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Predicting Carotid Artery Disease and Plaque Instability from Cell-derived Microparticles

A.L. Wekesa [a], K.S. Cross [b,c], O. O'Donovan [a], J.F. Dowdall [b], O. O'Brien [b], M. Doyle [b], L. Byrne [b], J.P. Phelan [a], M.D. Ross [a], R. Landers [b], M. Harrison [a,*]

[a] Biomedical Research Group, Schools of Health Science and Science, Waterford Institute of Technology, Waterford, Ireland
[b] Departments of Vascular Surgery and Histopathology, Waterford Regional Hospital, Waterford, Ireland
[c] Royal College of Surgeons in Ireland, Dublin, Ireland

WHAT THIS PAPER ADDS

Vascular cells shed membranous vesicles into the circulation associated with atherothrombotic processes that have the potential to act as disease biomarkers. This pilot study demonstrates the potential of microparticles shed from endothelial cells to predict plaque instability in patients undergoing carotid endarterectomy. The study adds to a previous study demonstrating similar predictive ability of leucocyte microparticles. Ultimately, microparticles may assist in the selection of carotid patients for surgical intervention.

Objectives: Cell-derived microparticles (MPs) are small plasma membrane-derived vesicles shed from circulating blood cells and may act as novel biomarkers of vascular disease. We investigated the potential of circulating MPs to predict (a) carotid plaque instability and (b) the presence of advanced carotid disease.

Methods: This pilot study recruited carotid disease patients (aged 69.3 ± 1.2 years [mean ± SD], 69% male, 90% symptomatic) undergoing endarterectomy ($n = 42$) and age- and sex-matched controls ($n = 73$). Plaques were classified as stable ($n = 25$) or unstable ($n = 16$) post surgery using immunohistochemistry. Blood samples were analysed for MP subsets and molecular biomarkers. Odds ratios (OR) are expressed per standard deviation biomarker increase.

Results: Endothelial MP (EMP) subsets, but not any vascular, inflammatory, or proteolytic molecular biomarker, were higher ($p < .05$) in the unstable than the stable plaque patients. The area under the receiver operator characteristic curve for $CD31^{+}41^{-}$ EMP in discriminating an unstable plaque was 0.73 (0.56–0.90, $p < .05$). $CD31^{+}41^{-}$ EMP predicted plaque instability (OR = 2.19, 1.08–4.46, $p < .05$) and remained significant in a multivariable model that included transient ischaemic attack symptom status. Annexin V^{+} MP, platelet MP (PMP) subsets, and C-reactive protein were higher ($p < .05$) in cases than controls. Annexin V^{+} MP (OR = 3.15, 1.49–6.68), soluble vascular cell adhesion molecule-1 (OR = 1.64, 1.03–2.59), and previous smoking history (OR = 3.82, 1.38–10.60) independently ($p < .05$) predicted the presence of carotid disease in a multivariable model.

Conclusions: EMP may have utility in predicting plaque instability in carotid patients and annexin V^{+} MPs may predict the presence of advanced carotid disease in aging populations, independent of established biomarkers.

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INTRODUCTION

The ability to identify carotid plaques that confer increased risk would greatly assist in the selection of patients for carotid artery endarterectomy or stenting. Simple quantification of carotid artery disease based on the degree of stenosis has limitations and there is a need to look beyond this parameter when assessing stroke risk.[1] As atherosclerosis is a multifocal systemic disease that is associated with diffuse lesions in the arterial tree, it is likely that plaque characteristics at one vascular site are reflective of plaque at other sites[2] with plaque instability potentially influenced by systemic processes.[3] Molecules associated with these atherothrombotic processes, released into the circulation, have the potential to identify the presence of atherosclerosis and the likelihood of an unstable plaque that would benefit from surgical intervention. Several molecular biomarkers of carotid artery disease, reviewed elsewhere,[1] have been identified that relate to the presence of disease, the degree of stenosis, plaque vulnerability, symptom status, and stroke risk. These include soluble cell adhesion molecules and selectins, inflammatory markers, cytokines, and matrix metalloproteinases (MMPs).

