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Characteristics of peripheral blood cells are independently related to major adverse cardiovascular events after carotid endarterectomy

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ABSTRACT

Background and aims: Patients who underwent carotid endarterectomy (CEA) still have a residual risk of 13% of developing a major adverse cardiovascular event (MACE) within 3 years. Inflammatory processes leading up to MACE are not fully understood. Therefore, we examined blood cell characteristics (BCCs), possibly reflecting inflammatory processes, in relation to MACE to identify BCCs that may contribute to an increased risk.

Methods: We analyzed 75 pretreatment BCCs from the Sapphire analyzer, and clinical data from the Athero-Express biobank in relation to MACE after CEA using Random Survival Forests, and a Generalized Additive Survival Model. To understand biological mechanisms, we related the identified variables to intraplaque hemorrhage (IPH).

Results: Of 783 patients, 97 (12%) developed MACE within 3 years after CEA. Red blood cell distribution width (RDW) (HR 1.23 [1.02, 1.68], $p = 0.022$), CV of lymphocyte size (LACV) (HR 0.78 [0.63, 0.99], $p = 0.043$), neutrophil complexity of the intracellular structure (NIMN) (HR 0.80 [0.64, 0.98], $p = 0.033$), mean neutrophil size (NAMN) (HR 0.67 [0.55, 0.83], $p < 0.001$), mean corpuscular volume (MCV) (HR 1.35 [1.09, 1.66], $p = 0.005$), eGFR (HR 0.65 [0.52, 0.80], $p < 0.001$); and HDL-cholesterol (HR 0.62 [0.45, 0.85], $p = 0.003$) were related to MACE. NAMN was related to IPH (OR 0.83 [0.71−0.98], $p = 0.02$).

Conclusions: This is the first study to present a higher RDW and MCV and lower LACV, NIMN and NAMN as biomarkers reflecting inflammatory processes that may contribute to an increased risk of MACE after CEA.

1. Introduction

Large clinical trials show that carotid endarterectomy (CEA) is a beneficial therapy for the treatment of symptomatic carotid stenosis to reduce the risk of recurrent stroke [1]. However, despite carotid revascularization and cardiovascular risk management, patients that underwent CEA still have a residual risk of 13% of developing a secondary major adverse cardiovascular event (MACE) consisting of (non)fatal myocardial infarction, (non)fatal ischemic or hemorrhagic stroke, and any cardiovascular death [2–4]. Currently, there is evidence that the residual risk of secondary MACE can be further reduced, not only by more aggressive lipid lowering strategies with ezetimibe or PCSK-9 inhibitors [5], but also with anti-inflammatory agents [6]. Recent large clinical trials targeting inflammation in cardiovascular disease have showed that the incidence of secondary MACE can be reduced by anti-inflammatory agents such as canakinumab and colchicine [7]. These findings underline the important role of inflammation in atherosclerosis and the potential of anti-inflammatory agents [8].

Abbreviations: CEA, Carotid Endarterectomy; MACE, Major Adverse Cardiovascular Events; UPOD, Utrecht Patient-Oriented Database; RSF, Random Survival Forest; RDW, Red Blood Cell Distribution Width; NAMN, Mean Neutrophil Size; IPH, Intraplaque Hemorrhage.

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However, inflammatory processes related to secondary MACE in patients who underwent CEA are not yet fully understood. Therefore, determining whether and how patients benefit from secondary prevention therapies remains challenging [2,9]. In previous studies by us, various biomarkers closely linked to or involved in inflammatory processes, including CD14, SG1, FABP4, Osteopontin (SPP1), and intraplaque hemorrhage (IPH), were associated with the risk of recurrent MACE [10–13]. Additionally, blood cell counts such as neutrophil and granulocyte counts have been associated with the incidence of cardiovascular disease [14]. More recent studies identified various blood cell characteristics, including characteristics of inflammatory and non-inflammatory cells such as plateletets, erythrocytes and leucocytes including their subpopulations, associated with recurrent vascular events in patients with clinically manifest vascular disease and secondary MACE in coronary angiography patients [15,16]. A major advantage of the blood cell characteristics described in both studies, is that the markers are readily available for clinical practice since they are routinely measured by most hematology analyzers. However, these routinely and easily measured blood cell characteristics have not been investigated in relation to secondary MACE in patients that underwent CEA and thus underwent surgery to reduce their risk for recurrent events.

