

# 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

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## WHAT THIS PAPER ADDS

In pursuit of improved risk stratification of patients with severe peripheral artery disease (PAD), higher levels of Serpin G1 and CD14 are independently associated with future major adverse cardiovascular events and major adverse limb events. Although validation in another cohort is necessary before clinical implementation, these biomarkers could potentially improve healthcare management by individualising drug regimens or improving informed decision making regarding revascularisations in patients with PAD.

**Objective:** Plasma extracellular vesicles (EV) are an emerging source of biomarkers for diagnosis and prognosis of cardiovascular disease (CVD). Risk stratification for common adverse events such as major adverse limb events (MALE) and major adverse cardiovascular events (MACE) by an EV blood sample could improve healthcare management by individualising drug therapy or improving informed decision making regarding revascularisations in patients with peripheral artery disease (PAD). As such, this study investigated the associations between plasma EV proteins and prospectively registered MALE and MACE in consecutive patients undergoing femoral endarterectomy.

**Methods:** Using the Athero-Express biobank study, four EV proteins (Cystatin C, CD14, Serpin C1, and Serpin G1) were measured in the high density lipoprotein 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 and MACE and MALE in the three year post-operative period.

**Results:** Most patients were treated for claudication (Fontaine II, 52.8%), although rest pain (Fontaine III, 30.1%) and ischaemic wounds (Fontaine IV, 17.1%) were common in this cohort. Within three years 51 patients died, amongst whom 25 deaths 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.

**Keywords:** Biomarkers, Extracellular vesicles, Major adverse cardiovascular events, Major adverse limb events, Peripheral artery disease, Risk stratification

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## INTRODUCTION

Peripheral artery disease (PAD) is considered one of the most prevalent vascular conditions, affecting over 202 million people worldwide in 2010.<sup>1</sup> Despite 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 who undergo a peripheral arterial intervention, up to 42% will have a MALE and 13% a MACE in the following three years.<sup>2</sup> Consequently, patients at high risk of CVE might benefit from add on therapies such as dual antiplatelet therapy, dual pathway inhibition, PCSK9 inhibition, or colchicine treatment.<sup>3–6</sup> In addition, improved risk stratification could support intervention decision making when the effectiveness of limb

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salvage is disputed or when additional arguments are warranted to select the mode of intervention.

Early identification of PAD patients with 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.<sup>7</sup> For this reason EVs are considered a “liquid biopsy” of numerous diseases.<sup>7,8</sup>

Levels of EV subgroups are increased in patients with acute coronary syndromes or ischaemic stroke, and in response to risk factors for cardiovascular disease, such as smoking, metabolic disease, and hypertension.<sup>9–12</sup> A study using Framingham Heart Study data showed that EV levels were associated with hypertension, dyslipidaemia, and metabolic syndrome.<sup>13</sup> 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 patients.<sup>14</sup> There is only one study focusing on the association between an EV protein (calprotectin) and future events (amputation) in a PAD population.<sup>15</sup> As such, the prognostic properties of these novel biomarkers are relatively unexplored territory. Recent research in patients undergoing carotid endarterectomy showed that EV proteins (Cystatin C, CD14, Serpin C1, and Serpin F2) were associated with future MACE.<sup>16</sup> Serpin G1 was associated with heart failure in patients with breathlessness.<sup>17</sup> Furthermore, Serpin C1, G1, and F2, and Cystatin C were associated with stress induced myocardial ischaemia in women.<sup>18</sup>

The current study hypothesised that these EV proteins may also be associated with future CVE in PAD patients. Hence, it investigated whether pre-operative levels of four EV proteins (Cystatin C, CD14, Serpin C1, and Serpin G1) were associated with MACE and MALE in patients after femoral endarterectomy. To explore potential pathophysiological mechanisms, histological plaque characteristics were related to EV protein levels.