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* Corresponding author.
E-mail address: mharrison@wit.ie (M. Harrison).

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Table 1. Descriptive characteristics (raw counts with percentages in parenthesis) of controls and cases, with cases subdivided by those with stable and unstable plaques.

	Controls ($n = 73$)	Cases ($n = 42$)	Stable ($n = 25$)	Unstable ($n = 16$)
Gender (%)				
Male	40 (55%)	29 (69%)	18 (72%)	11 (69%)
Age (years) mean ± SD	71.1 ± 0.9	69.3 ± 1.2	70.3 ± 1.6	68.1 ± 2.0
Smoking status (%)				
Former	24 (33%)	23 (55%)	14 (56%)	9 (56%)
Current	9 (12%)	11 (26%)	5 (20%)	6 (38%)
Never	40 (55%)	8 (19%)*	6 (14%)	1 (6%)
Medications (%)				
Anti-hypertensive	24 (33%)	37 (88%)*	22 (88%)	14 (88%)
Anti-platelet/anti-coagulant	14 (19%)	42 (100%)*	25 (100%)	16 (100%)
Lipid lowering	34 (45%)	39 (93%)*	23 (92%)	15 (94%)
Glucose control	3 (4%)	5 (12%)	5 (20%)	0
Cases				
Main diagnosis (%)				
Asymptomatic			4 (16%)	0
Amaurosis fugax			5 (20%)	1 (6%)
Transient ischaemic attack			16 (64%)	15 (94%)†
High creatinine (>90 µmol/L)			10 (40%)	6 (38%)

*$p < .05$ compared to control group.†$p < .05$ compared to stable plaque group. Comparisons evaluated using the chi-square test.

Cell-derived microparticles (MPs) are putative vesicular biomarkers of atherosclerotic disease. MPs are vesicles, 0.1–1.0 µm diameter, that are shed from the plasma membrane of vascular cells including platelets, endothelial cells, erythrocytes, and leucocytes. Triggers for vesicle budding and release include cell activation and apoptosis. Increased levels of endothelial microparticles (EMPs),[4] platelet microparticles (PMPs),[5] and leucocyte microparticles (LMPs)[6] have been reported in the plasma of patients with atherosclerotic diseases compared with matched controls. In addition, there is some evidence that MP can discriminate vascular disease severity and damage.[4,7–9] Only two studies to date have investigated MP in relation to carotid artery disease plaques. Using an indirect assessment of PMP, Michelsen and colleagues[10] reported no difference in PMPs between those with echogenic (highly fibrous calcified plaques) and echolucent plaques (plaques with high lipid content). In contrast, Sarlon-Bartoli and colleagues[11] demonstrated potential for a LMP subset in discriminating an unstable plaque.

The purpose of this study was to determine the ability of EMP and PMP to predict carotid plaque instability in patients undergoing endarterectomy and the presence of advanced carotid disease in a cohort of cases and matched controls. In addition to MP, a range of more frequently measured molecular biomarkers were measured to determine the additional value that might result from including MP in multivariable prediction models.

METHODS

Study overview

This prospective case–control study recruited men and women with (cases) and without (controls) documented carotid artery disease. The study set out to include all patients undergoing carotid endarterectomy at the regional vascular surgery unit over a 2-year period. Of these 46 patients, three could not be recruited for logistical reasons, and 42 of the remaining 43 patients consented to taking part. Post surgery, the excised plaques were graded as stable or unstable using immunohistochemistry based on the protocol of Redgrave et al.[12] that assesses inflammatory cell infiltration in the plaque and cap, evidence of plaque rupture, and surface thrombus. One plaque was incorrectly processed and lost to analysis. When carotid endarterectomy was performed on both the right and left sides ($n = 6$), only the first surgery data were included in the biomarker analyses, as surgical injury could potentially influence MP counts. Both plaques were graded however. The controls ($n = 73$) were matched for age and sex and recruited from a local general practice and the locality.