The use of hematology analyzers allows for the classification of platelets, erythrocytes and leucocytes and their counts, percentages, and morphological characteristics (e.g. cellular volume, granularity, and shape). On basis of these charactertics cells are automatically classified into different subpopulations (e.g. neutrophils, lymphocytes, eosinophils, basophils, and monocytes). In the current study, we combined 75 blood cell characteristics stored in the Utrecht Patient Oriented Database (UPOD) [17] with serum, plasma, and clinical data from CEA patients included in the Athero-Express biobank study [18]. We hypothesised that by examining associations between the blood cell characteristics and secondary MACE and correcting for possible clinical, serum and plasma confounders, we could identify blood cell characteristics that are closely related to inflammatory processes associated with an increased risk of MACE following CEA. Additionally, we investigated if the blood cells characteristics were associated with intraplaque hemorrhage, an important marker of plaque instability [4,19].

2. Materials and methods

2.1. Study population and data source

We included patients from the Athero-Express biobank, an ongoing prospective study of patients undergoing CEA for asymptomatic or symptomatic atherosclerotic carotid stenosis [18]. In short, consecutive patients were recruited from the St. Antonius Hospital Nieuwegein, Nieuwegein, the Netherlands and University Medical Center Utrecht in Utrecht, the Netherlands from 2002 onwards. Carotid endarterectomy was performed following recommendations by the ESVS guideline [1]. Patients that agreed to participate completed standardized baseline questionnaires about their medical history and medication use prior to surgery. Plaque samples were obtained during the CEA and analyzed as described below.

For this study, all patients that underwent CEA with available blood cell characteristic data from the Utrecht Patient Oriented Database (UPOD) were included (Fig. 1) [17]. The current study was conducted in accordance with the Declaration of Helsinki and was approved by the ethical boards of both hospitals. All patients provided written informed consent. The data that supports the findings of the current study are available through DataverseNL (https://dataverse.nl/dataverse/umculab).

2.2. Blood cell characteristics

Pre-operative blood samples were collected during hospital admission at baseline. Blood cell characteristics of the Sapphire routine blood cell analyzer were extracted (Abbott Diagnostics, Santa Clara, CA, USA). This analyzer allows for classification of platelets, erythrocytes and leucocytes and their counts, percentages, and morphological characteristics (e.g. cellular volume, granularity, and shape) on which basis cells are automatically classified into different subpopulations (e.g. neutrophils, lymphocytes, eosinophils, basophils, and monocytes). In total, 75 blood cell characteristics were available. A list of available blood cell characteristics is given in Supplemental Table S1.

2.3. Clinical data

Clinical parameters, including cardiovascular risk factors and medication use, were recorded at baseline through a questionnaire and from patient records. Coronary artery disease was defined as angina pectoris as defined by the Rose questionnaire, or history of myocardial infarction or coronary intervention [20]. Peripheral vascular disease was defined as intermittent claudication as defined by the Edinburgh questionnaire [21].

2.4. Outcome definition

MACE after CEA was defined as major adverse cardiovascular events: fatal or nonfatal myocardial infarction, fatal or nonfatal ischemic or hemorrhagic stroke, and any cardiovascular death, including sudden cardiac death, fatal rupture of an aortic aneurysm and fatal heart failure or other vascular death. These endpoints were assessed annually up to three years after CEA by means of questionnaires and review of medical records [18]. General practitioners or other treating physicians were contacted for follow-up data if necessary.

2.5. Histopathologic analysis of atherosclerotic plaque

Following CEA, the plaque samples were immediately transferred and processed according to a standardized protocol [22,23]. The culprit lesion was subjected to histopathological examination in 5 μm sections that were routinely stained [22,23]. Hematoxylin-Eosin (H&E) was used to obtain a general overview. IPH presence was defined as hemorrhage within the plaque tissue and was rated as being absent or present using H&E and Fibrin stainings [22,23]. Intra-observer and interobserver variability has been examined previously and showed good reproducibility (κ 0.6–0.9) for this semi-quantitative scoring method [23].

2.6. Modelling

2.6.1. Multicollinearity

The blood cell characteristic data contain multicollinearity as for example, white blood cell counts include neutrophil counts and segmented neutrophil counts, which are closely related. This may have implications for the analysis and should therefore be addressed [24–26]. Using the ClustOfVar R package [27], we identified clusters of blood cell characteristics within the data that were strongly related. We created synthetic variables using the first principal component that represented the variables in the clusters to eliminate multicollinearity. The suitable number of clusters was determined with a bootstrap approach, i.e. the stability function from the ClustOfVar package [27]. All variables were mean-centered.

