all baseline characteristics, with an alpha of 0.2 as a cutoff. For a literature model, risk factors derived from other research were implemented when these variables were available in our data. Our measured EV-proteins were implemented in these models and were then analysed for improvement by reviewing the Akaike Information Criteria (AIC).

The model improvement with the implementation of the EV protein was assessed by comparing the time-dependent Area Under the Curve (AUC) based on the model of Heagerty and Zheng.[22] A two-tailed alpha level of 0.05 was considered significant. Statistical analyses were performed using R version 4.1.2.

Results

Out of 3924 potentially eligible Athero-Express patients, 1034 patients underwent femoral endarterectomy. For this study, 643 patients completed the follow-up, and of these, 317 patients had enough citrate sample for analysis. These 317 patients were included. The study population encompassed patients of high age (mean 68.5 years old) with a male predominance (72.2%) (Table 1). The indication for surgery was either Fontaine stages II, III or IV in, respectively, 52.8%, 30.1% and 17.1% of these cases. Most participants had hypertension or received hypertensive medication (87.1%), and a cardiac comorbidity was common as 42.7% of patients had coronary artery disease (CAD). The prevalence of stroke and diabetes mellitus was lower, with respectively 18.4% and 28.7%.

EV-protein analysis

A multiplex assay was used for this research and included Cystatin C (CysC), CD14, Serpin C1 (SC1) and Serpin G1 (SG1). After the log transformation of these four proteins, their distribution was con-

Table 2: Baseline characteristics stratified for endpoints MACE and MALE

	No MACE 278	MACE 39	P	No MALE 192	MALE 125	P
Age [a]	68.20 (9.0)	70.46 (9.1)	.14	69.2 (9.4)	67.3 (8.4)	.069
Male	205 (73.7)	24 (61.5)	.16	140 (72.9)	89 (71.2)	.84
BMI [a]	26.2 (4.2)	26.3 (3.6)	0.91	25.9 (4.3)	26.7 (3.8)	.082
Smoking	106 (38.8)	18 (46.2)	.48	75 (39.9)	49 (39.5)	1
Fontaine Stage			0.14			.19
II	131 (54.8)	11 (36.7)		89 (57.4)	53 (46.5)	
III	70 (29.3)	11 (36.7)		41 (26.5)	40 (35.1)	
IV	38 (15.9)	8 (26.7)		25 (16.1)	21 (18.4)	
History of						
Coronary Artery Disease	119 (42.8)	16 (42.1)	1	78 (40.8)	57 (45.6)	.47
Stroke	41 (15.9)	13 (36.1)	.007	31 (17.5)	23 (19.7)	.76
Hypertension	239 (86.0)	37 (94.9)	.20	160 (83.3)	116 (92.8)	.022
Diabetes Mellitus	79 (28.4)	12 (30.8)	.91	57 (29.7)	34 (27.2)	.73
Medication use of						
Insulin	25 (9.0)	3 (7.7)	1	19 (9.9)	9 (7.2)	.54
Glucose inhibitors	63 (22.7)	9 (23.1)	1	45 (23.4)	27 (21.6)	.81
Antihypertensives	226 (81.3)	34 (87.2)	.50	149 (77.6)	111 (88.8)	.017
Anticoagulants	40 (14.4)	6 (15.4)	1	27 (14.1)	19 (15.2)	.91
Antiplatelets	242 (87.4)	31 (79.5)	.27	164 (85.4)	109 (87.9)	.64
Lipid Lowering Drugs	208 (74.8)	30 (76.9)	.93	139 (72.4)	99 (79.2)	.22
Laboratory Results						
GFR-MDRD [a]	79.7 (25.4)	79.7 (33.8)	.99	81.6 (27.7)	76.7 (24.4)	.12
LDL [a]	2.4 (0.89)	2.43 (0.91)	.88	2.38 (0.90)	2.44 (0.88)	.56
HDL [a]	1.12 (0.38)	1.05 (0.27)	.28	1.11 (0.37)	1.10 (0.37)	.68
Triglycerides [a]	2.09 (1.59)	1.98 (1.32)	.67	2.05 (1.36)	2.13 (1.84)	.68
Total Cholesterol [a]	4.38 (1.19)	4.34 (1.21)	.86	4.36 (1.20)	4.40 (1.18)	.77
EVs						
Cystatin C [a]	4.01 (0.424)	4.10 (0.43)	.26	4.06 (0.41)	3.965 (0.44)	.054
CD14 [a]	4.34 (0.189)	4.42 (0.19)	.011	4.36 (0.20)	4.331 (0.18)	.16
Serpin C1 [a]	6.38 (0.273)	6.37 (0.24)	.92	6.39 (0.26)	6.354 (0.28)	.27
Serpin G1 [a]	3.25 (0.267)	3.35 (0.32)	.046	3.23 (0.28)	3.315 (0.27)	.007
Plaque characteristics						
Fat >40%	14 (6.2)	2 (6.5)	1	13 (8.4)	3 (3.0)	.14
Fat >10%	62 (27.6)	9 (29.0)	1	46 (29.7)	25 (24.8)	.47
Intraplaque Haemorrhage	99 (47.1)	14 (48.3)	1	67 (47.2)	46 (47.4)	1
Smooth Muscle Cell	150 (72.5)	22 (75.9)	.87	96 (69.6)	76 (77.6)	.23
Calcification	131 (58.5)	19 (61.3)	.92	89 (57.8)	61 (60.4)	.78
Collagen	167 (80.7)	24 (82.8)	.99	108 (77.7)	83 (85.6)	.18

Data are presented as n (%), median [interquartile range] or mean ± standard deviation. BMI (Body Mass Index; ABI (Ankle-Brachial-Index; GFR-MDRD (Glomerular Filtration Rate -Modification of Diet in Renal Disease; LDL (Low-density lipoprotein; HDL (High-density lipoprotein; EVs (Extracellular Vesicles; SD (standard deviation).

sidered normal. Protein levels in relation to the time of blood sampling up until the time of analysis in the 317 patients showed no evident relation when examining distribution plots. Linear regression, however, demonstrated that all except CD14 were associated with the length of this interval, although

the direction was unambiguous and the effect was very minimal (Supplemental Table 1).

Outcomes

Three-year follow-up of the 317 PAD patients showed that 51 died, 25 of them from cardiovascular diseases. Thirty-nine patients had experienced MACE, and 125 patients experienced a MALE. These events seemed to unfold gradually over time (Supplemental Figure 1). For MACE, not many statistical differences in baseline characteristics were seen between the groups. Only a history of stroke was seen more often (15.9% vs 36.1%, P = .007) in patients with MACE (Table 2).

Equally, few distinguishable baseline characteristics were statistically different for MALE (Table 2). Only hypertension was reported more frequently in the group with MALE (92.8% vs 83.3%, P = .022), and (consequently) these patients were treated with antihypertensive drugs more often.

Association with outcomes

Statistical models

As a first step towards a complete multivariable model, univariable regression analysis was performed for all variables and both endpoints separately (Supplemental Table 2). This identified age, sex, Fontaine classification, history of stroke or TIA, hypertension, use of antiplatelets, CD14 and SG1 as potential predictors for MACE. Age, body mass index (BMI), hypertension, CysC and SG1 were potential predictors for MALE. Kaplan Meier curves are shown for quartiles of the significantly associated EV-proteins in Figures 1A-C.

Implementing EVs in multivariable models based on the statistical significance of covariates, CD14 and SG1 were both significant in the models for MACE, but only SG1 was significant for MALE (Table 3, full models in Supplemental Table 3). Stepwise regression for MACE selected CD14, SC1 and SG1 in its model (besides Stroke/TIA), whereas its equivalent for MALE only selected SG1 (alongside age, hypertension, Fontaine and eGFR) (Supplemental Table 4).

Table 3: Statistical multivariable cox regression models with EVs implemented.

	EV Protein	HR (95% CI)	P
MACE [a]			
	logCysC	1.16 (0.84 – 1.59)	.37
	logCD14	1.40 (1.03 – 1.90)	.029
	logSC1	0.97 (0.70 – 1.33)	.84
	logSG1	1.49 (1.08 – 2.06)	.016
MALE [b]			
	logCysC	0.69 (0.44 – 1.08)	.11
	logCD14	0.91 (0.76 – 1.09)	.31
	logSC1	0.91 (0.76 – 1.09)	.3
	logSG1	1.29 (1.07 – 1.57)	.009

[a] The model for Major Adverse Cardiovascular Events (MACE) includes age, sex, Fontaine, stroke, hypertension and antiplatelet therapy.
[b] The model for Major Adverse Limb Events (MALE) includes age, BMI and hypertension.

Literature model

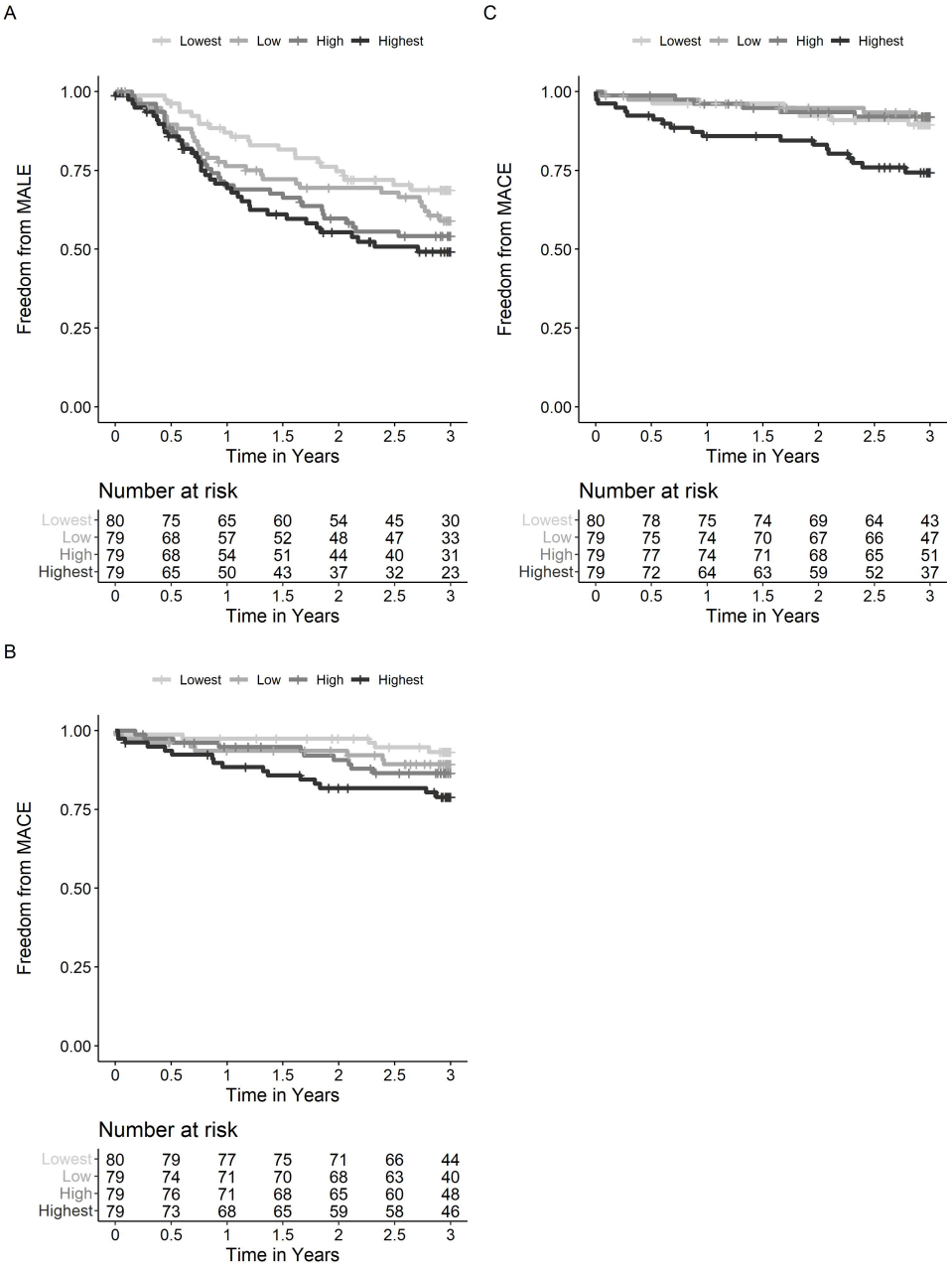
We established a collection of clinical risk factors that were described as relevant in today's literature. Both CD14 and SG1 were statistically significant when implemented in the two models for MACE. For MALE, SG1 was significant in all three models, whereas CysC was significant in only one (Table 4, full models in Supplemental Table 3).

Analysis of the AUC shows that these statistically significant EV proteins are able to improve these models. Especially the improvement with CD14 in MACE (AUCs of 0.626 to 0.682 and 0.638 to 0.685) and SG1 in MALE (AUCs of 0.602 to 0.636, 0.597 to 0.635 and 0.562 to 0.608) are noteworthy (Table 4).

Association with plaque characteristics

A total of 198 plaques were available for histological assessment. Semi-quantitative characteristics of the atherosclerotic plaque were related to continuous levels of EV proteins. Only two statistically significant associations were found for SC1 with the presence of macrophages (OR 0.19 [0.04 – 0.80] P = 0.029) and CysC with a lipid core (larger than 10% of the total plaque area) (OR 4.05 (1.0 – 16) P = 0.050) (Supplemental Table 5).

Figure 1: Kaplan Meier Curves with EV protein quartiles for MACE and MALE



Discussion

This study demonstrates that EV proteins are biomarkers associated with future MACE and MALE in patients with severe PAD. Both CD14 and SG1 were elevated preoperatively in patients who experienced MACE in the three years following femoral surgery, whereas SG1 was also higher in patients with postoperative MALE. Associations with these endpoints were confirmed in multiple multivariable models based on a statistical selection of covariates and clinical risk models derived from literature. For this, CD14 and SG1 may contribute to risk stratification of patients with severe PAD and thus facilitate personalised medicine when additional therapeutic options are considered. Pharmacological therapies might include further LDL-c lowering (with PCSK9 inhibitors), the use of colchicine, dual antiplatelet therapies and dual pathway inhibition.