Study population and ethics

The descriptive characteristics of the participants are summarized in Table 1. Clinical and paraclinical data for the cases was obtained from medical charts. Of the 41 cases, 37 were symptomatic with transient ischemic attack (TIA), the most common presenting symptom. The median lag time between the development of symptoms and surgery was 21 (interquartile range 11–58) days. The only exclusion criterion for carotid patients was an inability to consent because of greatly diminished cognitive function. The exclusion criteria for controls included a history of coronary artery disease, cerebrovascular disease, peripheral arterial disease, aneurysmal disease, or cancer. Suitable controls were identified by a general practitioner from a local practice, and the absence of exclusion criteria was also verified by self-report. Information on prescribed medication classes and smoking history was obtained from controls using a

short tick-box questionnaire, completed with the assistance of study investigators. Written informed consent was obtained from all participants, and the study was approved by an institutional and a hospital ethics committees.

Blood collection and processing

All blood samples were taken in the morning following an overnight fast, with cases sampled on the morning of surgery. The first 3.0 mL was discarded. Samples were kept at room temperature prior to centrifugation and processed within 2 hours of collection. Cases and controls continued to take prescribed medications in the days leading up to blood sampling. However, with the exception of aspirin, anti-platelet, and anti-coagulant medications ceased to be taken by cases 1 week prior to surgery.

The serum tubes were centrifuged for 15 minutes at 1500*g* at 20 °C. The upper part of the serum and plasma was collected down to 1 cm above the buffy coat layer. The sodium citrate tubes (3.2%), for MP analysis, underwent a double centrifugation protocol. They were first centrifuged for 15 minutes at 1500*g* at 20 °C, followed by 2 minutes at 13,000*g* at 20 °C to produce platelet poor plasma, with the pellet discarded. Aliquots of ~350 μL of serum and plasma were stored at −80 °C for later analysis.

Vascular imaging of arteries

The carotid arteries were examined for the presence of plaque in the common, internal and external vessels and at the bifurcation area bilaterally. Vertebral arteries were checked for patency. Blood flow velocity was measured at a standard 60° interrogation angle. The percentage degree of narrowing (stenosis) in the blood vessel was calculated based on the Strandness criteria. Control scans were reported as abnormal when the percentage vessel stenosis was >70%. One control volunteer with >70% stenosis was excluded from the study.

Immunohistochemical analysis of plaques

Plaques were collected immediately after excision, and preserved in 10% buffered formalin until processed. Processing of the plaques was undertaken not more than 24 hours after collection. All plaques were fixed in 10% formalin for 24 hours and decalcified using formic acid for a further 24 hours. The area of maximum disease was identified macroscopically, typically the thickest part of plaque, or where the lumen was most narrowed, or any obvious thrombus. Representative transverse sections were taken from these areas (3—4 mm thick each) and then embedded in paraffin wax in an automated embedder (Tissue-Tek AutoTEC Sakura). These blocks were sectioned at ~3 μm and immunostained on the Leica BOND-MAXTM automated immunostainer using the Bond Polymer Refine Detection kit. The sections were analysed for the presence of macrophage and T-cells using CD68 (Dako Denmark, clone PG-M1, dilution 1:50) and CD3 (Leica Clone LN-10, pre-diluted) antibodies respectively.

Grading of the plaques

Plaques were graded based on a previously published method, using semi-quantitative scales.[12] For this study, four immunohistological features were associated with unstable plaques. These were the presence of inflammatory cells (macrophage or lymphocytes stained respectively with CD68 or CD3 antibody) in the plaque core or plaque cap area, cap rupture (a break or erosion of the fibrous cap overlying the lipid core) or surface thrombus (identified adherent to the luminal surface of the plaque). The inflammatory cell proliferation in the plaque core and in the cap was graded separately on a scale of 1—3 for both CD68 and CD3 content. Grades 1, 2, and 3 represented occasional cells, one group of >50 cells, and two to five groups of >50 cells respectively. This gave a total inflammatory cell score of between 4 and 12, with 12 representing high CD68 and CD3 content in both the plaque core and the cap area. Plaques were regarded as unstable if they had any of the following: inflammatory cell score of between 9 and 12, evidence of cap rupture, or evidence of surface thrombus. The histopathologist classifying the plaques was blinded to the presenting symptom (if any) of the case and to all biomarker concentrations.