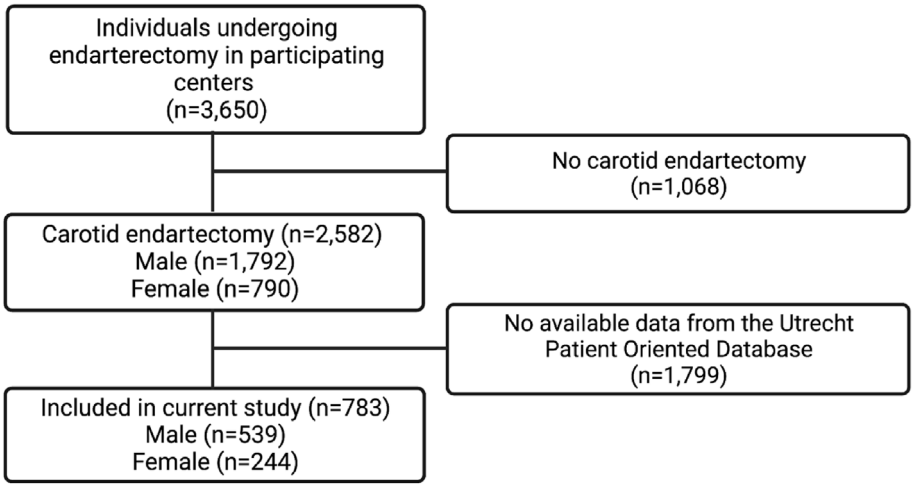

Fig. 1. Flowchart of number of individuals included in the analyses of the current study.

2.6.2. Variable selection

High-dimensional situations in combination with the strict proportional hazards assumption, makes Cox regression unsuitable for our research context. Instead, to perform an initial filtering of informative variables, we grew a Random Survival Forest (RSF), a nonlinear and nonparametric method that is suitable for high-dimensional settings [24,28] and which has proven its added value in clinical settings [29,30]. We trained an ensemble of tree-based survival models using the randomForestSRC R package [31], each tree (N = 500) was built on a different bootstrap sample (N = 50) of the training data, while leaving out an out-of-bag (OOB) sample. The default splitting rule (log-rank) was used. Variable importance was defined as the decrease in OOB C-index when a biomarker is randomly shuffled, which is indicative of how much the model depends on the biomarker. The entire procedure was repeated 100 times to calculate confidence intervals and standard errors for the variables importance's [32]. Variables with variable importances significantly larger than 0 were selected and used in further analyses.

2.6.3. Generalized additive model

Based on the selected variables from the previous steps, we used a generalized additive model (GAM) to incorporate other types of distributions in a survival model than strictly linear. This method is more flexible in capturing nonlinear relationships compared to the traditional proportional hazards regression model [33]. The cox.ph family within the gam() function from the mgcv R package was used to identify smooth functions for variables [34,35]. We applied this function to all the previously selected variables and determined whether the smooth term was significant with a p-value <0.05. Hazard ratios for the linear predictors were extracted by calculating the exponent of the beta coefficients and their 95% confidence intervals (CI).

2.6.4. Intraplaque hemorrhage

Given the previously described association between intraplaque hemorrhage (IPH) and increased risk of secondary MACE [13], we tested whether the variables significantly associated with secondary MACE in our study were also related to IPH using a logistic regression analysis.

3. Results

In total, 783 patients from the Athero-Express biobank with available blood cell characteristic data were included in the current study with an overall mean age of 69.5 years (SD 9.2 years), 31% was female (Table 1). Median follow-up was 3 years (IQR 2.8–3.0). In total, 97 patients (12%) developed MACE within 3 years of follow-up. An overview of the MACE component traits is given in Supplemental Table S2.

3.1. Random Survial Forest model selects twenty variables

The RSF model was used for initial variable selection based on the blood cell characteristics, serum and plasma makers and clinical data. The model consisted of twenty variables with variable importances larger than zero ($p < 0.05$) (Fig. 2). Of these twenty variables, two serum and plasma markers, i.e., total cholesterol, (CAD), high-density lipoprotein cholesterol (HDL-cholesterol), five clinical variables, i.e., history of peripheral intervention, Body Mass Index, eGFR (MDRD equation), history of coronary artery disease, and history of peripheral artery occlusive disease (PAOD) and thirteen blood cell characteristics were selected (Fig. 2).