CD14 is crucial in the innate immune response by monitoring pathogens and responding to bacterial lipopolysaccharide as a co-receptor of Toll-like receptor 4 (TLR4).[23] As such, CD14 stimulates a cascade of proinflammatory signalling pathways and has been established to influence cell metabolism (lipogenesis, insulin resistance).[23] CD14 activation can also increase the expression of cell adhesion molecules and procoagulant activity, which are often essential components of adverse cardiovascular events.[24] For these reasons, CD14 has gained interest as a potential cardiovascular risk factor and has thus far been associated with an increased risk of cardiovascular events in patients undergoing carotid endarterectomy and a mixed group of patients in cardiovascular cohorts.[16, 25, 26] With respect to PAD, plasma CD14 levels were elevated in patients with a combination of PAD and CAD, as well as patients with a higher PAD classification.[27]

Our study is the first to demonstrate that for patients with PAD, EV-CD14 is independently associated with a future event, MACE, and can, in fact, improve clinical risk models when implemented (AUC improvement of 0.059 and 0.046 for two existing clinical models).

No association of CD14 with MALE was established, which is in line with a study that demonstrated that CD14+ expressed monocytes were not associated with one-year revascularisation after percutaneous transluminal angioplasty (PTA).[28] Due to the pathophysiological mechanism, an association would be expected, and thus we believe that patient selection could be of influence. As the patients in our study had a severe PAD condition, reflected by a three-year MALE incidence of over 35% and a relatively high prevalence of higher Fontaine stages, the CD14 differences may have been too small but would have been found when comparing our cohort with a group with lower PAD severity. In addition, our data show that CD14 was not associated with major features of the vulnerable femoral plaque. Consequently, CD14 might have only a limited influence on the occurrence of new limb events. However, in contrast to other vascular territories, femoral occlusion is common in severe PAD patients leading to a more stable plaque phenotype that is not associated with the ongoing progression of atherosclerotic disease.[29] The absence of an association of our EV markers with plaque characteristics, although an association with MACE and/or MALE is proven, underlines this. Interpretation relating to the mechanistic and function of the markers with plaque pathology is, therefore, extremely difficult.

Regarding SG1, this complement 1 (C1)-inhibitor is an acute phase protein which regulates the vascular permeability and suppression of inflammation, effectively contributing to the neointimal plaque formation.[30] In addition, SG1 inhibits enzymes involved in fibrinolysis and intrinsic coagulation by targeting plasmin, factor XI and XII and plasma kallikrein.[31] Again, these processes are paramount for both MACE and MALE, as inflammation and coagulation are key drivers for atherosclerotic events.[32, 33] SG1 has been associated with an elevated risk of heart failure and stress-induced myocardial ischemia, but thus far, the prognostic capabilities are uncertain.[17, 18] Our study is the first to demonstrate the association of both clinically relevant endpoints with this marker. Furthermore, comparing the AUCs of available prognostic models, the addition of continuous SG1 levels demonstrates a modest improvement of between 0.025 and 0.05. Dichotomisation (low/high) of SG1 would probably lead to further improvement of these models, as a clear distinction was seen between the first three quartiles

Table 4: Literature multivariable cox regression models with EVs implemented

	EV protein	HR (95% CI)	P	AUC before	AUC after
MACE:					
Miao et al.[a]				0.626	
	logCysC	1.17 (0.85 – 1.60)	.344		0.631
	logCD14	1.46 (1.09 – 1.96)	.012		0.682
	logSC1	0.99 (0.72 – 1.35)	.933		0.628
	logSG1	1.40 (1.01 – 1.93)	.044		0.654
Berger et al. [b]				0.638	
	logCysC	1.12 (0.80 – 1.55)	.520		0.635
	logCD14	1.38 (1.03 – 1.86)	.033		0.685
	logSC1	0.95 (0.69 – 1.31)	.750		0.637
	logSG1	1.43 (1.03 – 1.98)	.033		0.658
MALE:					
Biscetti et al. c				0.602	
	logCysC	0.84 (0.70 – 1.02)	.073		0.615
	logCD14	0.91 (0.76 – 1.09)	.289		0.610
	logSC1	0.91 (0.76 – 1.09)	.293		0.609
	logSG1	1.30 (1.07 – 1.57)	.008		0.636
Zhang et al. [d]				0.597	
	logCysC	0.85 (0.70– 1.03)	.10		0.617
	logCD14	0.91 (0.76 – 1.09)	.286		0.609
	logSC1	0.92 (0.77 – 1.09)	.320		0.605
	logSG1	1.28 (1.06 – 1.56)	.011		0.635
Meltzer et al. [e]				0.562	
	logCysC	0.79 (0.65 – 0.96)	.018		0.592
	logCD14	0.86 (0.72 – 1.03)	.112		0.583
	logSC1	0.87 (0.73 – 1.05)	.144		0.572
	logSG1	1.40 (1.15 – 1.70)	.001		0.608

Two literature-based models for MACE, and three literature-based models for MALE, with all EV proteins implemented separately. EV-proteins are z-transformed, and thus the HR represents the HR per standard deviation increase. The two right columns indicate the Area Under the Curve (AUC) for each model before (thus without the EV-protein) and after implementation of the EV-protein. For AUC, higher levels indicate a better model performance.

[a] Includes coronary artery disease (CAD), stroke, Diabetes Mellitus, Hypertension, smoking, and use of insulin.

[b] Includes Age, BMI, chronic kidney disease (CKD), CAD, stroke, diabetes mellitus (DM) and smoking.

c Includes CAD, stroke, DM, hypertension, smoking, and age.

[d] Includes age, BMI, hypertension, DM, smoking, and sex.

[e] Includes Fontaine, DM, sex, smoking, CKD, CAD.

compared to the fourth quartile in the Kaplan-Meier curve, but this needs to be confirmed in future research.

This study is not without certain limitations. Inclusion criteria imply that our results and conclusions are only fit for patients with severe PAD of, at least the femoral arterial segment. It could be argued that patients with PAD without rest pain or ischemic wounds can be considered 'severe', but we tend to define significant atherosclerotic stenosis in combination with severely debilitating symptoms as such. The initial intervention was heterogenous, although a majority of patients underwent TEA. In

preliminary analyses, we scrutinised whether this affected the association of EV markers and MALE or MACE. We concluded that no effect on MACE was seen, whereas the association of SG1 gained very little with the addition of this variable. Consequently, we did not add the intervention category to our multivariable models.

Disease processes in patients of our cohort are most likely due to traditional risk factors, whereas in the coming years, we will probably see a shift towards diabetes as a cause of PAD. This population is relatively underrepresented in our cohort. It has also been suggested that ethnicity influences CD14's association with adverse outcomes, and thus our results and conclusions cannot be expanded to other races since patients enrolled in the AE are predominantly Caucasian. Although the use of anticoagulants/antiplatelets and lipid-lowering medication is high, the best medical treatment is not identical in these patients, as inclusion was performed between 2002 and 2016. Furthermore, in more recent years, the surgical indication has shifted to more severe stages, and consequently, fewer patients with Fontaine II are operated on. Linear regression shows that a potential time effect is very minimal and unambiguous or non-significant, and thus we believe that it is unlikely that patient collection and plasma storage would influence our results. Medication changes during follow-up were not recorded, so our baseline characteristics only provide medication use during the preoperative or direct postoperative period.

Although the power for MALE is adequate, the event rate of MACE is low and could lead to uncertainty. We have also not performed sex-adjusted analyses due to the low incidence of MACE, especially since 72% of this cohort is male. With regard to the improvement of existing models, we have not considered using net reclassification improvement (NRI) since this method is prone to overestimation in poorly fitted risk models.[34] Comparing the AUC of models has its limitations, as it is somewhat optimistic when case controls are imbalanced, it doesn't take the goodness-of-fit of the model into account, and it summarises the test performance across the whole reporter operating characteristic space, even when these are hardly applicable.[35]

We only analysed the HDL subfraction for EV analysis since this HDL subfraction contained the most differentially expressed proteins for MACE in carotid endarterectomy patients.[16]

Our results are the first critical step in risk prediction research regarding the use of EVs in PAD. As said, our results are not generalisable yet and need further validation in a larger cohort of patients with (severe) PAD.

In conclusion, we showed that increased levels of CD14 in the EV-HDL subfraction are associated with MACE in patients with severe PAD. Elevated levels of EV-HDL Serpin G1 are independently associated with both MACE and MALE following femoral endarterectomy.

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Supplemental Material

Table S1: Linear regression of protein levels influenced by sampling-analysis interval (in years)

	Time from blood sampling to analysis		
	HR	(95% CI)	P
logCysC	-0.01497	(-0.02875 – -0.00119)	.033
logCD14	0.00022	(-0.00592 – 0.00637)	.943
logSC1	0.01231	(0.00378 – 0.02083)	.005
logSG1	0.01589	(0.00725 – 0.02452)	< .001

Table S2: Univariable cox proportional hazard regression for all baseline characteristics

	MACE		MALE	
	HR (95% CI for HR)	P	HR (95% CI for HR)	P
Age	1.03 (0.99-1.1)	.11	0.99 (0.97-1)	.15
Male	0.62 (0.33-1.2)	.15	0.9 (0.61-1.3)	.61
BMI	1.03 (0.96-1.1)	.45	1.03 (0.99-1.1)	.15
Smoking	1.37 (0.73-2.6)	.33	1 (0.7-1.4)	.99
Fontaine III	2.24 (1.06- 4.74)	.034	1.38 (0.92 – 2.07)	.11
Fontaine IV	2.09 (0.82 – 4.73)	.078	1.19 (0.76 – 1.97)	.44
History of				
CAD	0.93 (0.49-1.8)	.82	1.16 (0.81-1.6)	.41
Stroke	2.29 (1.2-4.4)	.012	1.21 (0.8-1.8)	.36
Hypertension	2.77 (0.67-12)	.16	2.33 (1.2-4.6)	.015
Diabetes Mellitus	1.13 (0.57-2.2)	.73	0.87 (0.58-1.3)	.48
Use of				
Insulin	0.88 (0.27-2.9)	0.83	.77 (0.39-1.5)	.45
Glucose inhibitors	0.99 (0.47-2.1)	.98	0.83 (0.54-1.3)	.38
Anticoagulants	1.08 (0.45-2.6)	.86	1.03 (0.63-1.7)	.92
Antiplatelets	0.57 (0.26-1.3)	.16	1.21 (0.7-2.1)	.49
Lipid lowering drugs	1.09 (0.52-2.3)	.82	1.31 (0.85-2)	.22
Lab results				
eGFR	1 (0.99-1)	1.0	1 (0.99-1)	.16
LDL	1.13 (0.81-1.6)	0.48	1.04 (0.86-1.3)	.70
HDL	0.8 (0.34-1.9)	0.61	.85 (0.53-1.4)	.49
Triglycerides	0.92 (0.71-1.2)	.50	1.03 (0.92-1.1)	.64
Total cholesterol	1.05 (0.81-1.4)	.72	1.01 (0.88-1.2)	.88
EVs				
logCysC	1.21 (0.88-1.7)	.24	.83 (0.7-1)	.053
logCD14	1.45 (1.1-1.9)	.011	.89 (0.75-1.1)	.19
logSC1	0.98 (0.71-1.3)	.89	.88 (0.73-1.1)	.16
logSG1	1.44 (1-2)	.030	1.38 (1.1-1.7)	.0011

Table S3: A multivariable statistical model for MACE and MALE

	MACE		MACE		MALE		MALE	
Coefficient	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
Age	1.02 (0.98 – 1.06)	.41	1.03 (0.99 – 1.07)	.162	0.98 (0.96 – 1.00)	.074	0.98 (0.96 – 1.00)	.10
Male	0.75 (0.39 – 1.43)	.38	0.76 (0.39 – 1.46)	.40				
Stroke	2.18 (1.13 – 4.21)	.004	2.22 (1.15 – 4.28)	.018				
Hypertension	2.90 (0.69 – 12.36)	.15	2.39 (0.56 – 10.19)	.24	2.62 (1.30 – 5.29)	.007	2.35 (1.16 – 4.76)	.017
Fontaine III	1.83 (0.82 – 4.12)	.14	1.91 (0.86 – 4.27)	.113				
Fontaine IV	2.15 (0.97 – 4.78)	.061	2.43 (1.09 – 5.45)	.031				
BMI					1.02 (0.98 – 1.06)	.35	1.02 (0.98 – 1.06)	.35
Antiplatelet	0.72 (0.33 – 1.61)	.42	0.67 (0.30 – 1.48)	.33				
logCD14	1.4 (1.05 – 1.90)	.19						
logCysC					0.86 (0.71 – 1.03)	.105		
logSG1			1.49 (1.08 – 2.06)	.016			1.29 (1.07 – 1.56)	.009

For both MACE and MALE, variables were added to these models when they were statistically significant in a univariable model. We added the statistically significant biomarkers (CysC, CD14 and SG1) separately to these models in order to objectively view their own efficacy.