Microparticle and molecular biomarker enumeration

MPs were defined based on size (<1.0 μm) and fluorescence using flow cytometry. Annexin V binds to negatively charged phospholipids and annexin V^+ events within this size gate represent MP shed from various vascular cell types undergoing apoptosis. Annexin V^+ MP and two different subsets of PMP were enumerated, $CD41^+$ PMP and $AnnexinV^+CD41^+$ PMP, from thawed unwashed plasma. Plasma samples were washed twice in PBS-citrate prior to EMP enumeration. Two different EMP subsets were enumerated, MP expressing CD31 but not expressing the platelet marker CD41 ($CD31^+CD41^-$ EMP) and MP expressing one or more of the endothelial specific markers CD144, CD146, CD105 in a monochrome multimarker assay (mmEMP). This monochrome multimarker approach was previously employed[13] to improve the signal to noise ratio, given the low number of these endothelial specific markers on particles of small surface area. Soluble vascular injury, and angiogenic and proteolytic biomarkers were analysed in duplicate using commercially available immunoassays (Meso Scale Discovery, Rockville, MD, USA) employing multiplexing technology and electrochemiluminescence detection.

Statistics

As the majority of biomarkers were not normally distributed (Kolmogorov—Smirnov test), non-parametric statistics were employed. Data are presented as median (inter-quartile range). Between group differences were compared using the Mann—Whitney U test and the chi-square test for categorical variables. The discriminative ability of putative biomarkers was quantified with reference to the concordance statistic (C-statistic) of each receiver operator characteristic curve (ROC), which represents the area under the curve (AUC). Multiple

Table 2. Microparticle and molecular biomarkers in carotid artery disease cases and matched controls with cases subdivided into those with stable and unstable plaques.

Biomarker	Controls ($n = 73$)	Stable plaque ($n = 25$)	Unstable plaque ($n = 16$)	Combined cases ($n = 42$)
Microparticles				
Annexin V^+ MP (events/μL)	632 (377–812)	799 (477–1426)	1013 (381–1643)	805 (428–1427)*
$CD41^+$ PMP (events/μL)	358 (233–571)	600 (340–745)	535 (355–1369)	595 (34 2–933)*
AnnexinV+$CD41^+$ PMP (events/μL)	248 (164–450)	406 (220–686)	438 (132–1139)	413 (200–792)*
mmEMP* (events/μL)	1.13 (0.55–2.09)	0.73 (0.40–1.61)	1.88 (1.05–2.97)†	1.05 (0.53–2.08)
$CD31^+41^-$ EMP (events/μL)	0.75 (0.54–1.31)	0.62 (0.33–1.02)	1.30 (0.60–1.77)†	0.72 (0.41–1.33)
Molecular biomarkers				
C-reactive protein (mg/L)	1.49 (0.68–6.08)	3.3 (0.9–11.2)	4.2 (1.1–11.3)	3.06 (1.01–11.06)*
Serum amyloid A (ng/mL)	1617 (765–3748)	2166 (1091–5318)	1942 (1193–16182)	2102 (1167–5207)[a]
sVCAM-1 (ng/mL)	368 (270–472)	437 (353–567)	396 (285–529)	411 (338–550)[b]
sICAM-1 (ng/mL)	107 (84–152)	140 (104–166)	128 (99–158)	132 (101–162)
sE-selectin (ng/mL)	14.9 (10.4–18.0)	13.3 (10.6–15.1)	12.7 (6.1–17.1)	13.0 (9.6–16.5)
VEGF (pg/mL)		519 (288–869)	338 (234–926)	
VEGF-C (pg/mL)		453 (396–520)	410 (282–638)	
VEGF-D (pg/mL)		859 (699–1060)	882 (686–1224)	
PIFG (pg/mL)		23.8 (21.0–28.0)	19.2 (18.6–26.4)	
Tie-2 (pg/mL)		4598 (3765–5527)	5014 (4100–6132)	
sFlt-1 (pg/mL)		109 (85–134)	123 (98–175)	
bFGF (pg/mL)		6.4 (3.3–8.7)	3.8 (2.1–5.2)	
MMP-1 (pg/mL)		20213 (9802–24484)	20097 (9333–26587)	
MMP-2 (pg/mL)		83894 (73926–95042)	72666 (62181–90010)	
MMP-3 (pg/mL)		11926 (9393–16039)	13365 (9676–16756)	
MMP-9 (ng/mL)		210 (158–386)	197 (172–370)	
MMP-10 (pg/mL)		1349 (977–1915)	1738 (1330–2372)	