3.2. Seven variables are significantly related to MACE in a Generalized Additive Model

We did not observe nonlinear effects between the previously selected variables and MACE after CEA in a GAM, but we did observe various linear relationships. Of these twenty selected variables the following seven variables were significantly associated with MACE after CEA in a GAM: red blood cell distribution width (RDW) (HR 1.23; 95% CI 1.02, 1.68; $p = 0.022$), coefficient of variance of lymphocyte size (LACV) (HR 0.78; 95% CI 0.63, 0.99; $p = 0.043$), the neutrophil complexity of the intracellular structure (NIMN) (HR 0.80; 95% CI 0.64, 0.98; $p = 0.033$), mean neutrophil size (NAMN) (HR 0.67; 95% CI 0.55, 0.83; $p < 0.001$), red blood cell mean corpuscular volume (MCV) (HR 1.35; 95% CI 1.09, 1.66; $p = 0.005$), eGFR (HR 0.65; 95% CI 0.52, 0.80; $p < 0.001$); and HDL-cholesterol (HR 0.62, 95% CI = 0.45, 0.85; $p = 0.003$) (Table 2, Fig. 3).

3.3. Mean neutrophil size is related to intraplaque hemorrhage

Of the seven selected variables that were signficiantly associated with MACE, only mean neutrophil size was significantly associated with intraplaque hemorrhage in a multivariate logistic regression model (OR = 0.83; 95% CI 0.71, 0.98, $p = 0.023$) (Table 3). The other six variables were not significantly associated with the presence of IPH.

Table 1
Baseline characteristics.

		Full cohort	No Major Adverse Cardiovascular Events	Major Adverse Cardiovascular Events
Variable	N	N = 783[1]	N = 686	N = 97
Age in years — Median (IQR)	783	70 (64, 77)	70 (63, 76)	72 (65, 78)
Male - n (%)	783	539 (69%)	478 (70%)	61 (63%)
Body Mass Index in kg/m^2 - Median (IQR)	776	26.1 (24.0, 28.4)	26.1 (24.0, 28.4)	26.4 (24.1, 29.4)
Systolic blood pressure in mmHg - Median (IQR)	643	150 (134, 170)	150 (135, 170)	150 (131, 170)
Diastolic blood pressure in mmHg - Median (IQR)	643	80 (70, 90)	80 (70, 90)	79 (69, 90)
Current smoker n (%)	756	251 (33%)	228 (33%)	37 (38%)
Hypertension - n (%)	772	570 (74%)	505 (74%)	75 (77%)
Diabetes - n (%)	783	186 (24%)	154 (22%)	32 (33%)
History of coronary artery disease - n (%)	782	260 (33%)	214 (31%)	47 (48%)
History of transient ischemic attack/stroke - n (%)	783	626 (80%)	549 (80%)	77 (79%)
History of peripheral arterial occlusive disease - n (%)	782	168 (21%)	143 (21%)	26 (27%)
History of peripheral intervention(s) - n (%)	780	160 (21%)	130 (19%)	31 (32%)
Statin use - n (%)	781	659 (84%)	581 (85%)	80 (82%)
Blood pressure medication use - n (%)	781	602 (77%)	520 (76%)	84 (87%)
Antiplatelet use - n (%)	779	682 (88%)	602 (88%)	84 (87%)
Dual antiplatelet use - n (%)	779	450 (58%)	418 (61%)	44 (46%)
Oral anticoagulants - n (%)	781	89 (11%)	77 (11%)	13 (13%)
Total cholesterol in mmol/L - Median (IQR)	389	4.27 (3.59, 5.22)	4.29 (3.30, 5.51)	4.40 (3.40, 5.90)
Low-Density Lipoprotein in mmol/L - Median (IQR)	348	2.48 (1.80, 3.20)	2.49 (1.70, 3.37)	2.52 (1.77, 3.50)
High-Density Lipoprotein in mmol/L - Median (IQR)	377	1.09 (0.89, 1.35)	1.10 (0.89, 1.36)	1.01 (0.88, 1.29)
Triglycerides in mmol/L - Median (IQR)	374	1.30 (0.98, 1.87)	1.30 (0.95, 1.84)	1.40 (1.08, 2.19)
Estimated Glomerular Filtration Rate in mL/min/1.73/m^2 - Median (IQR)	720	69 (56, 82)	71 (59, 83)	60 (47, 74)
C-Reactive Protein (high sensitive, plasma, mg/L)	400	2 (1, 5)	2 (1, 4)	2 (1, 6)