Table S4: A multivariable literature model for MACE, with the addition of CD14 and SG1

		Miao et al.			**Miao et al.**			**Berger et al.**			**Berger et al.**	
Variable	HR	(95% CI)	P	HR	(95% CI)	P	HR	(95% CI)	P	HR	(95% CI)	P
logCD14	1.46	1.09 – 1.96	.012				1.38	1.03 – 1.86	.033			
logSG1				1.40	1.01 – 1.93	.044				1.43	1.03 – 1.98	.033
Age							1.02	0.97 – 1.06	.424	1.03	0.99 – 1.08	.154
Hypertension	2.78	0.65 – 11.80	.166	2.59	0.60 – 11.09	.201						
CAD	0.96	0.49 – 1.86	.899	0.83	0.43 – 1.61	.583	1.03	0.54 – 1.98	.927	0.91	0.47 – 1.75	.780
Stroke or TIA	2.27	1.18 – 4.36	.014	2.26	1.18 – 4.31	.014	2.37	1.23 – 4.59	.010	2.17	1.12 – 4.21	.021
Smoker	1.23	0.64 – 2.34	.536	1.22	0.64 – 2.34	.545	1.39	0.71 – 2.72	.33	1.44	0.73 – 2.84	.287
Diabetes Mellitus	1.16	0.54 – 2.49	.695	1.18	0.55 – 2.52	.669	1.07	0.53 – 2.15	.849	1.14	0.57 – 2.28	.712
Insulin	0.75	0.20 – 2.80	.668	0.77	0.20 – 2.86	.691						
CKD							1.41	0.67 – 2.99	.366	1.41	0.68 – 2.93	.361
BMI							1.05	0.98 – 1.13	.202	1.04	0.97 – 1.12	.283

We derived two risk models from literature that calculated an association of risk factors with MACE in people with PAD. These were:

[1] Miao B, Hernandez AV, Alberts MJ, Mangiafico N, Roman YM, Coleman CI. Incidence and Predictors of Major Adverse Cardiovascular Events in Patients With Established Atherosclerotic Disease or Multiple Risk Factors. J Am Heart Assoc. 2020;9(2):e014402. doi:10.1161/JAHA.119.014402

[2] Berger A, Simpson A, Bhagnani T, et al. Incidence and Cost of Major Adverse Cardiovascular Events and Major Adverse Limb Events in Patients With Chronic Coronary Artery Disease or Peripheral Artery Disease. Am J Cardiol. 2019;123(12):1893-1899. doi:10.1016/j.amjcard.2019.03.022

Table S5: A multivariable literature model for MALE, with the addition of CysC

	Biscetti et al.			Zhang et al.			Meltzer et al.		
Coefficient	HR	(95% CI)	P	HR	(95% CI)	P	HR	(95% CI)	P
logCysC	0.84	0.70 – 1.02	.073	0.85	0.70 – 1.03	.10	0.79	0.65 – 0.96	.018
Age	0.83	0.68 – 1.01	.068	0.85	0.69 – 1.03	.10			
Sex				0.97	0.64 – 1.47	.90	1.07	0.71 – 1.63	.74
CAD	0.99	0.68 – 1.44	.97				1.11	0.77 – 1.62	.58
Stroke / TIA	1.30	0.85 – 2.00	.23						
DM	0.83	0.55 – 1.23	.35	0.79	0.52 – 1.18	.24	0.92	0.62 – 1.39	.71
Hypertension	2.91	1.41 – 5.97	.004	2.77	1.37 – 5.62	.005			
Smoker	1.13	0.77 – 1.65	.53	1.19	0.81 – 1.76	.37	1.13	0.78 – 1.64	.53
CKD							1.47	0.95 – 2.26	.085
Fontaine III							1.51	1.00 – 2.28	.050
Fontaine IV							0.94	0.58 – 1.53	.81
BMI				1.12	0.95 – 1.31	.18			

We derived three risk models from literature that calculated an association of risk factors with MALE in people with PAD. These were:

[1] Biscetti F, Nardella E, Rando MM, et al. Outcomes of Lower Extremity Endovascular Revascularization: Potential Predictors and Prevention Strategies. Int J Mol Sci. 2021;22(4):2002. Published 2021 Feb 18. doi:10.3390/ijms22042002

[2] Zhang Y, Huang J, Wang P. A Prediction Model for the Peripheral Arterial Disease Using NHANES Data. Medicine (Baltimore). 2016;95(16):e3454. doi:10.1097/MD.0000000000003454

[3] Meltzer AJ, Evangelisti G, Graham AR, et al. Determinants of outcome after endovascular therapy for critical limb ischemia with tissue loss. Ann Vasc Surg. 2014;28(1):144-151. doi:10.1016/j.avsg.2013.01.018

Table S6: A multivariable literature model for MALE, with the addition of SG1

	Biscetti et al.			Zhang et al.			Meltzer et al.		
Coefficient	HR	(95% CI)	P	HR	(95% CI)	P	HR	(95% CI)	P
logSG1	1.30	1.07 – 1.57	.008	1.28	1.06 – 1.56	.011	1.40	1.15 – 1.70	.001
Age	0.84	0.69 – 1.01	.059	0.85	0.70 – 1.02	.079			
Sex				0.92	0.62 – 1.36	.66	0.97	0.65 – 1.45	.90
CAD	0.97	0.68 – 1.40	.88				1.06	0.74 – 1.53	.73
Stroke / TIA	1.27	0.84 – 1.92	.26						
DM	0.80	0.54 – 1.19	.27	0.77	0.51 – 1.16	.21	0.88	0.59 – 1.32	.55
Hypertension	2.54	1.24 – 5.22	.011	2.44	1.21 – 4.96	.013			
Smoker	0.91	0.63 – 1.33	.64	0.96	0.66 – 1.40	.84	0.90	0.62 – 1.31	.59
CKD							1.41	0.94 – 2.13	.099
Fontaine III							1.51	1.01 – 2.26	.046
Fontaine IV							1.01	0.63 – 1.63	.96
BMI				1.10	0.94 – 1.29	.24			

See footnote Table S5.

Table S7: Risk factors for MACE and MALE according to stepwise regression

	Stepwise Model for MACE			Stepwise Model for MALE		
Coefficient	HR	(95% CI)	P	HR	(95% CI)	P
logSG1	1.57	1.10 – 2.23	.012	1.31	1.07 – 1.60	.009
logSC1	0.63	0.39 – 1.02	.061			
logCD14	2.40	1.53 – 3.77	<.001			
Age				0.97	0.95 – 1.00	.023
Stroke or TIA	2.30	1.19 – 4.47	.014			
Hypertension				2.28	1.13 – 4.62	.022
Fontaine III				1.56	1.04 – 2.35	.033
Fontaine IV				1.01	0.62 – 1.65	.955
eGFR				0.99	0.98 – 1.00	.040

Analysis of risk factors for MACE and MALE according to automatic stepwise regression.

Figure S1: Kaplan-Meier Curve for MACE and MALE

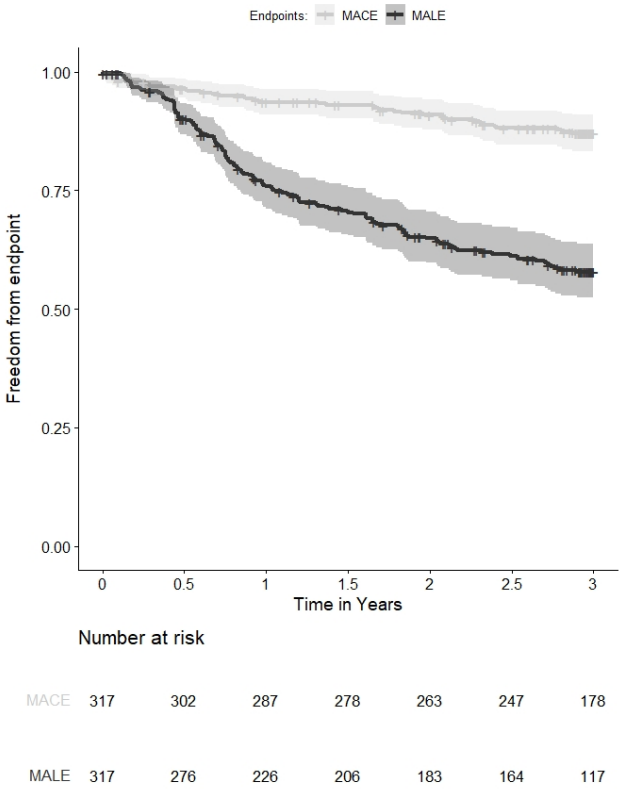

Table S8: Plaque characteristics associated with EV proteins

		logCysC			**logCD14**			**logSC1**			**logSG1**	
Predictors	OR	(95% CI)	P	OR	(95% CI)	P	OR	(95% CI)	P	OR	(95% CI)	P
Lipid >40%	3.04	0.85 – 11.6	.094	2.02	0.15 – 24.2	.59	1.52	0.22 – 9.98	.67	0.27	0.05 – 1.55	.13
Lipid >10%	2.42	1.22 – 4.94	.013	4.05	1.00 – 16.	.050	1.73	0.61 – 4.97	.30	0.60	0.22 – 1.59	.30
IPH	1.69	0.92 – 3.18	.095	2.15	0.58 – 8.20	.26	1.86	0.67 – 5.25	.24	0.49	0.18 – 1.30	.16
SMC	0.99	0.50 – 1.95	.97	1.32	0.30 – 6.02	.71	1.02	0.33 – 3.28	.97	1.98	0.67 – 5.94	.22
Calcification	1.29	0.71 – 2.36	.41	1.95	0.54 – 7.22	.31	1.78	0.68 – 4.75	.24	1.04	0.42 – 2.57	.93
Collagen	0.46	0.20 – 1.01	.058	0.31	0.06 – 1.63	.16	0.47	0.13 – 1.70	.25	1.37	0.40 – 4.65	.61
Macrophage	0.85	0.39 – 1.86	.68	1.00	0.18 – 5.29	.99	0.19	0.04 – 0.80	.029	2.51	0.71 – 9.32	.160

In this table, logistic regression results are given for the major histological plaque characteristics that were scored with the four tested extracellular vesicle proteins.

7

High lipoprotein(a) is associated with major adverse limb events after femoral artery endarterectomy

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Abstract

Backgrounds and aims: *Elevated lipoprotein(a) (Lp[a]) has been identified as a causal risk factor for cardiovascular disease, including peripheral arterial disease (PAD). Although Lp(a) is associated with the diagnosis of PAD, it remains elusive whether Lp(a) is associated with cardiovascular and limb events in patients with severe PAD.*

Methods: *Preoperative plasma Lp(a) levels were measured in 384 consecutive patients that underwent iliofemoral endarterectomy and were included in the Athero-Express biobank. Our primary objective was to assess the association of Lp(a) levels with Major Adverse Limb Events (MALE). Our secondary objective was to relate Lp(a) levels to Major Adverse Cardiovascular Events (MACE) and femoral plaque composition acquired from baseline surgery.*

Results: *During a median follow-up time of 5.6 years, 225 MALE were recorded in 132 patients. Multivariable analysis, including the history of peripheral intervention, age, diabetes mellitus, end-stage renal disease and PAD disease stages, showed that Lp(a) was independently associated with first (HR of 1.36 (95% CI 1.02–1.82) p = .036) and recurrent MALE (HR 1.36 (95% CI 1.10–1.67) p = .004). A total of 99 MACE were recorded, but Lp(a) levels were not associated with MACE. sLp(a) levels were significantly associated with a higher presence of smooth muscle cells in the femoral plaque, although this was not associated with MALE or MACE.*

Conclusions: *Plasma Lp(a) is independently associated with first and consecutive MALE after iliofemoral endarterectomy. Hence, in patients undergoing iliofemoral endarterectomy, Lp(a) could be considered a biomarker to enhance risk stratification for future MALE.*

Introduction

Patients with peripheral artery disease (PAD) are treated with lifestyle management and an appropriate medication regimen, such as antithrombotic or anticoagulant drugs combined with lipid-lowering therapies, to reduce the risk of future cardiovascular events (CVE). In addition, patients with severe symptoms often require vascular intervention to restore adequate perfusion. Although this will relieve most symptoms in the short term, the chronic nature of atherosclerosis will persist, and the risk of future CVE remains extremely high.[1] Major Adverse Limb Events (MALE), a combination of lower limb amputation and peripheral vascular intervention, and Major Adverse Cardiovascular Events (MACE), a composite of non-fatal stroke/myocardial infarction and cardiovascular death, are two important categories of CVE that reflect more localised and systemic disease progression and are used as objective performance goals after revascularisation.[2]

Up to 42% and 13% of PAD patients will have a MALE and MACE within three years following a peripheral intervention, respectively, and consequently, improvement of tertiary prevention is warranted to reduce this residual risk.[3, 4] Patients at high risk for CVE may benefit from novel therapies such as dual antiplatelet therapy, the addition of direct oral anticoagulants, PCSK9 inhibition, or colchicine therapy.[5–7] Moreover, insight into the individual risk of MALE could guide the preferred mode of intervention or may substantiate treatment decisions when the efficacy of limb salvage is disputed. Unfortunately, early identification of these high-risk PAD patients is still lacking. Prediction models that incorporate clinical risk factors have so far been inconclusive with regard to individual risk and are consequently not widely used in PAD.[8, 9] In order to determine which PAD patients are at elevated risk, biomarkers associated with future CVE are needed.

Lipoprotein(a) is a polymorphic lipoprotein with much resemblance to low-density lipoprotein (LDL), with apolipoprotein(a) [apo(a)] covalently linked to ApoB100. From a biological and physiological point of view, Lp(a) exhibits several features that could render it a reliable biomarker. Independent of external factors like age, sex and fasting state, Lp(a) plasma levels are primarily genetically determined, which implies that plasma concentrations are fairly stable throughout life.[10] Lp(a) accumulates in the subendothelial space and interferes with fibrinolytic cascades, promotes expression of proinflammatory cytokines, enhances endothelial cell permeability, increases their proinflammatory phenotype and stimulates both smooth muscle cell migration and monocyte recruitment, all pivotal processes in atherosclerosis progression.[10–13]

In carotid and coronary artery disease (CAD), Lp(a) has been shown to be a reliable marker for cardiovascular disease (CVD) progression.[11, 14, 15] With regards to the lower limbs, Lp(a) has primarily been investigated as a diagnostic marker for PAD.[16] Other studies revealed that higher levels of Lp(a) are associated with higher PAD classifications, limb amputation, loss of patency and ankle-brachial-index (ABI) values.[16–20] The association of Lp(a) and MALE has not been investigated in surgical patients with severe PAD. Based on the association of Lp(a) in other cardiovascular areas and its involvement in processes contributing to progressive atherosclerosis, we hypothesize that high plasma levels of Lp(a) are associated with future MALE or MACE. This could improve the identification of patients at increased risk for secondary CVE and enhance treatment strategies for these vulnerable patients.

In this study, we investigated the association of plasma Lp(a) levels with the risk of (recurrent) MALE and MACE in a cohort of patients undergoing iliofemoral endarterectomy.

Methods

Study population

The Athero-Express (AE) (www.atheroexpress.nl) is an ongoing prospective biobank study (2002 – present) in which consecutive patients scheduled for carotid endarterectomy (CEA) or thromboendarterectomy (TEA) in two referral hospitals in the Netherlands (the St. Antonius Hospital Nieuwegein

and the University Medical Center Utrecht) are included. The detailed protocol has been published before.[21] In short, preoperative blood and perioperative atherosclerotic plaque samples are collected from all patients undergoing CEA or iliofemoral endarterectomy. All patients were medically treated according to the latest guidelines, either in collaboration with the general practitioner or specialists from (vascular) internal medicine.[22] Baseline patient characteristics were acquired by standardised preoperative questionnaires and by examination of medical records. For the first three consecutive years after the intervention, all patients received a questionnaire annually to collect follow-up data with regard to cardiovascular events and cardiovascular-related hospital admissions. These endpoints are verified by a medical professional with relevant correspondence from either the general practitioner or (referring) hospital. For this study, all patients that underwent iliofemoral endarterectomy, with available lipid profile measurements, were included. Follow-up was extended by examination of medical records, and information about new peripheral procedures was recorded in more detail (side, target vessel, type of peripheral intervention). The medical ethics committee of both hospitals approved the study, and all participants gave informed consent in writing. The study was carried out in accordance with the Helsinki Declaration.