Note. Values are median (inter-quartile range). MP = microparticles; PMP = platelet microparticles; EMP = endothelial microparticles; mmEMP, monochrome multimarker EMP, MP positive for one or more of the endothelial markers CD144, CD146, or CD105; ICAM = intercellular adhesion molecule; MMP = matrix metalloproteinase; sFlt-1 = soluble fms-like tyrosine kinase-1; VEGF = vascular endothelial growth factor; sVCAM-1 = soluble vascular cell adhesion molecule-1, bFGF = basic fibroblast growth factor, PlGF = placental growth factor. *$p < .05$ cases vs. controls; [a]$p = 0.067$ cases vs. controls; [b]$p = 0.087$ cases vs. controls; †$p < .05$ stable vs. unstable.

logistic regression analysis with forward stepwise entry of variables (likelihood ratio) was used to identify the independent predictors of unstable plaques and carotid artery disease. Into the model to predict unstable plaques were entered eligible ($p < .10$) biomarkers and categorical variables ($CD31^+41^-$ EMP and TIA symptom status) following single logistic regression analyses. Into the model to predict carotid artery disease cases were entered eligible ($p < .10$) biomarkers (annexin V^+ MP, $CD41^+$ PMP, annexin V^+CD41^+ PMP, mm EMP, CRP, SAA and sVCAM-1 and smoking history) from single logistic regression analyses. This multivariable analysis was undertaken with and without the entry of aspirin (other anti-platelet agents had been withdrawn), anti-lipaemic medications, and anti-hypertensive medications as confounding variables. Odds ratios (ORs) and 95% confidence intervals (CIs) for these logistic regression analyses are expressed per standard deviation biomarker increase. The study was designed to detect a 25% difference in annexin V^+ MP counts with 40 cases and 60 controls, assuming a statistical power of 0.80 and $p < .05$, based on previous laboratory data. Significance was set as $p < .05$.

RESULTS

Of the 41 carotid endarterectomy cases, 37 (90%) were symptomatic and four (10%) asymptomatic, 25 (61%) with stable and 16 (39%) with unstable plaques (Table 1). All of the asymptomatic cases had stable plaques. In five of the six cases where carotid artery endarterectomy was required on the left and right sides, the carotid plaques had the same stable/unstable classification. The percentage of cases with a primary diagnosis of TIA was higher ($p = .03$) in the unstable plaque group (Table 1).

$CD31^+CD41^-$ EMP and mmEMP were 2.1- and 2.6-fold higher ($p < .05$) respectively in patients with unstable compared with stable plaques (Table 2). Annexin V^+ MP, $CD41^+$ PMP, and annexin V^+CD41^+ PMP were not different in the unstable compared to the stable plaque group (Table 2). None of the vascular, inflammatory, angiogenic, or proteolytic markers measured were different between these groups (Table 2). $CD31^+41^-$ EMPs were also higher in patients with TIA symptoms than in those who were asymptomatic (Fig. 1). No MP subset was different in patients that underwent endarterectomy within 21 days (median lag time) of developing symptoms compared to those with later surgeries. The C statistics for the ROC curves based on $CD31^+41^-$ EMP and mmEMP were 0.73 (CI 0.56–0.90, $p = 0.016$) and 0.73 (CI 0.58–0.89, $p = 0.012$) respectively (Fig. 2). Using single logistic regression analysis, only $CD31^+41^-$ EMP (OR = 2.34, CI 1.18–4.66, $p = .015$) significantly predicted an unstable plaque, though TIA