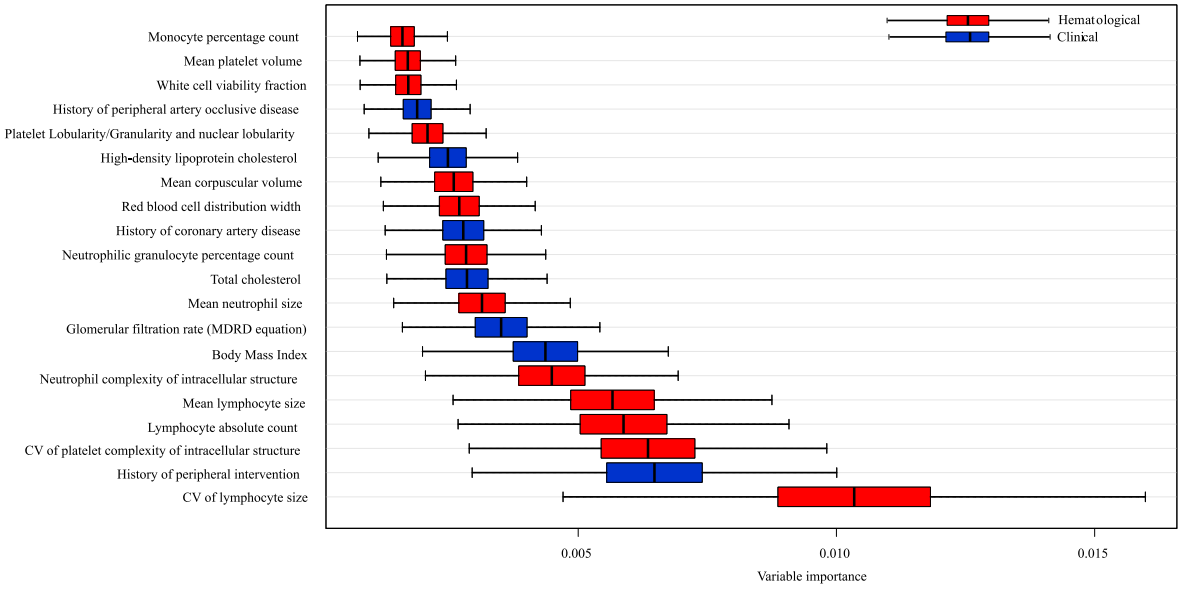

Fig. 2. Variable importances (VIMP) derived from the random forest survival analysis.
Clinical variables are shown in blue, hematological variables in red. VIMPs were calculated using the permutation method on out-of-bag (OOB) samples and defined as the drop in Harrell's C-index when the variable is shuffled randomly. Delete-d jackknife 99% asymptotic normal confidence intervals are shown. CV = coefficient of variance. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

In this study, we showed that multiple blood cell characteristics, including characteritics of non-inflammatory and inflammatory cell types were associated with an increased risk for MACE after CEA. We found that a higher red blood cell distribution width (RDW), a lower coefficient of variation of lymphocyte size (LACV), lower neutrophil complexity of the intracellular structure (NIMN), lower mean neutrophil size (NAMN), and higher red blood cell mean corpuscular volume (MCV) were independenty associated with an increased risk for MACE after CEA in multivariable analyses. Additionally, a lower NAMN was associated with the presence of IPH, which is a potent plaque marker for secondary MACE [22,36]. Thus taken together, a lower NAMN may represent a marker of

Table 2
Results of fitted Generalized Additive Model applied to a survival context.

Variable	HR[a]	95% CI[b]	p-value
CV of lymphocyte size	0.78	0.63, 0.96	**0.021**
History of peripheral intervention			
No			
Yes	1.42	0.80, 2.52	0.2
CV of platelet complexity of intracellular structure	1.09	0.88, 1.35	0.4
Lymphocyte absolute count	0.98	0.78, 1.23	0.9
Mean lymphocyte size	1.01	0.81, 1.27	>0.9
Neutrophil complexity of intracellular structure	0.80	0.64, 0.98	**0.033**
Body Mass Index	1.13	0.93, 1.39	0.2
Glomerular filtration rate (MDRD equation)	0.65	0.52, 0.80	**<0.001**
Mean neutrophil size	0.67	0.55, 0.83	**<0.001**
Total cholesterol	1.24	1.00, 1.55	0.054
Neutrophilic granulocyte percentage count	0.93	0.74, 1.18	0.6
History of coronary artery disease			
No			
Yes	1.53	0.98, 2.39	0.061
Red blood cell distribution width	1.23	1.02, 1.68	**0.022**
Mean corpuscular volume	1.35	1.09, 1.66	**0.005**
High-density lipoprotein	0.62	0.45, 0.85	**0.003**
Platelet lobularity/granularity and nuclear lobularity	0.97	0.74, 1.26	0.8
History of peripheral artery occlusive disease			
No			
Yes	0.86	0.48, 1.54	0.6
White cell viability fraction	1.00	0.81, 1.23	>0.9
Mean platelet volume	1.10	0.89, 1.35	0.4
Monocyte percentage count	1.17	0.97, 1.41	0.092

[a] HR = exp(Beta).
[b] CI = Confidence Interval.

higher risk for MACE after CEA and is associated with plaque hemorrhage.