Laboratory measurements

Preoperative blood samples were collected during hospital admission, processed and stored at minus 80 °C until use. Lp(a) was measured in nanomole (nmol) per litre (L) by a particle-enhanced immunoturbidimetric assay (the Cobas c702 (Roche) and the LPA2 Tina-quant Lp(a) Gen.2 kit from Cobas (LPA2: CAN 8723)) in which Lp(a) agglutinates with latex particles coated with anti-Lp(a) antibodies. The precipitate is determined turbidimetrically at 800/660 nm. The measuring range of this assay was between 7 and 240 nmol/L. Standard lipid profile measurements (cholesterol, triglycerides and HDL) were performed, and LDL-c was calculated using the Dahlen formula.

Atherosclerotic plaque assessment

For histological assessment of the atherosclerotic plaque, a standardised protocol was used that has previously been described in detail.[23] In short, plaques were stained with alpha-actin for smooth muscle cells (SMC), CD68 for macrophages, CD34 for microvessels, picrosirius red for collagen and lipid content, and hematoxylin-eosin and fibrin for intraplaque haemorrhage (IPH). Two experienced independent observers semi-quantitatively scored the stainings as no/minor (0) and moderate/heavy. Lipid content was estimated as a percentage of total plaque area and stratified into higher and lower than 10% and 40%. Intraplaque haemorrhage was rated as absent or present. Intraobserver and interobserver variability showed good reproducibility in a study performed previously (κ, 0.6–0.9).[24] Finally, SMC and macrophage content were quantitatively scored using the computerised analysis software AnalySIS 3.2 (Soft Imaging Systems GmbH, Münster, Germany). The content of SMCs and macrophages was expressed as the average percentage of positive staining of the plaque area from three representative areas of interest in the plaque, selected by an experienced technician at 40x magnification.

Outcomes

Our primary outcome of interest was MALE. MALE was defined as a composite of (new) infrainguinal (endo)vascular interventions that were performed due to a loss of patency or novel stenosis/occlusion in other ipsilateral segments. These included: percutaneous transluminal angiography (PTA), stent, drug-coated balloon (DCB), drug-coated stent (DCS), mechanical thrombectomy, atherectomy, thrombolytic (urokinase or alteplase) treatment, bypass surgery and major (above-the-ankle) amputations. Short-term reinterventions due to hemorrhagic bleeding of the patch, bypass, or endovascular puncture site were excluded, as well as surgical site infections that required surgery. Diagnostic angiography with the intent of endovascular treatment and failed endovascular procedures were defined as periph-

Table 1: Baseline characteristics, overall and stratified by dichotomous Lp(a)

	Overall 384	Below median Lp(a) 192	Above median Lp(a) 192	P
Age – years	68.6 (8.9)	68.7 (9.1)	68.6 (8.7)	.89
Gender – male	281 (73.2)	138 (71.9)	143 (74.5)	.65
BMI – kg/m²	26.1 (4.0)	25.8 (4.1)	26.3 (3.9)	.21
Smoking	147 (38.9)	75 (39.3)	72 (38.5)	.96
Fontaine stage				.44
II	224 (58.3)	114 (59.4)	110 (57.3)	
III	99 (25.8)	52 (27.1)	47 (24.5)	
IV	61 (15.9)	26 (13.5)	35 (18.2)	
ABI	0.58 (0.2)	0.57 (0.2)	0.58 (0.2)	.75
History of				
Peripheral intervention	159 (41.4)	75 (39.1)	84 (43.8)	.41
Coronay artery disease	165 (43.1)	75 (39.3)	90 (46.9)	.16
Stroke	23 (6.6)	15 (8.5)	8 (4.6)	.20
Hypertension	270 (72.8)	131 (70.1)	139 (75.5)	.28
Diabetes mellitus	103 (26.8)	55 (28.6)	48 (25.0)	.49
Medication				
Insulin	32 (8.4)	15 (7.9)	17 (8.9)	.85
Glucose inhibitors	78 (20.4)	46 (24.1)	32 (16.7)	.094
Anticoagulants	55 (14.4)	27 (14.1)	28 (14.6)	1.00
Antiplatelets	325 (85.1)	162 (84.8)	163 (85.3)	1.00
Lipid lowering drugs	283 (73.9)	140 (73.3)	143 (74.5)	.88
Statins	281 (73.4)	139 (72.8)	142 (74.0)	.88
Laboratory results				
eGFR - ml/min/1,73 m²	80.6 (26.7)	81.3 (25.8)	80.0 (27.6)	.59
Triglycerides - mmol/L	1.8 [1.3, 2.4]	1.9 [1.4, 2.5]	1.7 [1.2, 2.4]	.037
Lp(a) - nmol/L	25.9 [7.9, 128.3]	8.0 [7.0, 13.9]	128.4 [49.8, 201.5]	<.001
LDL - mmol/L	2.4 (0.9)	2.3 (0.9)	2.5 (0.8)	.13
LDL corrected - nmol/L	1.1 (0.4)	1.1 (0.4)	1.1 (0.3)	.65
HDL - mmol/L	4.4 (1.2)	4.3 (1.2)	4.5 (1.2)	.23

Data are presented as n (%), median [interquartile range] or mean ± standard deviation. MALE = Major adverse Limb Events; BMI = Body Mass Index; ABI = Ankle-Brachial Index; eGFR = Estimated Glomerular Filtration Rate; Lp(a) = Lipoprotein(a); LDL = Low-density lipoprotein; HDL = High-density lipoprotein.

eral intervention, whereas a fully diagnostic angiography without the intent to treat was not. Objective loss of patency without subsequent intervention was not scored or included in the composite definition. The secondary endpoint of interest was MACE, a composite of non-fatal stroke, myocardial infarction, and death from all cardiovascular causes. Sudden death was categorised as cardiovascular death if no other explicit factors were found.

Statistical analyses

Quantitative data were expressed as mean (±standard deviation (SD)) or as median (interquartile range, (IQR)) as appropriate to their distribution and were compared with a Student t-test and a Mann-Whitney U test, respectively. Discrete data were presented as frequencies and percentages and were compared using the $\chi 2$-test or Fisher exact test. A comparison of baseline characteristics was performed for two groups stratified by the outcome.

Lipoprotein(a) levels were transformed logarithmically for normalization and dichotomised (based

on median values) for discrete analysis. Freedom from our primary endpoints was estimated using Kaplan-Meier survival analysis on dichotomised Lp(a) and included log-rank testing to calculate a statistical difference. Cox Proportional Hazard (PH) regression was used to calculate the hazard ratio (HR) with a 95% confidence interval (CI) for the association between quantitative Lp(a) and the primary outcome during follow-up. Lp(a) was added to risk factors of several clinical models that were derived from available literature to eliminate potential confounding and give an overview of the potential incremental value of Lp(a) in addition to these models. Missing data were imputed by predictive mean matching or were discarded when these exceeded 25%. By assessing the Schoenfeld residuals, the PH assumption was tested. When a time-dependent variable is present, a deterministic function of time will be included in the model for this variable.

For recurrent event analysis, three extensions of the Cox PH model were used. The Andersen-Gill (AG) and two variants of the Prentice-Williams-Peterson (PWP) models, namely the total-time (TT) model and gap-time (GP) model.[25]

Akaike Information Criteria (AIC) were used to assess the goodness-of-fit and whether a risk factor should be used in a model. Stepwise Cox PH regression analysis was performed to see whether Lp(a) would be implemented in an automatically generated model free from potential investigator bias. Univariable logistic regression was used to find whether Lp(a) levels were associated with these plaque characteristics. All P values were 2-tailed, with a $P < .05$ considered statistically significant. Statistical analyses were performed with R version 4.0.4 inside an R Studio 1.4.1103 environment.

Results

Baseline characteristics

Association of lp(a) and male

A total of 384 unique patients that underwent iliofemoral endarterectomy were included from the Athero-Express biobank. General baseline characteristics show that patients were predominantly male (73%), with a mean age of 69 (±SD 8.9) years and were slightly overweight (BMI 26 (±SD 4)) (Table 1). Intermittent claudication (IC), rest pain and ischemic wounds were indications for surgery in descending frequencies (58%, 26% and 16%, respectively). About 41% of the patients had a history of an infrainguinal peripheral intervention before baseline surgery, and 43% were previously diagnosed with CAD. The prevalence of diabetes mellitus (DM) was 27%. The median follow-up time in 384 patients was 5.6 years (IQR, 3.45–6.78); 146 patients died (all-cause mortality) during follow-up.

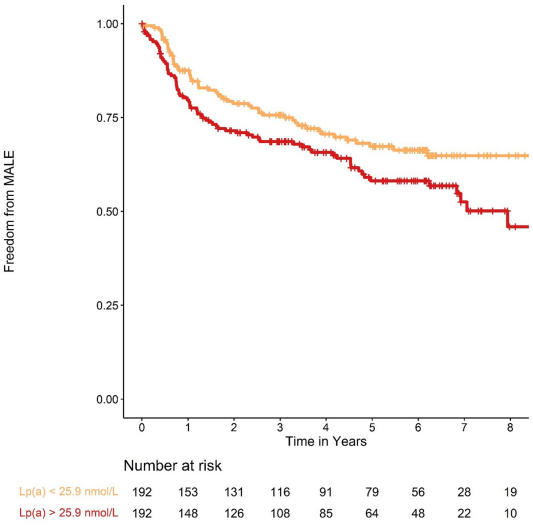

Figure 1: Freedom from major adverse limb events (MALE) in patients who underwent iliofemoral endarterectomy in relation to serum lipoprotein(a), below or above the median. Censoring includes all-cause death and loss to follow-up.

Baseline characteristics stratified by lp(a)

Lipoprotein(a) levels ranged from 7 to 566 nmol/L with a median of 25.9 nmol/L (IQR 8.0, 128.3). For a comparison of Lp(a) and baseline characteristics, dichotomization of Lp(a) was performed based on the median (Table 1). Patients with higher levels of Lp(a) were more likely to have a statistically significant lower triglyceride level (1.7 mmol/L [IQR 1.2, 2.4] versus 1.9 mmol/L [IQR 1.4, 2.5]); p =

Table 2: Baseline characteristics stratified by MALE.

	No MALE 252	MALE 132	P
Age – y	69.8 (9.0)	66.6 (8.4)	.001
Gender – male	190 (75.4)	91 (68.9)	.21
BMI – kg/m ²	25.9 (4.0)	26.4 (4.0)	.23
Smoking	92 (37.1)	55 (42.3)	.38
Fontaine stage			.31
II	154 (61.1)	70 (53.0)	
III	60 (23.8)	39 (29.5)	
IV	38 (15.1)	23 (17.4)	
ABI	0.56 (0.20)	0.61 (0.21)	.064
History of			
Peripheral intervention	98 (38.9)	61 (46.2)	.20
Coronary artery disease	105 (41.8)	60 (45.5)	.57
Stroke	19 (8.3)	4 (3.3)	.11
Hypertension	181 (74.2)	89 (70.1)	.47
Diabetes mellitus	64 (25.4)	39 (29.5)	.45
Medication			
Insulin	21 (8.4)	11 (8.3)	1.00
Glucose inhibitors	49 (19.5)	29 (22.0)	.67
Anticoagulants	33 (13.1)	22 (16.7)	.44
Antiplatelets	212 (84.5)	113 (86.3)	.75
Lipid lowering drugs	182 (72.5)	101 (76.5)	.47
Statins	182 (72.5)	99 (75.0)	.69
Laboratory results			
eGFR - ml/min/1,73 m²	80.571 (26.013)	80.8 (27.9)	.93
Triglycerides - mmol/L	1.750 [1.290, 2.415]	1.8 [1.2, 2.5]	.96
Lp(a) - nmol/L	19.4 [7.0, 97.4]	37.2 [10.3, 155.1]	.017
LDL - mmol/L	2.4 (0.92)	2.4 (0.83)	.76
LDL - corrected, nmol/L	2.21 (0.92)	2.12 (0.87)	.34
HDL - mmol/L	1.1 (0.36)	1.1 (0.37)	.67
Cholesterol - mmol/L	4.4 (1.3)	4.3 (1.1)	.57

Data are presented as n (%), median [interquartile range] or mean ± standard deviation. MALE = Major adverse Limb Events; BMI = Body Mass Index; ABI = ankle brachial Index; eGFR = Estimated Glomerular Filtration Rate; Lp(a) = Lipoprotein(a); LDL = Low-density lipoprotein; HDL = High-density lipoprotein.

0.037. LDL-C was not significantly different (1.1 mmol/L (±SD 0.4) versus 1.1 mmol/L (±SD 0.3)); p = 0.65. No other significant differences in baseline characteristics were found when stratified for high and low Lp(a) plasma levels.

A total of 132 patients had a first MALE with a median time of 381 days (IQR, days, 204–928). These MALE consisted of amputations above (5) and amputations below the knee (5), bypass surgeries (28), redo-ilio-femoral endarterectomies (12), thrombolyses (8) or endovascular interventions (73). Only seven patients recorded a first MALE after five years from inclusion. Baseline characteristics, when stratified for MALE, are listed in Table 2. In short, patients with MALE were younger, more likely to have a history of peripheral intervention(s) and had a significantly higher plasma Lp(a): 19.4

nmol/L (IQR 7.0, 97.4) versus 37.2 nmol/L (IQR 10.3, 115); p = 0.017. History of CAD was equally prevalent in the group with MALE and in the group without MALE, at 41.8% and 45.5%, respectively (p = 0.568).

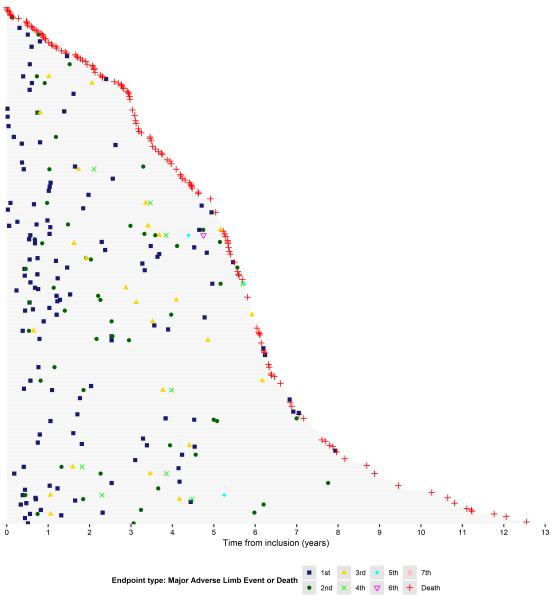

Figure 2: A plot of recurrent major adverse limb events and terminal events. On the y-axis, all participants are shown in ascending order for total follow-up length. For each participant, a horizontal grey line corresponds to the length of follow-up. Consecutive MALEs are shown on this line, with a gradient from yellow to red indicating the first till seventh MALE during follow-up. A red solid triangle indicates death.