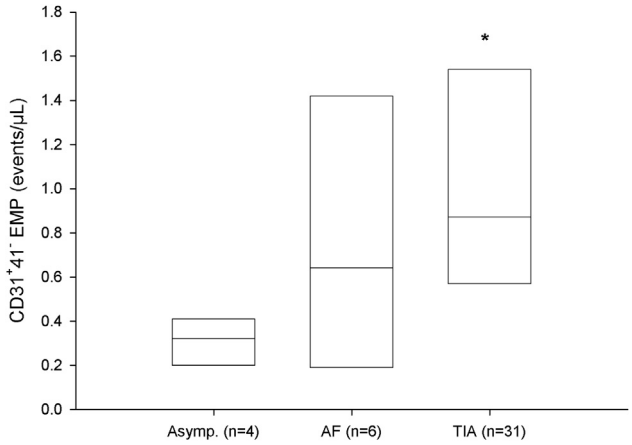

Figure 1. $CD31^{+}41^{-}$ endothelial microparticles in carotid artery disease patients who presented with no symptoms, amaurosis fugax, and transient ischemic attack. *Note*. Values are median (interquartile range). Asymp. = no symptoms; AF = amaurosis fugax; TIA = transient ischaemic attack. *$p < .05$ compared to asymptomatics.

Table 3. Predicting carotid artery disease from microparticle and molecular biomarkers.

Biomarker	ROC AUC (95% CI)	*p*
Microparticles		
Annexin V^{+} MP	0.68 (0.57—0.79)	0.002
$CD41^{+}$ PMP	0.68 (0.57—0.79)	0.002
$AnnexinV^{+}CD41^{+}$ PMP	0.66 (0.55—0.77)	0.01
mm EMP	0.53 (0.41—0.65)	0.60
$CD31{+}41^{-}$ EMP	0.52 (0.41—0.64)	0.70
Molecular biomarkers		
C-reactive protein	0.63 (0.52—0.74)	0.02
sVCAM-1	0.61 (0.50—0.72)	0.06
Serum amyloid A	0.60 (0.49—0.71)	0.08
sICAM-1	0.59 (0.47—0.70)	0.14
sEselectin	0.41 (0.30—0.53)	0.14

ROC AUC = receiver operator characteristic curve area under curve; CI = confidence interval; MP = microparticles; PMP = platelet microparticles; EMP = endothelial microparticles; mmEMP = monochrome multimarker EMP, MP positive for one or more of the endothelial markers CD144, CD146, or CD105.

symptom status (OR = 8.45, CI 0.95—74.85, $p = 0.055$) met the criterion for inclusion in the multivariable model. Using multiple logistic regression analysis, $CD31^{+}41^{-}$ EMP remained a significant predictor of an unstable plaque (OR = 2.19, CI 1.08—4.46, per SD increase, $p = .03$) even with TIA symptom status in the model.

Annexin V^{+} MP, $CD41^{+}$ PMP, annexin $V^{+}CD41^{+}$ PMP, and C-reactive protein (CRP) were higher ($p < 0.05$) in cases than controls (Table 2). $CD31^{+}CD41^{-}$ EMP, mmEMP, soluble E-selectin (sE-selectin), soluble intercellular adhesion molecule (sICAM-1), soluble vascular cell adhesion molecule (sVCAM-1), and serum amyloid A (SAA) ($p = 0.067$) were not different in cases and controls (Table 2). The prevalence of previous smoking history and of anti-lipaemic, anti-hypertensive, and anti-platelet therapy was higher ($p < .05$) in the cases than controls (Table 1). The ability of MPs and molecular biomarker variables to discriminate carotid artery disease cases from controls is shown in Table 3. The C statistic for the ROC curve was significant ($p < .05$) for annexin V^{+} MP, $CD41^{+}$ PMP, and CRP and marginally higher for the MPs (0.66—0.68) than the molecular (0.61—0.63) markers. Annexin V^{+} MP (OR = 3.15, 1.49—6.68, $p = .003$), sVCAM-1 (OR = 1.64, 1.03—2.59, $p = .03$), and previous smoking history (OR = 3.82, 1.38—10.60, $p = .01$) were significant in a multiple logistic regression model to predict carotid artery disease. When aspirin, anti-lipaemic medications, and anti-hypertensive medications were entered into the model, annexin V^{+} MPs (OR = 10.56, 2.07—53.75, $p = .005$) and CRP (OR = 2.44, 1.17—5.11, $p = .018$) emerged as the independent biomarker predictors.