## 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) were eligible for inclusion in the Athero-Express (AE). This biobank was established to collect important biological material, such as atherosclerotic plaque and pre-operative blood, which can be used for research into pathophysiology, predictive biomarkers, and

other applications. The AE has therefore been extensively used and previously described in greater detail.<sup>19</sup> The study was approved by the Institutional Review boards of both hospitals and written informed consent was obtained from all patients. The study was conducted in accordance with the Declaration of Helsinki.<sup>20</sup>

For this research, PAD patients who underwent femoral endarterectomy with complete three year follow up data were selected. Of these, 218 patients underwent thromboendarterectomy (TEA), 69 patients underwent endarterectomy with a ring strip cutter (RSC), and 30 patients underwent bypass surgery after endarterectomy. Two patients underwent RSC in addition to the TEA, 11 underwent stenting in addition to TEA, and five underwent RSC and stenting in addition to TEA. Patients with insufficient biomarker material (citrate plasma), patients who died during surgery, or patients who underwent lower limb amputation were excluded from the analysis.

### *Blood collection and processing*

In the 24 hours before surgery, venous blood was collected in citrate tubes. These were centrifuged (10 minutes, 1 850 ×g at room temperature) within 30 minutes of collection. Plasma was aliquoted and directly stored at −80 °C.

### *Isolation of extracellular vesicle plasma subfractions and Meso Scale immunoassay*

The high density lipoprotein (HDL) subset of plasma EVs, a subset that co-precipitates with HDL particles, was isolated according to previously published protocols.<sup>18</sup> This subset was chosen because this subfraction contained the most differentially expressed proteins for MACE in patients undergoing carotid endarterectomy.<sup>16</sup> In short, solutions of dextran sulphate (MP Biomedicals, Santa Ana, CA, USA) and manganese (II) chloride (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). Serpin F2 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 that was used and extracellular vesicle characterisation have been described in detail in two previously published papers (especially in the supplementary materials of Zhang *et al.*).<sup>17,21</sup> In short, this study used density gradient centrifugation of the HDL plasma subfractions, with density gradient fractions characterised by CD9 Western blot analysis as the 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 gain easy

access to these data an EV track ID was created, EV200044, in which the data were structured in a uniform way.

### Histological atherosclerotic plaque examination

The atherosclerotic plaque was processed and immunohistochemically analysed for the number of macrophages (CD68 stain), smooth muscle cells (alpha actin stain), microvessels (CD34, endothelial stain), amount of collagen (picosirius), intraplaque haemorrhage (haematoxylin and eosin stain and elastic van Gieson), lipid core (picosirius and haematoxylin and eosin stain), and calcifications by the standardised AE protocol.<sup>19</sup> Two experienced observers examined plaque and scored macrophage, smooth muscle cell, calcification, and collagen content as no or minor staining or moderate or heavy staining. The lipid core was estimated and categorised as <10%, >10%, and <40%, >40% of the total plaque area.

### Follow up and clinical outcome measures

Following surgery, patients included in the AE underwent three years of follow up, which consisted of an annual questionnaire. Important outcomes described in the original AE were validated, which required official letters describing these events.<sup>19</sup> Two important endpoints (MACE and MALE) were used. Major adverse cardiovascular events (MACE) are a composite of non-fatal myocardial infarction or stroke, and death attributed to cardiovascular disease. The latter was defined as fatal myocardial infarction, fatal stroke (both haemorrhagic or ischaemic), fatal ruptured abdominal aneurysm, fatal heart failure, and sudden cardiac death. Major adverse limb events (MALE) are 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 *t* test, Mann–Whitney U test, Chi square, or Fisher's exact test, depending on the type of variable and respective distribution.

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

improvement with implementation of the EV protein was assessed by comparing time dependent Area Under the Curve (AUC) based on the model of Heagerty and Zheng.<sup>22</sup>

A two tailed alpha level of 0.05 was considered statistically significant. Statistical analyses were performed using R version 4.1.2.

## RESULTS

Out of 3 924 potentially eligible Athero-Express patients, 1 034 patients underwent femoral endarterectomy. For this study, 643 patients completed the follow up; 317 of them had enough citrate sample for analysis and were included. The study population mainly included older patients (mean aged 68.5 years) who were mainly male (72.2%) (Table 1). The indication for surgery was either Fontaine stages II, III, or IV in 52.8%, 30.1%, and 17.1% of these cases, respectively. Most participants had hypertension or received hypertensive medication (87.1%), and a cardiac comorbidity was common, as 42.7% of patients had coronary artery

**Table 1. Baseline characteristics for 317 femoral endarterectomy patients included in the Athero-Express biobank study, in the Netherlands**

Variable	Patients (n = 317)
Age – y	68.5±9.0
Male	229 (72.2)
BMI – kg/m <sup>2</sup>	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 – mL/min/1.73 m <sup>2</sup>	79.7±26.4
LDL – mmol/L	2.41±0.89
HDL – mmol/L	1.11±0.37
Triglycerides – mmol/L	2.08±1.56
Total cholesterol – mmol/L	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)

Data are presented as n (%) or mean ± standard deviation. BMI = body mass index; GFR-MDRD = glomerular filtration rate - modification of diet in renal disease; LDL = low density lipoprotein; HDL = high density lipoprotein.

disease (CAD). The prevalence of stroke and diabetes mellitus was lower, at 18.4% and 28.7%, respectively.