4.1. Mean neutrophil size

Unfortunately the literature regarding neutrophil size in relation to atherosclerosis and MACE is scarce. However, increased counts of neutrophils have been significantly associated with cardiovascular mortality, even after correcting for smoking and other known risk factors [37]. In this study, we observed no significant association between neutrophil count and secondary MACE, neither between neutrophil count and mean neutrophil size in blood. We suggest that mean neutrophil size might be a more precise marker for neutrophil activation and processes leading to secondary MACE in this study population. On the contrary, absolute neutrophil count might be of more interest to differentiate between a healthy population versus a population with atherosclerosis and/or other inflammatory conditions but further research is required to confirm this. Aside from their counts and size, the actual contribution of neutrophils to the progression of atherosclerosis has long been unclear, partly because they are barely studied in human plaques and partly due to technical issues such as the lack of specificity of most antibodies to neutrophils and the fact that neutrophils have a very short lifespan due to their intrinsic nature [38]. However, there is increasing evidence that neutrophils play a role in atherosclerosis [39]. The contribution of neutrophils in atherosclerotic disease in humans appears to be primarily a feature of complicated advanced plaques and not, or at least to a much lesser extent, of intact or early lesions [40]. In response to injury, neutrophils secrete inflammatory mediators and produce threads of chromatin coated with granule-derived peptides and proteolytic enzymes; these are called neutrophil extracellular traps (NETs) [41]. The release of NETs is called netosis, which is an alternative form of cell death besides other types such as necrosis or apoptosis [42]. The presence of NETs appear to be mostly associated with eroded or erosion-prone carotid plaques [43]. Both neutrophils and NETs are present in plaques and their numbers are significantly higher in plaques with ongoing plaque hemorrhages [44]. In addition, elevated NET levels were associated with increased carotid fragility in patients who were not taking statins or antithrombotic medications prior to the index event [45]. Other analyses showed that this association was driven by IPH, lipid-rich necrotic core and ulceration [45]. One hypothesis could be, that the association of lower mean neutrophil size in relation to IPH could be explained by increased adhesion of circulating mature and larger neutrophils to plaque endothelium during plaque hemorrhage. Moreover, lower NAMN was also associated with an increased risk of MACE after CEA. Other studies suggest that the formation of NETs may promote thrombus mass growth after plaque rupture or erosion by providing a scaffold for erythrocyte binding and platelet aggregation [46,47]. These results and the possible migration of circulating neutrophils into plaque neutrophils might be an underlying plausible explaination for our finding. However, these hypotheses need scientific scrutiny in future studies.

4.2. Leucocyte markers

In the current paper we identified two other leucocyte markers, that were independently associated with secondary MACE. In general, the recruitment of leukocytes by attaching to activated endothelial cells and the release of pro-inflammatory cytokines characterize early processes involved in atherogenesis [48]. However leucocytes are also involved in inflammatory processes at later stages of atherogenesis [49]. Moreover, inflammatory pathways are intertwined with thrombosis, which is ultimately responsible for the late manifestations of atherosclerotic disease, such as myocardial infarction and stroke [50]. Studies on leucocyte characteristics and CVD have largely focused on blood cell counts and their association with the incidence of CVD risk [49]. For instance, previous studies have found that neutrophil counts correlate with severity of CAD and secondary MACE [14,51,52]. Unfortunately, the evidence regarding the association of lymphocyte size, and the neutrophil