A Kaplan-Meier curve for the first MALE stratified for low and high Lp(a) based on the median demonstrated that the majority of the first MALE took place within the first year after iliofemoral endarterectomy (Figure. 1). Analysis of Lp(a) quartile levels show that the lowest and the highest quartiles offer the greatest difference in hazard (Supplemental Figure 1). Logrank test (p = 0.039) indicated that there is a statistical difference between the high and low Lp(a) groups. The association of Lp(a) and MALE was further investigated in multiple univariable analyses, where quantitative Lp(a) was found to be associated with MALE with an HR of 1.37 (95% CI, 1.0–1.8); p = 0.030 (Supplementary Table 1). Age (HR 0.98 (0.96–1); p = 0.040) and Fontaine stages were also associated with MALE. Of note, Fontaine stages were dependent on time and thus violated the PH assumption: meaning that the HR of Fontaine classification for MALE is declining over time, turning below 1 at 1.5 years after surgery.

In a multivariable model, which includes the risk factors history of peripheral interventions, age, Fontaine stages corrected by time, diabetes, and end-stage renal disease (ESRD), Lp(a) was associated with MALE with an HR of 1.36 (95% CI 1.02–1.82); p = 0.036 (Table 3). Furthermore, Lp(a) remained associated with MALE when risk factors of other existing PAD-related risk models were used (Supplementary Table 2).[9, 26, 27]. Likewise, automatic stepwise regression analysis selected Lp(a) as an independent factor in its multivariable model.

Association of lp(a) and recurrent male

Having established that plasma Lp(a) levels were associated with the first MALE after iliofemoral endarterectomy, we investigated whether Lp(a) was also associated with recurrent MALE. A total of 225 MALE were recorded in 132 patients with successive frequencies for 2nd -7th MALE: 54, 26, 9, 3, 1 and 1. (Figure 2). We adopted the same risk factors as with the first MALE in a multivariable multiple event models, showing that Lp(a) was also significantly associated with recurrent MALE with an HR of 1.36 (95% CI 1.10–1.67) p = 0.004 (Table 3). Other multivariable models offered a similar conclusion with corresponding HR of 1.30 (95% CI 1.06–1.61) p = 0.014 and 1.45 (95% CI 1.12–1.87) p = 0.005

(Supplementary Table 3). Stepwise regression analysis included Lp(a) alongside age, Fontaine stage, smoking status, history of CAD and eGFR (Supplementary Table 4).

Association of Lp(a) and mace

A total of 99 patients had a MACE, with a median time of 1156 days (IQR 490–1985). Stratified for MACE, baseline characteristics were not significantly different (Supplementary Table 6). Furthermore, no association of Lp(a) and MACE was found in regression analysis HR 0.88 (95% CI 0.63–1.23); p = 0.448.

Association of Lp(a) and plaque characteristics

Since Lp(a) is involved in processes of atherosclerotic plaque progression, we investigated the association of Lp(a) with plaque characteristics (Supplementary Table 5). Lipoprotein(a) levels were positively associated with moderate/heavy staining of SMC: OR 1.85 (1.14–3.07); p = 0.014. A trend toward significance was observed between the association of Lp(a) with IPH (OR 1.49 (95% CI 0.99–2.26); p = 0.06). Univariable and multivariable Cox PH regression analysis indicated that plaque composition was not associated with (first or recurrent) MALE and MACE.

Table 3: Exemplary multivariable Cox PH model for first and recurrent Major Adverse Limb Events.

	Exemplary model for first MALE			**Exemplary model for recurrent MALE**		
Variable	HR	95% IC)	P	HR	(95% IC)	P
Lp(a)	1.36	1.02–1.82	.036	1.36	1.10–1.67	.004
History of peripheral intervention	1.28	0.90–1.82	.178	1.17	0.89–1.55	.28
Age	0.98	0.96–1.00	.036	0.98	.97–0.99	.006
Diabetes mellitus	1.05	0.71–1.55	.812	0.96	0.70–1.33	.81
ESRD	14.80	3.30–66.31	.001	5.00	2.37–10.58	<.001
Fontaine III 1.5Y	2.19	1.33–3.63	.002	1.65	1.05–2.60	.029
Fontaine IV <1.5Y	1.89	1.02–3.53	.044	1.74	0.98–3.05	.056
Fontaine III >1.5Y	0.32	0.13–0.77	.011	0.53	0.28–1.00	.051
Fontaine IV >1.5Y	0.47	0.18–1.25	.131	0.65	0.26–1.59	.34

HR = Hazard Ratio; CI = Confidence Interval; MALE = Major Adverse Limb Events; Lp(a) = Lipoprotein(a); ESRD = End Stage Renal Disease). A multivariable Cox Proportional Hazard regression model to demonstrate risk factors and their relation to first and recurrent MALE by time. The Prentice-Williams-Peterson Total-Time regression model is used for this multivariable recurrent event analysis. A Fontaine was corrected for time (below and above 1.5 years).

Discussion

In this study, we showed that in 384 unique patients who underwent iliofemoral endarterectomy, elevated plasma Lp(a) levels were associated with an increased risk of first and recurrent MALE during a median follow-up of 5.6 years. The composite MALE has been frequently used as a relevant clinical endpoint in large clinical trials and is considered an objective performance goal as it provides a benchmark of symptoms in combination with failed patency or ongoing atherosclerotic disease in other arterial segments.[2, 5, 28] Since the incidence of MALE in patients with PAD is high, improvement of tertiary prevention would potentially benefit many patients, but increasing costs and elevated risk of adverse events (often attributed to this improved treatment) impede a roll-out of these add-on therapies for all PAD patients.[29] Additionally, enhanced knowledge about disease progression and need for (extensive) (endo-)vascular therapies could provide valuable information when limb salvage is questionable and objective substantiation is required before drastic measures such as amputation are undertaken. A risk model, which could include Lp(a), could aid in the allocation of preventive and therapeutic applications.

The Athero-Express patients included in this study were predominantly male, and the overall prevalence of CAD was high, which resembles other cohorts of Western European PAD patients.[30] Our results indicated that neither gender nor age, risk factors found to be related to Lp(a) levels in other research, were associated with Lp(a).[31, 32] The mortality rate observed in our study (38%) appears high but is consistent with rates described in comparable studies; all-cause mortality after hospitalisation for IC and Chronic Limb-Threatening Ischemia (CLTI) are 31.6% and 57.5%, respectively.[3]

With regards to our primary endpoints, the prospects of these patients are considered poor, as one-third of patients required a second intervention of the index limb within our follow-up time, and one in four patients experienced a MACE. This course of events is consistent with the findings of others, as 20% of CLTI patients experience a MALE in the first year after surgery, and this rate is about 35% at 5 years following open surgery of femoropopliteal lesions.[33, 34] According to another study, MACE occurred in 20% of patients, three years after intervention.[35]

Our analysis showed that Lp(a) is consistently and independently associated with MALE and recurrent MALE within a median follow-up of 5.6 years. As the HR of Lp(a) is consistent with both first MALE and recurrent MALE, we believe that our analysis of recurrent data strengthens the evidence for the relationship of Lp(a) with MALE. To the best of our knowledge, we present the first study to analyze the association of Lp(a) in iliofemoral endarterectomy patients with (recurrent) MALE, and therefore no direct comparison of our results and conclusions can be made.

In a retrospective study of 189 Japanese patients who underwent aortoiliac endovascular therapy, Lp(a) levels >40 mg/dL were associated with MALE.[36] However, differences in participants' race, treated vascular segment and mode of intervention prevent the extension of these conclusions to our patients. A prospective Spanish registry (FRENA) of stable out-clinic patients concluded that in their PAD subgroup of 528 patients, Lp(a) was associated with ischemic events, including lower limb amputation.[18] According to another study (41 limbs), p(a) levels >30 mg/dL were associated with restenosis at 6 months after infrainguinal PTA. However, the small study size and the perhaps short time frame are potential pitfalls that render the conclusions unsure.[37] In addition, a recent study investigating PAD patients concluded that Lp(a) levels >30 mg/dL were associated with the requirement for a peripheral artery operation, but a model with a cutoff point at 50 mg/dL was not.[35] However, most patients in this study were referred with an abdominal aortic aneurysm, and only a smaller proportion of patients were referred for lower limb PAD (CI and CLI). Unfortunately, no subgroup analysis based on these interventions was performed. Furthermore, their outcome (revascularisation of the lower extremities) did not include amputation. Although they briefly touched on the subject of recurrent outcomes, no further regression analysis regarding Lp(a) was performed with these data. The same study found no association of Lp(a) with MACE, which is in line with our results. However, since an association with Lp(a) and MACE has been found in major trials on other cardiovascular territories, our results might have been influenced by a smaller sample size and a smaller event rate for MACE.[38]

Lipoprotein(a) has been associated with arterial inflammation, thrombosis and progressive atherosclerosis, and thus we examined the association of Lp(a) with the composition of femoral atherosclerotic plaque.[13] Semi-quantitative analysis of 196 atherosclerotic femoral plaques demonstrated that moderate/heavy staining of SMC in the plaque was related to higher Lp(a) levels. This is in accordance with both human and animal studies, showing that Lp(a) is associated with the proliferation of (vascular) SMC in the atherosclerotic lesion.[39, 40] Since synthetic SMC present in atherosclerotic plaques contain a lower amount of alpha-smooth-muscle actin, the number of SMC might be underestimated. As this would proportionally be the case in all atherosclerotic plaques, this would probably not influence the association with Lp(a) levels. We found that the association of Lp(a) and IPH had a trend towards significance. The association of Lp(a) with IPH cannot be substantiated by studies on plaque histology, although radiological IPH presence has been associated with Lp(a) in carotid plaques.[41] For the relationship of Lp(a) and IPH, several mechanisms have been suggested, including impairment of

fibrinolysis due to the structural similarity of Lp(a) and plasminogen, the precursor of plasmin.[42] However, it remains unclear whether such interaction exists and whether this is relevant for lower limb PAD.[43] Although the increase of (semi-quantitative) SMC staining, as a substrate for progressive atherosclerosis, was associated with higher levels of Lp(a), the quantitative measurement was not, and both characteristics were not related to (recurrent) MALE and MACE according to our analyses.

Strengths and limitations

The Athero-Express is a highly regarded biobank that has produced a wealth of research. However, as a consequence of its broad inclusion period, preventive measures and therapeutic options have been improved over time and could potentially lead to a different prevalence of risk factors. Furthermore, the follow-up of early participants is potentially longer than that of more recent patients. However, we ensured that the minimum theoretical follow-up was five years and found no association between time of inclusion and Lp(a) levels and MALE.

The Athero-Express has a successful inclusion rate beyond 95%, limiting the chance of selection bias within both hospitals. Race is not formally registered, but our experience with these patients indicates that an overwhelming majority is of Caucasian descent. Since Lp(a) mass concentrations depend on race, we emphasize that our conclusions are only appropriate to patients of similar descent and should not be applied to other races without further investigation.[44–46] The Lp(a)-levels of some samples exceeded the 240 nmol/L, the upper level of the measuring range of the essay used. By dilution, we confirmed that these samples were indeed elevated beyond the calibration curve. Consequently, these corrected values were used in our analysis. On another note, the validity of the LDL-C values used in our analysis is open for debate, as these levels were calculated rather than measured.

With regards to our endpoint, some studies use the objective loss of patency, without correlation with symptoms, as a component of MALE. We believe that this constituent, without further consequences for treatment, is of lesser clinical relevance, although we understand that it could be considered pertinent in terms of disease progression. Due to the heterogeneity of standard clinical follow-up beyond one year after surgery, diagnostic tests for loss of patency are not performed in the same way in all patients, resulting in selection bias. Given these arguments, we opted to exclude the loss of patency from our definition of MALE.

In this study, no attempt has been made to establish a definitive cutoff point for Lp(a), although several levels have been proposed as such. [47, 48] Because we investigated a specific high-risk subgroup, such a cutoff point would offer little benefit to other populations and could potentially lead to an overestimation of the predictive efficacy of Lp(a). We believe that the use of quantitative Lp(a) is more transparent when looking for an independent association. Before Lp(a) can be used as a reliable biomarker for risk stratification and treatment allocation, future studies are required to create and validate a model that incorporates Lp(a) for predicting clinically relevant outcomes such as MALE.

On a similar note, we provided various statistical models in our analysis. It was not our intent to provide the best prediction model, but we sought to show the predictive performance of Lp(a) in relation to different, commonly used risk factors (Supplementary Table 3). The analysis of recurrent event data was performed with the same concept in mind. All three models treat recurring data differently and could potentially result in a significant association of Lp(a) in one model but a non-significant in another. By including these approaches, we offered a transparent result substantiating our conclusion.

In conclusion, this is the first study to demonstrate that Lp(a) is independently associated with both first MALE and recurrent MALE after iliofemoral endarterectomy in a population of Western-European patients with severe PAD. This identifies Lp(a) as a potential blood biomarker for subsequent lower limb events in high-risk patients, which can aid the allocation of preventive and therapeutic treatments.