### Extracellular vesicle protein analysis

A multiplex assay was used for this research and included CysC, CD14, SC1, and SG1. After log transformation of these four proteins, their distribution was considered normal. Protein levels in relation to the time of blood sampling up to time of analysis in the 317 patients showed no evident relationship when examining distribution plots. However, linear regression demonstrated that all except CD14 were associated with length of this interval, although the direction was unambiguous and the effect was very minimal (Supplementary Table S1).

### Outcomes

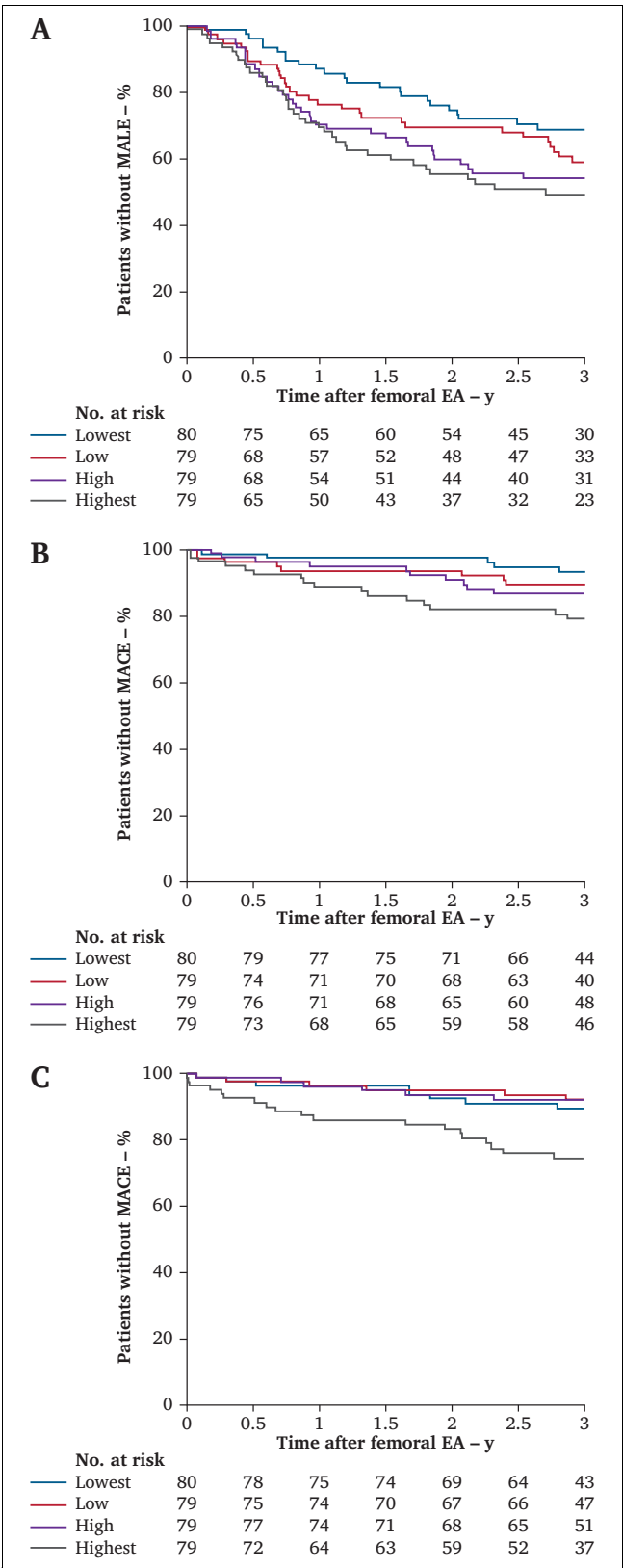
The three year follow up of the 317 PAD patients showed that 51 patients died, 25 of them from cardiovascular diseases; 39 patients had experienced MACE and 125 patients MALE, and these events seemed to gradually unfold over time (Supplementary Fig. S1). Not many statistical differences in baseline characteristics were seen between the groups for MACE. Only a history of stroke was seen more often (15.9% vs. 36.1%;  $p = .007$ ) in patients with MACE (Table 2).