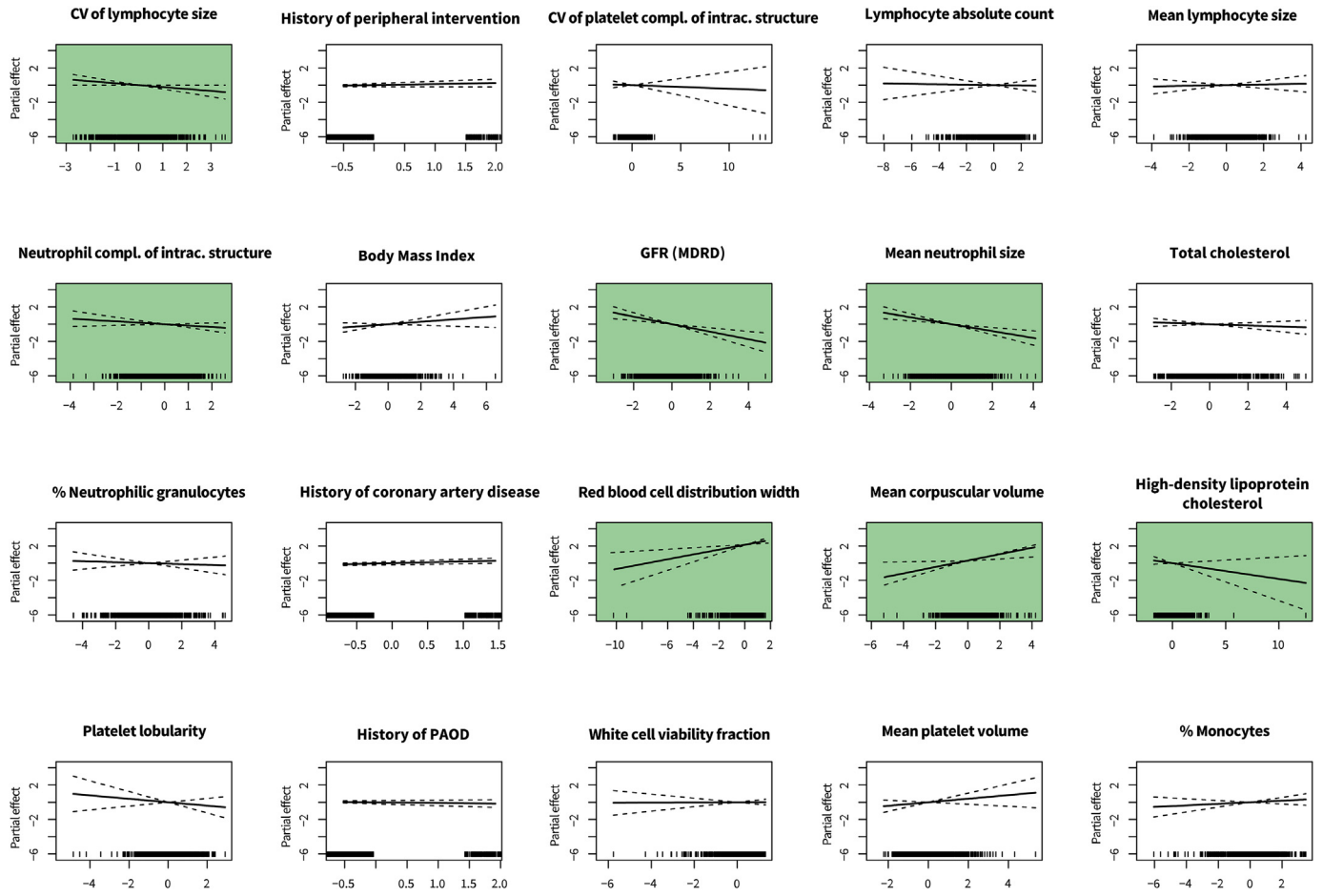

Fig. 3. Partial effects of selected explanatory variables on the occurrence of major adverse cardiovascular events after carotid endarterectomy.
The tick marks on the x-axis are observed data points. The y-axis represents the partial effect of each variable. The shaded areas indicate the 95% confidence intervals. The green colored plots represent the significant effects at a $p < 0.05$ level. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Results of logistic regression analysis to identify associations between markers related to MACE and intraplaque hemorrhage.

Variable	OR[a]	95% CI[a]	p-value
Coefficient of variation of lymphocyte size	1.02	0.87, 1.19	0.8
Glomerular filtration rate (Modification of Diet in Renal Disease equation)	0.91	0.77, 1.06	0.2
Neutrophil complexity of intracellular structure	1.11	0.95, 1.30	0.2
Mean neutrophil size	0.83	0.71, 0.98	**0.023**
Mean corpuscular volume	1.02	0.87, 1.19	0.8
High-density lipoprotein cholesterol	1.06	0.91, 1.25	0.4

[a] OR = Odds Ratio, CI = Confidence Interval.

complexity of the intracellular structure with secondary MACE is scarce. They might be manifestations of aberrant hematopoiesis, which was found to be associated with atherosclerotic CVD [53], and thus these leucocyte markers deserve more research.