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Supplemental Material

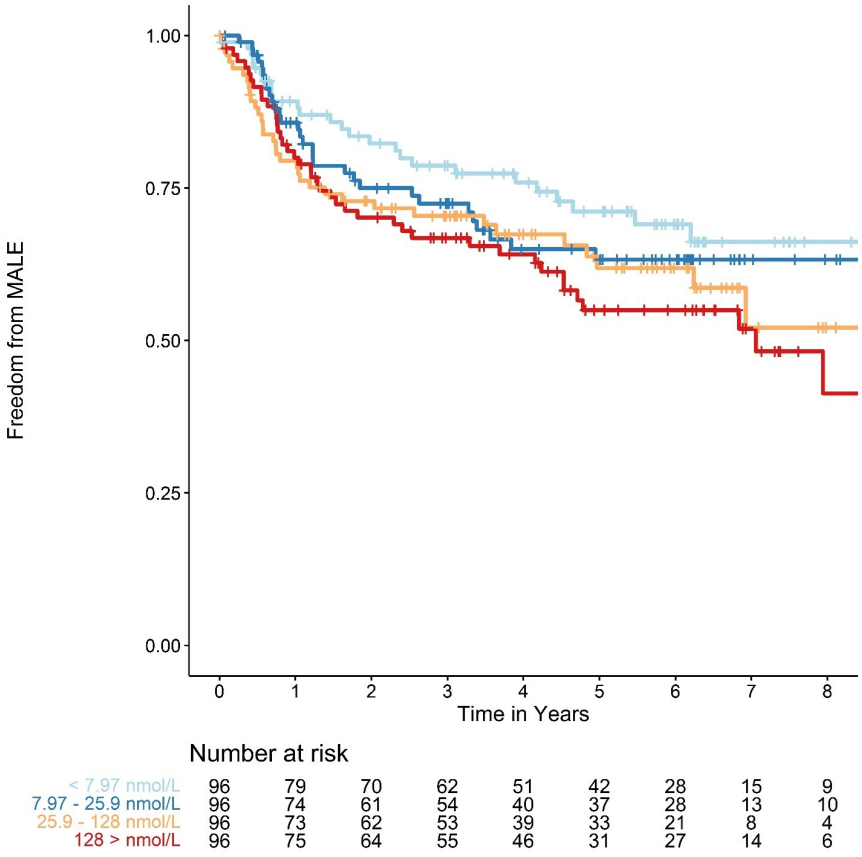

Figure S1: Quartiles of Lp(a) and probability of MALE

Table S1: Multiple Univariable Analysis for MALE

	HR (95% IC)	P
Age	0.98 (0.96-1)	.040
Gender	0.76 (0.53-1.1)	.15
BMI	1.01 (0.97-1.1)	.57
Smoking	1.19 (0.84-1.7)	.32
Fontaine III 1.5Y	2.39 (1.45-3.91)	<.001
Fontaine IV 1.5Y	2 (1.09-3.69)	.026
Fontaine III >1.5Y	0.3 (0.13-0.73)	.008
Fontaine IV >1.5Y	0.42 (0.15-1.15)	.091
ABI	1.49 (0.68-3.3)	.32
History of		
Peripheral Intervention	1.3 (0.92-1.8)	.13
CAD	1.2 (0.85-1.7)	.30
Stroke	0.4 (0.15-1.1)	.070
Hypertension	0.95 (0.65-1.4)	.78
Diabetes Mellitus	1.14 (0.78-1.7)	.50
Medication		
Insulin	0.95 (0.51-1.8)	.87
Oral Glucose inhibitors	1.03 (0.68-1.6)	.88
Anticoagulants	1.32 (0.84-2.1)	.23
Antiplatelets	1.08 (0.66-1.8)	.76
Statins	1.11 (0.74-1.7)	.62
Lipid Lowering Drugs	1.04 (0.7-1.5)	.86
Laboratory Results		
eGFR	1 (0.99-1)	.54
ESRD	14.55 (3.50-60.5)	<.001
Triglycerides	0.98 (0.88-1.1)	.70
Lp(a)	1.37 (1-1.8)	.030
LDL	0.98 (0.81-1.2)	.83
LDL corrected	0.92 (0.76-1.12)	.42
HDL	0.89 (0.55-1.4)	.62
Total Cholesterol	0.96 (0.83-1.1)	.56

HR = Hazard Ratio; CI = Confidence Interval; BMI = Body Mass Index; ABI = Ankle-Brachial Index; CAD = Coronary Artery Disease; eGFR = Estimated Glomerular Filtration Rate; ESRD = End Stage Renal Disease [eGFR < 15]; Lp(a) = Lipoprotein(a); LDL = Low-Density Lipoprotein; HDL = High-Density Lipoprotein.

Table S2: Exemplary regression models for MALE with Lp(a) incorporated

	BASIL			Nhanes			Meltzer		
	HR	(95% CI)	P	HR	(95% CI)	P	HR	(95% CI)	P
Lp(a)	1.40	1.05 – 1.87	.021	1.37	1.03 – 1.83	.031	1.34	1.01 – 1.79	.046
Age	1.30	0.82 – 2.06	.26	1.23	0.78 – 1.95	.37	1.34	0.84 – 2.13	.22
eGFR	1.10	0.75 – 1.63	.63	1.08	0.73 – 1.59	.71	1.13	0.76 – 1.68	.55
History of CAD	0.73	0.50 – 1.06	.10						
Tissue Loss	1.14	0.80 – 1.61	.48				1.15	0.79 – 1.68	.47
Diabetes Mellitus	17.37	3.99 – 75.75	.001	15.87	3.67 – 68.64	.001			
Currently Smoking				0.43	0.16 – 1.18	.10			
ABI							0.80	0.66 – 0.97	.026
BMI							0.86	0.71 – 1.06	.15
Hypertension							1.23	0.87 – 1.75	.25
Gender (male)							1.12	0.94 – 1.33	.21
End Stage Renal Disease							1.01	0.85 – 1.21	.89

HR = Hazard Ratio; CI = Confidence Interval; Lp(a) = Lipoprotein(a); eGFR = Estimated Glomerular Filtration Rate; CAD = Coronary Artery Disease; ABI = Ankle-Brachial Index; BMI = Body Mass Index; ESRD = End Stage Renal Disease [eGFR < 15]. Lipoprotein(a) is implemented in three risk models for PAD; BASIL (a model for mortality); Nhanes (a model for mortality); Meltzer (a reduced model for peripheral interventions).

Table S3: Two other recurrent Cox Proportional Hazard regression models

	Prentice-Williams-Peterson Gap-Time			Andersen-Gill		
	HR	(95% CI)	P	HR	(95% CI)	P
Lp(a)	1.30	1.06 – 1.61	.014	1.45	1.12 – 1.87	.005
History of peripheral intervention	1.16	0.88 – 1.55	.30	1.21	0.85 – 1.73	.30
Age (years)	0.98	0.97 – 0.99	.007	0.98	0.96 – 0.99	.012
Diabetes Mellitus	0.93	0.67 – 1.30	.67	0.95	0.63 – 1.45	.83
ESRD	5.07	2.71 – 9.49	.001	10.73	6.34 – 18.18	<.001
Fontaine III 1.5Y	1.59	1.08 – 2.34	.019	1.76	1.10 – 2.81	.018
Fontaine IV 1.5Y	2.10	1.28 – 3.45	.004	1.96	1.09 – 3.52	.024
Fontaine III >1.5Y	0.49	0.26 – 0.95	.034	0.57	0.31 – 1.05	.073
Fontaine IV >1.5Y	0.34	0.16 – 0.72	.005	0.59	0.25 – 1.40	.23

HR = Hazard Ratio; CI = Confidence Interval; Lp(a) = Lipoprotein(a); ESRD = End Stage Renal Disease [eGFR < 15]. A Prentice-Williams-Peterson Gap-Time and Andersen-Gill model, two recurrent event regression models, are demonstrated. The first model assumes an event as a renewal process, after which all 'time of risk' is reset, but previous events are still associated with the hazard of a new event. The latter model only considered baseline variables relevant to the hazard for recurrent events and assumed that previous events are not related to the hazard of a new event. Fontaine was corrected for time (below and above 1.5 years).

Table S4: Stepwise Prentice-Williams-Peterson Total-Time model for recurrent MALE

	HR	(95% CI)	P
Lp(a)	1.31	1.06 – 1.61	.014
Age (years)	0.98	0.97 – 1.00	.022
History of CAD	1.32	0.99 – 1.75	.058
ESRD	4.65	2.48 – 8.69	<.001
Smoking	1.28	0.96 – 1.71	.095
Fontaine III (1.5Y)	1.59	1.07 – 2.35	.021
Fontaine IV (1.5Y)	2.10	1.32 – 3.34	.002
Fontaine III (>1.5Y)	0.50	0.26 – 0.95	.035
Fontaine IV (>1.5Y)	0.35	0.17 – 0.73	.005

HR = Hazard Ratio; CI = Confidence Interval; Lp(a) = Lipoprotein(a); ESRD = End Stage Renal Disease [eGFR < 15]. This stepwise multivariable regression model based on the Prentice-Williams-Peterson Total-Time model demonstrates lipoprotein (a) is incorporated in a model for recurrent Major Adverse Limb Events.

Table S5: Lp(a) levels based on presence of plaque characteristics, and logistic regression of these.

	Lp(a) level			**Regression analysis for plaque and Lp(a)**		
	No	Yes	P	Odds Ratios	(95% CI)	P
Lipid >40%	23.3	30.2	0.51	0.73	0.25 – 1.92	.53 [a]
Lipid >10%	22.9	30.2	0.64	0.97	0.59 – 1.56	.89 [a]
Collagen	18.7	27.5	0.20	1.38	0.81 – 2.40	.25 [a]
SMC	12.9	31.15	0.006	1.85	1.14 – 3.07	.014 [a]
SMC staining %				0.01	0.02 - 0.03	.45 [b]
IPH	18.1	36.9	0.11	1.49	0.99 – 2.26	.060 [a]
Macrophages	23.9	29.8	0.85	1.11	0.65 – 1.90	.70 [a]
Macrophage staining %				-0.06	-0.15 – 0.03	.20 [b]

[a] Logistic regression
[b] Linear regression
Plaque characteristics are (semi-)quantitatively rated and related to Lp(a) levels.
Lp(a) = Lipoprotein(a); CI = Confidence Interval; SMC = Smooth Muscle Cell; IPH = Intraplaque Hemorrhage.

Table S6: Baseline characteristics stratified by major adverse cardiovascular events

	No MACE	MACE	P
N	285	99	
Age – y	68.540 (8.723)	69.020 (9.484)	.65
Gender - Male	217 (76.1)	64 (64.6)	.036
BMI - kg/m²	26.078 (3.792)	26.064 (4.533)	.98
Smoking	101 (36.1)	46 (46.9)	.075
Fontaine Stage			.92
II	167 (58.6)	57 (57.6)	
III	72 (25.3)	27 (27.3)	
IV	46 (16.1)	15 (15.2)	
ABI	0.586 (0.211)	0.555 (0.176)	.27
History of			
Peripheral Intervention	116 (40.7)	43 (43.4)	.72
Coronary Artery Disease	122 (42.8)	43 (43.9)	.95
Stroke	14 (5.3)	9 (10.1)	.19
Hypertension	202 (73.5)	68 (70.8)	.72
Diabetes Mellitus	80 (28.1)	23 (23.2)	.42
Medication			
Insulin	24 (8.5)	8 (8.1)	1.00
Glucose inhibitors	61 (21.5)	17 (17.2)	.44
Anticoagulants	41 (14.4)	14 (14.1)	1.00
Antiplatelets	241 (85.2)	84 (84.8)	1.00
Lipid Lowering Drugs	211 (74.3)	72 (72.7)	.86
Statins	210 (73.9)	71 (71.7)	.77
Laboratory Results			
eGFR - ml/min/1,73 m²	80.388 (25.609)	81.416 (29.520)	.75
Triglycerides - mmol/L	1.750 [1.260, 2.540]	1.700 [1.295, 2.315]	.35
Lp(a) - nmol/L	29.800 [7.900, 139.200]	19.000 [8.700, 99.400]	.57
LDL - mmol/L	2.403 (0.858)	2.440 (0.986)	.73
HDL - mmol/L	1.113 (0.379)	1.076 (0.315)	.38
Cholesterol - mmol/L	4.406 (1.172)	4.356 (1.325)	.72

Data are presented as n (%), median [interquartile range] or mean ± standard deviation. MALE = Major Adverse Limb Events; BMI = Body Mass Index; ABI = Ankle Brachial Index; eGFR = Estimated Glomerular Filtration Rate; Lp(a) = Lipoprotein(a); LDL = Low-density lipoprotein; HDL = High-density lipoprotein.

IV

Discussion and summary

8

Summary, General Discussion and Future Perspectives

General issues of peripheral artery disease

Patients with cardiovascular disease, and peripheral artery disease (PAD) in particular, remain at increased risk of cardiovascular adverse events. Paradoxically, research in the last few decades has not led to the anticipated enhancements.[1, 2] As a result, PAD still puts a significant strain on patients, healthcare providers, and resources. By focusing on each patient's individual needs, patient-tailored medicine can potentially improve outcomes and reduce the burden of PAD. Novel add-on therapies could benefit these patients but are unfortunately accompanied by potentially hazardous side effects, high costs, and the requirement of more healthcare resources.[3–6]

Knowledge about the progression of the disease and its symptoms could substantiate the decision-making processes clinicians have to perform on a regular basis. Deciding whether to treat and choosing the best method of intervention are important considerations for healthcare professionals that sometimes lack appropriate rationale now.

An estimate of disease progression is no easy feat, as PAD is a complex multifactorial disease that is influenced by a wide range of variables, including genetics, lifestyle, and comorbidities. Even though international cardiovascular guidelines support various risk-modifying algorithms, it remains difficult to distinguish between those who are at elevated risk for atherosclerotic complications and those who are not.[7, 8]

The world thus needs better tools to overcome these gaps in knowledge. An excellent place to start would be to look at patients with an aggravated risk of adverse events.

Increased risk in pad subtypes and present-day model performance in these patients

This thesis covered at least two specific patient groups with a high a priori chance of adverse events due to the extent of disease progression or its pathophysiological mechanisms.

First, in *Chapter 2*, our research indicated that patients with non-revascularisable (NR) chronic limb-threatening ischemia (CLTI) had an amputation-free survival of 43% after five years, which was driven by an equal rate of all-cause mortality and amputation. Amputation after one year from inclusion was rare, as if the viability of an extremity is determined by the initial (acute) phase and a relatively stable plateau phase is reached thereafter. Although the five-year prognosis for these patients is poor, their prospects are not (much) worse than those of patients with revascularisable CLTI.[9] Whether these relatively benign results could be attributed to the extensive care and strict surveillance applied in this cohort remains uncertain, but this could be a relevant explanation that justifies the costly care of these ill-fated patients.

In conclusion, we concluded that an NR status in CLTI does not drive towards immediate amputation per se if the best medical/wound treatment can be applied, and neither does this amputation lead to premature death (compared to revascularisable CLTI patients). Since this NR-CLTI population is underrepresented in research, our article addresses a long-standing paucity of information concerning their prognosis. Although an NR status is rare in literature, its current real-world prevalence is probably higher than one would expect. And perhaps more importantly, it could be argued that a peripheral intervention is sometimes pursued against all odds, as a final NR status is generally considered a death sentence. Hopefully, our data will refute this general perception slightly.

Survival predictions in these patients may seem less relevant as surgical revascularization strategies are not expected to be successful or patients are deemed too frail to be exposed to intervention risks. However, a reliable estimation of the prognosis of no-option CLTI patients could be relevant, especially in light of future therapeutic options (like biologics) and prevention strategies, in studies as well as in daily practice.

Using the same cohort of NR-CLTI patients, we validated a novel survival risk model in *Chapter 3*. This model was created with the Vascular Quality Initiative (VQI), an extensive North American registry that includes over one million procedures.[10] Our analysis demonstrated a C-statistic of 0.86,

which indicated that the combined hazards of twelve clinical covariates provided excellent discrimination in our cohort, even though the distribution of comorbidities in our cohort was different from that in the VQI cohort. As a result, this model can be used to identify no-option CLTI patients who are at high risk of (premature) death.