Equally, there were not many distinguishable baseline characteristics that were statistically different for MALE (Table 2). Only hypertension was more frequently reported in the group with MALE (92.8% vs. 83.3%;  $p = .022$ ) and,

**Table 2.** Baseline characteristics stratified for endpoints major adverse cardiovascular events (MACE) and major adverse limb events (MALE) in 317 femoral endarterectomy patients included in the Athero-Express biobank study, in the Netherlands

Variable	No MACE (n = 278)	MACE (n = 39)	p value	No MALE (n = 192)	MALE (n = 125)	p value
Age – y	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 – kg/m <sup>2</sup>	26.2±4.2	26.3±3.6	.91	25.9±4.3	26.7±3.8	.082
Smoking	106 (38.8)	18 (46.2)	.48	75 (39.9)	49 (39.5)	1.0
Fontaine stage			.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)	1.0	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)	1.0	19 (9.9)	9 (7.2)	.54
Glucose inhibitors	63 (22.7)	9 (23.1)	1.0	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)	1.0	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 – mL/min/1.73 m <sup>2</sup>	79.7±25.4	79.7±33.8	.99	81.6±27.7	76.7±24.4	.12
LDL – mmol/L	2.4±0.89	2.43±0.91	.88	2.38±0.90	2.44±0.88	.56
HDL – mmol/L	1.12±0.38	1.05±0.27	.28	1.11±0.37	1.10±0.37	.68
Triglycerides – mmol/L	2.09±1.59	1.98±1.32	.67	2.05±1.36	2.13±1.84	.68
Total cholesterol – mmol/L	4.38±1.19	4.34±1.21	.86	4.36±1.20	4.40±1.18	.77
Extracellular vesicles						
Cystatin C	4.01±0.424	4.10±0.43	.26	4.06±0.41	3.965±0.44	.054
CD14	4.34±0.189	4.42±0.19	.011	4.36±0.20	4.331±0.18	.16
Serpine C1	6.38±0.273	6.37±0.24	.92	6.39±0.26	6.354±0.28	.27
Serpine G1	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)	1.0	13 (8.4)	3 (3.0)	.14
Fat > 10%	62 (27.6)	9 (29.0)	1.0	46 (29.7)	25 (24.8)	.47
Intraplaque haemorrhage	99 (47.1)	14 (48.3)	1.0	67 (47.2)	46 (47.4)	1.0
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 (%) 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.



**Figure 1.** Cumulative Kaplan–Meier estimates of major adverse cardiovascular events (MACE) and major adverse limb events (MALE) in 317 femoral endarterectomy (EA) patients stratified by plasma extracellular vesicle protein quartiles: (A) Serpin G1 (SG1) quartiles for MALE, (B) SG1 quartiles for MACE, and (C) CD14 quartiles for MACE.

consequently, these patients were more often treated with antihypertensive drugs.

**Association with outcomes**

**Statistical models.** As a first step towards a complete multivariable model, univariable regression analysis was separately performed for all variables and both endpoints (Supplementary Table S2). This identified age, sex, Fontaine classification, history of stroke or transient ischaemic attack (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 Figure 1A–C.

Implementing EVs in multivariable models based on the statistical significance of covariables, CD14 and SG1 were both statistically significant in the models for MACE, but only SG1 was statistically significant for MALE (Table 3, full models in Supplementary Table S3).

Stepwise regression for MACE selected CD14, SC1, and SG1 in its model (besides stroke or TIA), whereas its equivalent for MALE only selected SG1 (alongside age, hypertension, Fontaine, and eGFR) (Supplementary Table S4).

**Literature model.** A collection of clinical risk factors that were described as relevant in today’s literature was established. 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 one (Table 4, full models in Supplementary Table S3). Analysis of the AUC showed that these statistically significant EV proteins were able to improve these models. The improvement with CD14 in MACE (AUCs of 0.626 – 0.682 and 0.638 – 0.685) and SG1 in MALE (AUCs of 0.602 – 0.636, 0.597 – 0.635 and 0.562 – 0.608) are particularly noteworthy (Table 4).