4.3. Erythrocyte markers

Among the red blood cell (RBC) markers, we identified MCV and RDW as independent markers for secondary MACE. An increase in MCV, as a measure of the size of RBCs, has been previously identified as an independent predictor of compositie cardiovascular outcomes [54]. In addition, higher MCV was found to be associated with angiographically determined disease severity in patients with PAD [55]. The mechanism by which MCV may contribute to the presence and severity of the disease has not yet been determined. One possible route may be through an unhealthy lifestyle with associated low vitamin intake contributing to the development and progression of atherosclerotic disease. This notion is supported by studies showing that either vitamin B12 or folate deficiency impairs DNA synthesis, resulting in a decrease in cell divisions, which causes red blood cells to be larger than normal [56]. Both deficiencies have been related to atherosclerotic disease [57]. Moreover, smoking, a major risk factor for cardiovascular disease, was found to be associated in a dose-dependent manner with decreased circulating vitamin B12 and folate concentrations [58]. This indicates that MCV may be a surrogate marker for a lifestyle that increases the risk of cardiovascular events in general. Closely related to MCV, RDW was also identified as an independent marker

for MACE after CEA in the current paper. RDW has been linked to poor outcome in patients with coronary artery disease, stroke and peripheral arterial disease [16,59–62]. Ineffective erythropoiesis or a reduced life span of red blood cells may result in an increased RDW. Whether RDW is a cause or a consequence of CVD is unclear and needs further scrutiny, however, RDW may reflect low grade inflammation in atherosclerosis [63] or impaired kidney function [64].

4.4. Serum and plasma markers

Besides two hematological markers, two serum and plasma markers were associated with the risk for secondary MACE, i.e. the well-known cardiovascular risk factors impaired kidney function, and low HDL-cholesterol [10,65]. In a population of CEA patients, higher HDL-cholesterol levels were associated with a reduction in the risk of MACE after CEA, after adjusting for confounding variables [66].

4.5. Limitations and strengths

Limitations of the current study need to be addressed. First, since we only examined the blood cell characteristics data at one time point (baseline), we do not know whether blood cell abnormalities lead to plaque abnormalities or vice versa. Given the interaction of these blood cell characteristics with lifestyle-associated risk factors, it will be challenging to tease apart cause and effect. Second, tree-based models are able to pick up small signals from the data and effects may be caused by a small proportion of patients. Because we were specifically interested in identifying biomarkers associated with secondary MACE, which may ultimately provide clues about underlying biological processes of secondary MACE, we used the GAM model to filter out non-robust variables from the RSF model. A disadvantage of this is, that it is done on the same dataset (as to the best of our knowledge a similar, independent dataset does not exist), this may limit the generalizability of the results. However, we used cross-validation procedures within the modeling to avoid overfitting as much as possible. Ignoring multicollinearity can have a major impact on the results, especially on the calculation of permutation imports, and is not usually considered in many studies in a tree-based (survival) context, yet strongly recommended [67]. We paid attention to and eliminated multicollinearity in our data, which represents a methodological strength of this study. Third, we refrained from including individual outcomes in the composite endpoint MACE because of power limitations. To have sufficient discriminatory power, we chose the composite variable MACE during the first 3 years as the endpoint variable. Future studies should address the individual composite traits of MACE, to better understand the effects between hematologic variables and these individual composite traits. Finally, little research has been done with such an extensive dataset of blood cell characteristics, making it challenging to link biological mechanisms to blood cell characteristics. Therefore, more research is needed to gather substantiated evidence, particularly on average neutrophil size in relation to MACE in CEA patients. A strength of the blood cell characteristics used in the current study is that they are readily measurable with standard hematological analyzers and thus routinely available, which may facilitate the application of blood cell characteristics in daily clinical practice.

4.6. Future perspectives

Since the hematological markers in this study are often readily measurable with standard hematological analyzers, they offer potential for both clinical application and further research. The current study suggests that leucocyte and erythrocyte markers, such as mean neutrophil size and mean corpuscular volume contain valuable information about the clinical status of CEA patients. These markers should be further investigated to study the underlying biological mechanisms involved and to see if they can help identify patients at high residual risk.

5. Conclusion

This is the first study to present routinely measured blood cell characteristics, including leucocytes and erythrocytes, such as higher RDW and MCV and lower LACV, NIMH, NAMN as novel, independently associated biomarkers reflecting inflammatory processes that may contribute to increased risk of MACE after CEA.

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Authorship contribution statement

Conception and design: LMO, JMM, SWvdL, SH.
Analysis and interpretation: LMO, JMM, SdJ, SWvdL, SH.
Data extraction: CHV.
Drafting of manuscript: LMO, JMM, SWvdL, SH.
Critically revising of manuscript: SWvdL, SH, WvS, SdJ, CHV, GJdB, DPVdK, IEH.
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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.athplu.2023.05.003.

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