Another cardiovascular minority with a high risk of PAD (complications) are patients with pseudoxanthoma elasticum (PXE), who are discussed in *Chapter 4*. Our research, with the highest inclusion of PXE patients thus far, indicated that half of these patients meet the criteria for PAD, which is seven times the expected prevalence compared to the general population.[11] This aggravated risk cannot be attributed to age, as this increased prevalence was demonstrated in all age groups. General practitioners and clinicians should thus be aware of PAD in patients with PXE, and consider PXE as a potential cause of premature PAD.

Although PAD is common in PXE, progression to higher ischemic stages is much rarer, and peripheral interventions are generally not performed very often (2.25 per 1,000 patient-years). However, the reintervention rate is quite unfavourable in most patients undergoing interventions, with multiple reinterventions occurring within one year after the primary intervention (21 out of 35). Our research is not suited to elucidate the mechanism for this observation, and thus future studies should investigate this. This would not only benefit patients with PXE but could provide new insights into atherosclerosis, as PXE has been proposed as a good model for this disorder.[12]

In conclusion, caution should be exercised when undertaking peripheral interventions in patients with PXE. More so than in regular PAD patients, conservative treatments such as supervised walking therapy should be performed for as long as possible in patients with PXE before advancing to (endo)vascular interventions that could ultimately lead to more invasive procedures than one would have preferred.

Biomarker discovery

As the above-mentioned chapters of this thesis scrutinised existing risks for adverse events in PAD, the following chapters are more future-oriented. All chapters involve biomarker studies, in which biological markers predictive of adverse outcomes are explored.

Starting with *Chapter 5*, differences between biomarker sources were investigated. We found that the correlation between plasma and EVs is, on average moderate. However, when proteins are considered individually, the range of correlations is extremely broad, as demonstrated by another study.[13] Hypothetically, some well-correlating proteins might reflect the same processes in an equal origin in plasma and EVs, whereas poorly-correlating proteins indicate that origins or pathophysiological mechanisms differ in these sources. In contrast, both of these circulating sources have a very limited correlation with atherosclerotic plaque, which might suggest that neither of them originates to a great extent from this segment of atherosclerotic plaque. Plaque proteins correlated quantitatively better with EV-proteins than with their plasma protein counterparts. Since EVs have also been proven to emanate from atherosclerotic plaque, it could be hypothesized that EV proteins originate from plaque to a greater extent compared to plasma and consequently reflect its pathophysiological state better, but our study is not fit to prove this.[14, 15]

When investigating a possible relationship with important endpoints, by a large margin, more plaque-derived proteins were differentially expressed when stratifying for a recent (presurgical) stroke compared to the circulating markers. This is not surprising given that carotid plaque is thought to be the cause of (ischemic) stroke (and is thus removed). In contrast, proteins from plasma and EVs were more often differentially-expressed when stratifying for future major adverse cardiovascular events (MACE) compared to proteins from plaque. As MACE is a composite of adverse events in multiple vascular territories, this implies that circulating markers reflect systemic pro-atherogenic processes better than local plaque does. Of note, EVs contained the most significantly higher proteins in patients with MACE and could therefore be of more use when it comes to risk stratification. According

to our research, EVs appear to provide additional information about the severity and progression of systemic atherosclerosis than can be obtained from plasma or atherosclerotic plaque alone. Although plasma is an excellent source for prognostic biomarkers of (systemic) events, EVs should be investigated, preferably in combination with plasma markers.

In *Chapter 6*, we thus explored the prognostic features of four EV-proteins, cystatin C (CysC), CD14, serpin C1 (SC1) and serpin G1 (SG1), in patients undergoing femoral endarterectomy. Our results indicated that CD14 was independently associated with MACE, and SG1 was independently associated with both MACE and major adverse limb events (MALE) in these patients. Using these risk factors within (existing) risk models led to a modest improvement in these models' efficacy. Furthermore, not only are these proteins associated with outcome, but other research indicates that a causal link exists, too, further underlining the relationship between EV and endpoint.[16–19]

In *Chapter 7*, an equivalent investigation was performed with plasma lipoprotein(a) (Lp[a]). This biomarker is a reliable marker for cardiovascular disease (CVD) progression in carotid and coronary artery disease (CAD); other studies have found a cause-and-effect relationship with CVD.[20–23] Our study showed that in patients undergoing femoral endarterectomy, high Lp(a) levels were consistently associated with first MALE and recurrent MALE. Our data failed to demonstrate an association with MACE, possibly due to a lower incidence compared to MALE, although bigger studies came to the same conclusion.[24]

Both these chapters could, in the future, help establish a risk model that could stratify patients according to their risk of clinically important adverse events. As part of the CLTI framework, such stratification enables the patient-tailored management of preventive treatments, future revascularizations and limb salvage.

Future perspective and conclusions

In stark contrast to the health gains for individuals with coronary artery disease, comparable progress has not been achieved for patients with PAD.[1, 7] It is unclear whether this is due to the smaller investment in clinical research by governmental agencies and the pharmaceutical industry or a lack of awareness about PAD and its detrimental effects.[25] Non-controlled or underpowered trials have served as the hallmark for many PAD investigations thus far, but the foreseeable future should provide more opportunities to initiate extensive clinical studies or assess treatment efficacy in large cohorts. The VQI, as used in *Chapter 3*, and the *Athero-Express*, with which we were able to conceive *Chapter 5, 6 and 7*, are some of those much-needed large-scale registries and biobanks that can elucidate about the pathophysiology and risks in (specific subtypes of) PAD. Large studies require collaborations, and these bonds can strengthen the scientific revolution that is much needed.

personalised medicine is essential in modern-day healthcare but challenging in PAD due to its multifactorial nature and heterogeneity. It is thus essential to distinguish "syndromes" within PAD. This includes rare subtypes of PAD, which should be scrutinised to expand our knowledge of the full spectrum of PAD as we did in *Chapter 2 and 4*.

Finally, classical risk factors are the most frequently used components in current general risk models for PAD, although the application of these prediction tools is hardly part of the daily workflow. Classifications such as the Wound, Ischemia, and foot Infection Classification System and the Global Limb Anatomic Staging System are good examples of new methods to objectively grade disease severity in PAD.[7] While in other fields of medicine, researchers have found ways to stratify risk using tissue profiles, no biologically available markers are used in PAD that can truly show the severity of the disease. Thus, as we studied in *Chapter 5, 6 and 7*, biomarkers should be explored as perfect candidates for risk stratification, as they can be objective, quantifiable characteristics of biological processes. A countless number of different bioactive markers from a multitude of different sources render the possibilities limitless. If reliable research is conducted in a large group with relevant and well-defined endpoints, it is only a matter of time before the holy grail is discovered.

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9

Nederlandse samenvatting

Algemene problematiek van perifeer arterieel vaatlijden

Patiënten met cardiovasculaire ziekten (CVD) en perifeer arterieel vaatlijden (PAV) in het bijzonder, blijven een verhoogd risico lopen op complicaties als gevolg van de aandoening. Paradoxaal genoeg heeft het onderzoek van de afgelopen decennia niet geleid tot de verwachte vooruitgang in de behandeling of preventie van CVD, terwijl het aantal patiënten met PAV blijft toenemen.[1, 2] Hierdoor vormt PAV nog steeds een niet te onderschatten belasting voor patiënten, maar ook voor zorgverleners en zorgmiddelen. Door te richten op specifieke behandeling en preventie van de individuele patiënt heeft deze op maat gemaakte zorg het potentieel om behandelresultaten te verbeteren en dus de last van PAV te verminderen. Nieuwe aanvullende therapieën zouden deze patiënten ten goede kunnen komen, echter gaan zij ook gepaard met potentieel gevaarlijke bijwerkingen, hoge kosten en in sommige gevallen een grotere aanspraak op medische zorg.[3–6]

Kennis van het toekomstige ziektebeloop en de daaraan gerelateerde symptomen zou de dagelijkse besluitvorming van clinici kunnen onderbouwen. De beslissing om al dan niet te behandelen en de keuze van de beste interventiemethode zijn belangrijke overwegingen voor zorgprofessionals die op dit moment niet altijd objectief onderbouwd zijn.

Een inschatting van de ziekteprogressie is niet eenvoudig, aangezien PAV een complexe multifactoriële aandoening is die wordt beïnvloed door een groot aantal variabelen, waaronder genetica, levensstijl en comorbiditeiten. Hoewel internationale cardiovasculaire richtlijnen diverse risico verlagende algoritmen ondersteunen, blijft het moeilijk een onderscheid te maken tussen patiënten met een verhoogd risico op complicaties van atherosclerose en patiënten die dat niet hebben.[7, 8]

Er zijn dus betere instrumenten nodig om deze tekortkomingen te verhelpen. Een goed uitgangspunt is het onderzoeken van patiënten met een extra hoog risico op ongewenste complicaties.

Verhoogd risico en prestaties van voorspellende modellen in subtypes van perifeer arterieel vaatlijden

Dit proefschrift heeft betrekking op ten minste twee specifieke patiëntengroepen met een hoge a priori kans op cardiovasculaire complicaties. Dit is ten gevolge van de ziekteprogressie dan wel de pathofysiologische mechanismen waarmee deze gepaard gaan. Allereerst heeft ons onderzoek in *hoofdstuk 2* aangetoond dat patiënten met niet-revasculariseerbaar (NR) chronisch ledemaat-bedreigende ischemie (de internationale term chronic limb-threatening ischemia (CLTI) wordt voortaan gebruikt) een amputatie-vrije overleving hadden van 43% na vijf jaar. Dit werd bepaald door een gelijk percentage mortaliteit en amputatie. Een late amputatie (later dan een jaar na opname voor CLTI) was zeldzaam, alsof de vitaliteit van de extremiteiten wordt bepaald door de initiële (acute) fase en daarna een relatief stabiele plateaufase wordt bereikt. Hoewel de vijfjaarsprognose voor deze patiënten slecht is, zijn hun vooruitzichten niet (veel) slechter dan die van patiënten met CLTI die wel een revascularisatie ondergaan.[9] Of deze relatief gunstige resultaten kunnen worden toegekend aan de uitgebreide zorg en het strikte toezicht die in dit cohort werden toegepast blijft onzeker, maar dit zou een relevante verklaring kunnen zijn die de dure en intensieve zorg voor deze noodlijdende patiënten rechtvaardigt.

Wij concludeerden dat een NR-status bij CLTI niet per se leidt tot onmiddellijke amputatie als de beste medische- en wondbehandeling kan worden toegepast en dat deze amputatie evenmin leidt tot vroegtijdig overlijden (in vergelijking met revascularisseerbare CLTI-patiënten). Aangezien deze NR-CLTI-populatie ondervertegenwoordigd is in onderzoek, richt ons artikel zich op een langdurig gebrek aan informatie over hun prognose. Hoewel een NR-status volgens de literatuur zelden voorkomt, is de prevalentie ervan in de praktijk waarschijnlijk hoger dan men zou verwachten. En wellicht nog belangrijker, men zou kunnen stellen dat een perifere interventie soms tegen alle vooruitzichten in wordt nagestreefd, aangezien een definitieve NR-status over het algemeen als een soort doodvonnis wordt beschouwd bij een gebrek aan vertrouwen in het natuurlijke ziektebeloop. Hopelijk weerleggen onze bevindingen deze algemene opvatting enigszins.

Een betrouwbare schatting van de prognose van NR CLTI-patiënten zou relevant kunnen zijn,

vooral in het licht van toekomstige therapeutische opties (zoals biologische geneesmiddelen) of nieuwe tertiaire preventie, zowel in studieverband als in de dagelijkse praktijk.

Met hetzelfde cohort NR CLTI-patiënten hebben we in *hoofdstuk 3* een nieuw model gevalideerd dat kans op overleven inschat. Dit model werd ontwikkeld met het Vascular Quality Initiative (VQI), een groot Noord-Amerikaans register dat meer dan een miljoen procedures omvat.[10] Onze analyse toonde een C-statistiek van 0.86, wat aantoont dat de gecombineerde hazards van twaalf klinische variabelen een uitstekende discriminatie opleverde in ons cohort, onafhankelijk van het feit dat de verdeling van comorbiditeiten verschilde met die in het VQI-cohort. Derhalve kan dit model worden gebruikt om NR CLTI-patiënten, die een hoog risico op (vroegtijdige) dood hebben, te identificeren.

Een andere cardiovasculaire minderheid met een hoog risico op PAV (-complicaties) zijn patiënten met pseudoxanthoma elasticum (PXE), die worden besproken in *hoofdstuk 4*. Ons onderzoek, met de grootste inclusie van PXE-patiënten tot nu toe, wees uit dat de helft van deze patiënten voldoet aan de criteria voor PAV, wat zeven keer de verwachte prevalentie is in vergelijking met de algemene bevolking.[11] Dit verhoogde risico kan niet worden toegeschreven aan leeftijd, aangezien deze verhoogde prevalentie in alle leeftijdsgroepen werd aangetoond. Huisartsen en clinici moeten zich dus bewust zijn van PAV bij patiënten met PXE, en PXE beschouwen als een mogelijke oorzaak van vroegtijdige PAV.

Hoewel de prevalentie van PAV hoog is bij PXE, is progressie naar hogere ischemische stadia veel zeldzamer en zijn perifere interventies over het algemeen zeldzaam (2,25 per 1.000 patiëntjaren). Het percentage reinterventies is echter vrij ongunstig bij de meeste patiënten die een vasculaire operatie ondergaan, met meerdere reinterventies binnen een jaar na de primaire operatie (21 van de 35). Ons onderzoek is niet geschikt om het mechanisme achter deze waarneming te ontrafelen en toekomstige studies moeten dit nader bestuderen. Omdat PXE is gesuggereerd als een goed model voor atherosclerose zou dit niet alleen patiënten met PXE ten goede komen, maar zou dit ook nieuwe inzichten kunnen verschaffen voor alle patiënten met CVD. [12]

Concluderend stellen wij dat voorzichtigheid geboden is bij perifere ingrepen bij patiënten met PXE. Meer nog dan bij gewone patiënten met PAV moeten bij deze populatie zo lang mogelijk conservatieve behandelingen worden toegepast, zoals gesuperviseerde looptraining, alvorens over te gaan tot (endo)vasculaire ingrepen die uiteindelijk kunnen leiden tot verdergaande invasieve procedures dan aanvankelijk de intentie was.

Ontdekking van biomarkers

Terwijl in de bovengenoemde hoofdstukken van dit proefschrift bestaande risico's voor cardiovasculaire complicaties bij PAV onder de loep zijn genomen, zijn de volgende hoofdstukken meer toekomstgericht. Deze hoofdstukken hebben betrekking op biomarkerstudies, waarin biologische indicatoren worden onderzocht die voorspellend (kunnen) zijn voor atherosclerotische complicaties.