**Association with plaque characteristics**

A total of 198 plaques were available for histological assessment. Semiquantitative characteristics of the atherosclerotic plaque were related to continuous levels of EV proteins. Two statistically significant associations were found: SC1 with presence of macrophages (OR 0.19; 0.04 – 0.80;  $p = .029$ ) and CysC with a lipid core (>10% of the total plaque area) (OR 4.05; 1.0 – 16;  $p = .050$ ) (Supplementary Table S5).

**DISCUSSION**

This study has demonstrated that EV proteins are biomarkers associated with future MACE and MALE in patients with severe PAD. Both CD14 and SG1 were elevated pre-operatively in patients who experienced MACE in the three years following femoral surgery, whereas SG1 was also higher in patients with post-operative MALE. Associations with these endpoints were confirmed in multiple multivariable models, based on a statistical selection of



**Table 3. Statistical multivariable Cox regression models with plasma extracellular vesicles (EVs) implemented in 317 femoral endarterectomy patients included in the Athero-Express biobank study, in the Netherlands**

EV protein for each model	HR (95% CI)	p value
<b>MACE<sup>*</sup></b>		
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
<b>MALE<sup>†</sup></b>		
logCysC	0.69 (0.44–1.08)	.11
logCD14	0.91 (0.76–1.09)	.31
logSC1	0.91 (0.76–1.09)	.30
logSG1	1.29 (1.07–1.57)	.009

Cox regression analysis when EV proteins are implemented in a model that involves risk factors that have  $p < .2$  according to univariable Cox regression analysis. CysC = Cystatin C; SC1 = Serpin C1; SG1 = Serpin G1.

\* The model for major adverse cardiovascular events (MACE) included: age, sex, Fontaine, stroke, hypertension, and antiplatelet therapy.

† The model for major adverse limb events (MALE) included: age, body mass index, and hypertension.

covariables and clinical risk models that were 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), 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.<sup>23</sup> As such, CD14 stimulates a cascade of pro-inflammatory signalling pathways and has been established to influence cell metabolism (lipogenesis, insulin resistance).<sup>23</sup> CD14 activation can also increase the expression of cell adhesion molecules and procoagulant activity, which in turn are often essential components of adverse cardiovascular events.<sup>24</sup> For these reasons, CD14 has gained interest as a potential cardiovascular risk factor, and has thus far been associated with increased risk of cardiovascular events in patients undergoing carotid endarterectomy and a mixed group of patients in cardiovascular cohorts.<sup>16,25,26</sup> With respect to PAD, plasma CD14 levels were elevated in patients with a combination of PAD and CAD, as well as those with a higher PAD classification.<sup>27</sup> The current study is the first to demonstrate that EV-CD14 is independently associated with a future event, MACE, and can improve clinical risk models when implemented for patients with PAD (AUC improvement of 0.059 and 0.046 for two existing clinical models).

No association between CD14 and 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.<sup>28</sup> Due to the pathophysiological mechanism, an association would be expected, and thus it is believed that patient selection could be of influence. As the patients in this study had severe PAD conditions, reflected by a three year MALE incidence of >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 the cohort with a group with lower PAD severity. In addition, the data show that CD14 was not associated with major features of the vulnerable femoral plaque; consequently, CD14 might have a limited influence on the occurrence of new limb events. However, in contrast to other vascular territories, femoral occlusion was common in severe PAD patients, leading to a more stable plaque phenotype that was not associated with ongoing progression of atherosclerotic disease.<sup>29</sup> The absence of an association between the current EV markers and plaque characteristics, although an association with MACE and or MALE is proven, underlines this. Therefore, interpretation related to mechanism and function of the markers with plaque pathology is extremely difficult.

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

This study had some limitations. The inclusion criteria imply that the 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 ischaemic wounds can be considered “severe”, but significant atherosclerotic stenosis tends to be defined in combination with severely debilitating symptoms as such. The initial intervention was heterogeneous, although most patients underwent TEA. The preliminary analyses scrutinised whether this influenced the association between EV markers and MALE or MACE. It was concluded that no effect on MACE was seen, whereas the association with SG1 gained very little with the addition of this variable. Consequently, the intervention category was not added to the multivariable models. Disease processes in the current

**Table 4. Literature multivariable Cox regression models with plasma extracellular vesicles (EVs) implemented**

Literature based models	EV protein	HR (95% CI)	p value	AUC before	AUC after
<b>MACE</b>					
Miao <i>et al.</i> <sup>*</sup>				.626	
	logCysC	1.17 (0.85–1.60)	.34		.631
	logCD14	1.46 (1.09–1.96)	.012		.682
	logSC1	0.99 (0.72–1.35)	.93		.628
	logSG1	1.40 (1.01–1.93)	.044		.654
Berger <i>et al.</i> <sup>†</sup>				.638	
	logCysC	1.12 (0.80–1.55)	.52		.635
	logCD14	1.38 (1.03–1.86)	.033		.685
	logSC1	0.95 (0.69–1.31)	.75		.637
	logSG1	1.43 (1.03–1.98)	.033		.658
<b>MALE</b>					
Biscetti <i>et al.</i> <sup>‡</sup>				.602	
	logCysC	0.84 (0.70–1.02)	.073		.615
	logCD14	0.91 (0.76–1.09)	.29		.610
	logSC1	0.91 (0.76–1.09)	.29		.609
	logSG1	1.30 (1.07–1.57)	.008		.636
Zhang <i>et al.</i> <sup>§</sup>				.597	
	logCysC	0.85 (0.70–1.03)	.10		.617
	logCD14	0.91 (0.76–1.09)	.29		.609
	logSC1	0.92 (0.77–1.09)	.32		.605
	logSG1	1.28 (1.06–1.56)	.011		.635
Meltzer <i>et al.</i> <sup>  </sup>				.562	
	logCysC	0.79 (0.65–0.96)	.018		.592
	logCD14	0.86 (0.72–1.03)	.11		.583
	logSC1	0.87 (0.73–1.05)	.14		.572
	logSG1	1.40 (1.15–1.70)	.001		.608

Two literature based models for major adverse cardiovascular events (MACE), and three literature based models for major adverse limb events (MALE), with all EV proteins implemented separately. The EV proteins are z transformed and thus the HR represents the HR per standard deviation increase. The right two 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. CysC = Cystatin C; SC1 = Serpin C1; SG1 = Serpin G1.

\* Included: coronary artery disease (CAD), stroke, diabetes mellitus (DM), hypertension, smoking, and use of insulin.

† Included: age, body mass index (BMI), chronic kidney disease (CKD), CAD, stroke, DM, and smoking.

‡ Included: CAD, stroke, DM, hypertension, smoking, and age.

§ included: age, BMI, hypertension, DM, smoking, and sex.

|| Included: Fontaine, DM, sex, smoking, CKD, and CAD.

patients were most likely due to traditional risk factors, whereas a shift towards diabetes as a cause of PAD will probably be seen in coming years; this population was relatively underrepresented in the cohort. It has also been suggested that ethnicity influences the association between CD14 and adverse outcomes, and thus the current results and conclusions cannot be expanded to other races, since patients enrolled in the AE are predominantly Caucasian. Although use of anticoagulants, antiplatelets and lipid lowering medication is high, best medical treatment was 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 showed that a potential time effect was very minimal and unambiguous or non-significant, and thus it is thought that it is unlikely that patient collection and plasma storage would have influenced the results. Medication changes during follow up were not recorded, so the baseline characteristics only

provided medication use in the pre-operative or direct post-operative period.

Although the power for MALE was adequate, the event rate of MACE was low and could have led to uncertainty. Sex adjusted analyses were not performed due to the low incidence of MACE, especially since 72% of this cohort was male. Regarding the improvement in existing models, the use net re-classification improvement was not considered since this method is prone to overestimation in poorly fitted risk models.<sup>34</sup> Comparing the AUC of models has its limitations too, as it is somewhat optimistic when case controls are imbalanced, it does not take 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.<sup>35</sup> The HDL subfraction for EV analysis was only analysed, since this HDL subfraction contained the most differentially expressed proteins for MACE in carotid endarterectomy patients.<sup>16</sup>

These results are a first and critical step into risk prediction research when it comes to the use of EVs in PAD.

However, the results are not yet generalisable and need further validation in a larger cohort of patients with (severe) PAD.

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

## APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejvs.2022.10.045>.

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