Om te beginnen werden de verschillen tussen de biomarkerbronnen onderzocht in *hoofdstuk 5*. Wij vonden een gemiddeld matige correlatie tussen plasma en extracellulaire vesicles (EV's). Wanneer de eiwitten echter afzonderlijk worden beschouwd is het spectrum van correlaties zeer breed, zoals ook is aangetoond in een andere studie.[13] Sommige goed gecorreleerde eiwitten zouden hypothetisch dezelfde (patho-)fysiologische processen kunnen weerspiegelen uit eenzelfde origine, terwijl slecht gecorreleerde eiwitten erop wijzen dat de herkomst van de marker, of het pathofysiologische mechanismen in deze bronnen, verschillen. Beide circulerende biomarkerbronnen (EV en plasma) hebben daarentegen een zeer beperkte correlatie met de atherosclerotische plaque, wat erop zou kunnen wijzen dat geen van beide in hoge mate afkomstig is uit (dit segment van) de atherosclerotische plaque. Plaque-eiwitten correleerden kwantitatief beter met EV-eiwitten dan met hun plasma-eiwit equivalent. Aangezien ook is aangetoond dat EV's afkomstig zijn van atherosclerotische plaque, zou men kunnen veronderstellen dat EV-eiwitten in hogere mate afkomstig zijn van plaque dan plasma, en derhalve de pathofysiologische toestand ervan beter weerspiegelen.[14, 15] Onze studie is

echter niet geschikt om dit te bewijzen.

Bij nader onderzoek naar een mogelijk verband met relevante eindpunten bleek dat bij stratificatie voor een recente (preoperatieve) beroerte meer eiwitten afkomstig van plaques verschillend tot expressie werden gebracht vergeleken met de circulerende markers. Dit is niet verwonderlijk, aangezien men veronderstelt dat carotisplaque de veroorzaker is van een ischemische beroerte (en derhalve chirurgisch wordt verwijderd als risico reductie). Daarentegen werden eiwitten uit plasma en EV's vaker verschillend gemeten bij stratificatie voor toekomstige belangrijke eindpunten op cardiovasculair gebied (major adverse cardiovascular events (MACE)), vergeleken met eiwitten uit plaque. Aangezien MACE een samenstelling is van complicaties in meerdere vasculaire gebieden impliceert dit dat circulerende markers systemische pro-atherogene processen beter weergeven dan lokale plaque. Opmerkelijk is dat EV's de meeste eiwitten bevatten die significant hoger waren bij patiënten met MACE en daarom nuttiger zouden kunnen zijn voor risicostratificatie dan plasma alleen. Volgens ons onderzoek blijken EV's dus extra informatie te verschaffen over de ernst en progressie van systemische atherosclerose dan kan worden verkregen uit plasma of atherosclerotische plaque alleen. Hoewel plasma een uitstekende bron is voor prognostische biomarkers van (systemische) gebeurtenissen, moeten EV's in de toekomst zeker ook worden onderzocht, bij voorkeur in combinatie met plasmamarkers.

In *hoofdstuk 6* onderzochten wij dus de prognostische kenmerken van vier EV-eiwitten (cystatine C (CysC), CD14, serpin C1 (SC1) en serpin G1 (SG1)) bij patiënten die een femorale endarterectomie ondergingen. Onze resultaten toonden aan dat CD14 onafhankelijk geassocieerd was met MACE, en SG1 onafhankelijk geassocieerd was met zowel MACE als major adverse limb events (MALE), een uitkomstmaat voor perifere interventies en amputaties. Het gebruik van deze risicofactoren binnen (bestaande) risicomodellen leidde tot een bescheiden verbetering van de effectiviteit van deze modellen. Deze eiwitten zijn niet alleen geassocieerd met de uitkomst, maar uit ander onderzoek blijkt dat er ook een oorzakelijk verband bestaat, hetgeen de relatie tussen EV en deze eindpunten nog verder onderstreept.[16–19]

In *hoofdstuk 7* werd een soortgelijk onderzoek uitgevoerd met plasma lipoproteïne(a) (Lp[a]). Deze biomarker is een betrouwbaar prognostisch eiwit voor de progressie van CVD bij coronaire hartziekten (CAD) en atherosclerose van de carotis. Andere studies vonden tevens een oorzakelijk verband met CVD.[20–23] Onze studie toonde aan dat bij patiënten die femorale endarterectomie ondergingen, hoge Lp(a)-niveaus consistent geassocieerd waren met primaire MALE en recidiverende MALE. Onze resultaten toonden geen verband aan met MACE, mogelijk als gevolg van een lagere incidentie in vergelijking met MALE, alhoewel ook grotere studies tot dezelfde conclusie kwamen.[24]

Deze beide hoofdstukken zouden in de toekomst kunnen bijdragen tot de totstandkoming van een risicomodel dat patiënten kan stratificeren volgens hun risico op klinisch belangrijke complicaties van atherosclerose. Als onderdeel van het PLAN (Patiënt risico, Ledemaat risico, ANatomisch risico) maakt deze stratificatie een op de patiënt afgestemd beleid ten aanzien van preventieve behandelingen en (toekomstige) revascularisaties mogelijk, hetgeen leidt tot het redden van ledematen en minder complicaties in andere vasculaire segmenten.[7]

Toekomstperspectieven en conclusies

In schril contrast met de gezondheidswinst voor personen met coronaire hartziekten, is er geen vergelijkbare vooruitgang geboekt voor patiënten met PAV.[1, 7] Het is onduidelijk of dit te wijten is aan de kleinere investeringen in klinisch onderzoek door overheidsinstanties en de farmaceutische industrie, of dat er een gebrek aan bewustzijn is rond PAV en de schadelijke effecten van deze ziekte.[25] Niet-gecontroleerde of underpowered studies zijn tot dusver het voornaamste kenmerk geweest van veel PAV-onderzoeken. De nabije toekomst zou meer mogelijkheden moeten bieden om grote klinische studies te starten of de doeltreffendheid van de behandeling in grote cohorten te beoordelen. De VQI, zoals gebruikt in *hoofdstuk 3*, en de Athero-Express, waarmee we *hoofdstuk 5, 6 en 7* konden verrichten, zijn enkele van die noodzakelijke grootschalige registers en biobanken die duidelijkheid kunnen

verschaffen over de pathofysiologie en de risico's bij (specifieke subtypes van) PAV. Grote studies vereisen samenwerkingsverbanden en deze relaties kunnen de broodnodige wetenschappelijke revolutie versterken.

Gepersonaliseerde geneeskunde is essentieel in de hedendaagse gezondheidszorg, maar blijft een uitdaging bij PAV vanwege de multifactoriële aard en de heterogeniteit ervan. Het is dus essentieel om clusters of syndromen binnen PAV te onderscheiden. Dit omvat ook zeldzame subtypes van PAV, die onderzocht moeten worden om de kennis van het volledige spectrum van PAV uit te breiden, zoals wij in *hoofdstuk 2 en 4* hebben gedaan.

Ten slotte zijn klassieke risicofactoren de meest gebruikte componenten in de huidige algemene risicomodellen voor PAV, hoewel de toepassing van deze voorspellingsinstrumenten nauwelijks deel uitmaakt van de dagelijkse workflow. Classificaties zoals het Wound, Ischemia, and foot Infection Classification System en het Global Limb Anatomic Staging System zijn goede voorbeelden van nieuwe methoden om de ernst van de ziekte bij PAV objectief te beoordelen en zullen hopelijk langzaam hun intrede doen in de dagelijkse praktijk.[7]

Terwijl onderzoekers op andere gebieden van de geneeskunde manieren hebben gevonden om het risico te stratificeren aan de hand van weefselprofielen, worden bij PAV nog geen modellen gebruikt met biologisch beschikbare markers die de ernst van de ziekte echt kunnen aantonen. Zoals wij in *hoofdstuk 5, 6 en 7* hebben onderzocht, moeten nieuwe biomarkers worden bekeken als perfecte kandidaten voor risicostratificatie, aangezien zij objectieve, kwantificeerbare kenmerken van biologische processen kunnen zijn. Een ontelbaar aantal verschillende bioactieve markers uit een groot aantal verschillende bronnen maken de mogelijkheden hiertoe onbeperkt. Als betrouwbaar onderzoek wordt verricht in een grote groep patiënten en er relevante en goed gedefinieerde eindpunten worden gekozen, is het slechts een kwestie van tijd voordat de heilige (prognostische) graal wordt ontdekt.

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Curriculum Vitæ

Maarten Cees Verwer

Maarten Cees Verwer was born in Deventer, the Netherlands. He was raised in Zutphen and graduated in 2010 at the Baudartius College (Atheneum, Profiles: Nature & Technology, Nature & Health). He worked full-time for six months in order to travel to Nepal to volunteer in a local Nepalese Hospital (Alka Hospital, Kathmandu), before enrolling in medical school at the Erasmus University in Rotterdam in 2011.

During his first two years as a student, he joined A.R.S.R Skadi as a lightweight rower, participating in a training program exercising up to nine times a week. In 2014, he proceeded with his interest in rowing as a coach for the lightweight freshmen of A.R.S.R Skadi. Meanwhile, he attended several extracurricular courses (Tropical Medicine Course, Mastercourse Clinical Reasoning I & II, ErasmusMC Anatomy Research Project) and completed medical school in 2018.

To celebrate his graduation, he completed a long-cherished dream by cycling an extensive, independently travelled route of 7000 kilometres from Rotterdam through Europe and Turkey to Teheran, Iran. Returning home, he started working as a surgical resident not in training (ANIOS) at Ikazia Hospital as his interest in surgery was sparked there during his internship. After a year of clinical work, in 2019, he commenced as a PhD student at the University Medical Centre Utrecht under the guidance of Prof. dr. Gert Jan de Borst and Prof. dr. de Kleijn. In two and a half years, he laid the groundwork for his dissertation called **Peripheral Artery Disease** *Evaluation of Novel Biomarkers and Adverse Events in Unexplored Subtypes*.

Yearning for the daily clinical practice, in 2022 he re-enlisted as a surgical ANIOS at the IJsselland Hospital, in Capelle aan de IJssel. On January 1st of 2023 he commenced with his surgical residency training in the Reinier de Graaf Hospital, in Delft.

List of Publications

1. **Maarten C. Verwer**, Joep G.J. Wijnand, Martin Teraa, Hendrik Gremmels, Jessica P. Simons, Michael S. Conte, Marianne C. Verhaar, and Gert J. de Borst. External validation of the vascular quality initiative prediction model for survival in no-option chronic limb-threatening ischemia patients. *Journal of Vascular Surgery*, 72(5):1659–1666.e1, nov 2020. doi: 10.1016/j.jvs.2020.02.018
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3. **Maarten C. Verwer**, Martin Teraa, Luuk van Eijk, C. E. V. B. (Stijn) Hazenberg, and Gert J. de Borst. Peripheral artery disease and diabetes: complex multidisciplinary care for an increasing number of patients. *Nederlands Tijdschrift voor Geneeskunde*, 165(D5560), 2021
4. **Maarten C. Verwer**, Joost M. Mekke, Nathalie Timmerman, Qiu Y. Van Der Pol, Claire Frissen, Gerard Pasterkamp, Gert J. De Borst, Constantijn E.V.B. Hazenberg, and Dominique P.V. De Kleijn. Plasma extracellular vesicle serpin g1 and CD14 levels are associated with major adverse cardiovascular events and major adverse limb events in patients undergoing femoral endarterectomy. *European Journal of Vascular and Endovascular Surgery*, nov 2022. doi: 10.1016/j.ejvs.2022.10.045
5. **Maarten C. Verwer**, Farahnaz Waissi, Joost M. Mekke, Mirthe Dekker, Erik S.G. Stroes, Gert J. de Borst, Jeffrey Kroon, Constantijn E.V.B. Hazenberg, and Dominique P.V. de Kleijn. High lipoprotein(a) is associated with major adverse limb events after femoral artery endarterectomy. *Atherosclerosis*, 349:196–203, may 2022. doi: 10.1016/j.atherosclerosis.2021.11.019
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7. **Maarten C. Verwer**, Joost Mekke, Nathalie Timmerman, Farahnaz Waissi, Arjan Boltjes, Gerard Pasterkamp, Gert J. de Borst, and Dominique P. V. de Kleijn. Comparison of cardiovascular biomarker expression in extracellular vesicles, plasma and carotid plaque for the prediction of MACE in CEA patients. *Scientific Reports*, 13(1), jan 2023. doi: 10.1038/s41598-023-27916-6
8. **Maarten C. Verwer**, Constantijn E.V.B. Hazenberg, Wilko Spiering, and Gert J. de Borst. Peripheral interventions in patients with pseudoxanthoma elasticum (PXE). *European Journal of Vascular and Endovascular Surgery*, 65(1):142–148, jan 2023. doi: 10.1016/j.ejvs.2022.08.009
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10. Lotte Slenders, Lennart P L Landsmeer, Kai Cui, Marie A C Depuydt, **Maarten Verwer**, Joost Mekke, Nathalie Timmerman, Noortje A M van den Dungen, Johan Kuiper, Menno P J de Winther, Koen H M Prange, Wei Feng Ma, Clint L Miller, Redouane Aherrahrou, Mete Civelek, Gert J

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11. Michal Mokry, Arjan Boltjes, Lotte Slenders, Gemma Bel-Bordes, Kai Cui, Eli Brouwer, Joost M. Mekke, Marie A. C. Depuydt, Nathalie Timmerman, Farahnaz Waissi, **Maarten C. Verwer**, Adam W. Turner, Mohammad Daud Khan, Chani J. Hodonsky, Ernest Diez Benavente, Robin J. G. Hartman, Noortje A. M. van den Dungen, Nico Lansu, Emilia Nagyova, Koen H. M. Prange, Jason C. Kovacic, Johan L. M. Björkegren, Eleftherios Pavlos, Evangelos Andreakos, Heribert Schunkert, Gary K. Owens, Claudia Monaco, Aloke V. Finn, Renu Virmani, Nicholas J. Leeper, Menno P. J. de Winther, Johan Kuiper, Gert J. de Borst, Erik S. G. Stroes, Mete Civelek, Dominique P. V. de Kleijn, Hester M. den Ruijter, Folkert W. Asselbergs, Sander W. van der Laan, Clint L. Miller, and Gerard Pasterkamp. Transcriptomic-based clustering of human atherosclerotic plaques identifies subgroups with different underlying biology and clinical presentation. *Nature Cardiovascular Research*, 1(12):1140–1155, dec 2022. doi: 10.1038/s44161-022-00171-0