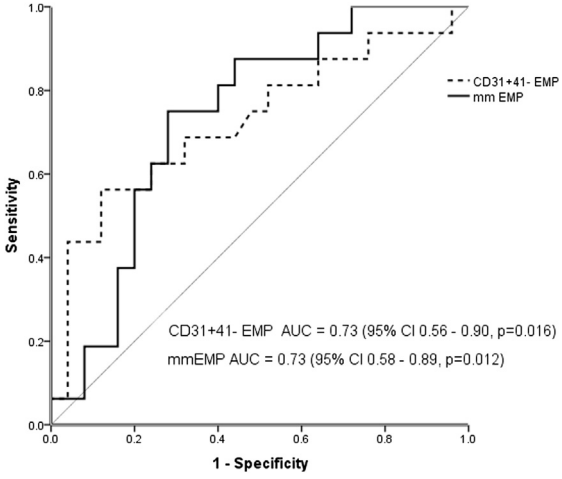

Figure 2. Receiver operator characteristic curve to discriminate an unstable carotid plaque based on $CD31^{+}41^{-}$ EMP and mmEMP. *Note*. EMP = endothelial microparticles; mmEMP = monochrome multimarker EMP, MPs positive for one or more of the endothelial markers CD144, CD146, or CD105.

DISCUSSION

This was a pilot study to examine the predictive potential of MPs, novel biomarkers, in carotid artery disease. When patients with stable and unstable carotid plaques were compared, the $CD31^{+}41^{-}$ EMP and the mmEMP subsets were higher in the unstable plaque group. The area under the ROC curve was similar for both EMP subsets. $CD31^{+}41^{-}$ EMPs were also significantly higher in patients presenting with TIA than those who were asymptomatic, albeit with a very small sample size of asymptomatics. $CD31^{+}41^{-}$ EMPs emerged as the single significant biomarker of an unstable plaque in a multivariable analysis, and remained significant even after adjusting for TIA symptom status. In contrast, none of the vascular inflammatory, proteolytic, or angiogenic factors measured were different between these groups. When biomarker concentrations in carotid artery disease cases and matched controls were compared, annexin V^{+} MPs, $CD41^{+}$ MPs, annexin $V^{+}CD41^{+}$ MPs, and the inflammatory marker CRP were all higher in the cases.

Annexin V^+ MPs and sVCAM-1 independently predicted advanced carotid artery disease, but not the specific PMP subsets. This is not surprising as the majority of annexin V^+ events are derived from platelets.[14]

Different EMP and PMP subsets were enumerated in this study, an approach not uncommon in the MP literature. A limitation of MP research is the wide range of definition markers and marker combinations that are used in clinical studies to identify MPs, particularly EMPs. Indeed, there is evidence that specific EMP subsets can reflect different pathophysiological processes.[15] Both EMP subsets but not PMPs were higher in those with unstable than stable plaques whereas both PMP subsets and annexin V^+ MPs (mainly PMPs) but not EMPs were higher in cases than controls. $CD31^+41^-$ EMPs emerged in this study as the predictor of an unstable plaque, and annexin V^+ MP as the most consistent predictor of advanced carotid disease present in two different models. Ideally, the same MP subset would predict an unstable plaque and the presence of advanced carotid disease. However, biomarkers need not have universal utility and different cardiovascular biomarkers may be needed to screen for a disease, identify unstable patients and monitor treatment.[16]

Mechanistic studies clearly indicate that MPs are more than cellular debris shed from diseased vascular tissue. EMPs harbour functional molecules that are involved in inflammation, coagulation, endothelial cell function, and angiogenesis.[17] EMPs generated in vitro expose MMP-2 and MMP-9, potentially contributing to plaque destabilization.[18] EMPs also promote premature endothelial cell senescence.[19] Thus, in addition to their biomarker role, MPs may be mediators of disease progression in the carotid artery and also at other sites throughout the vascular tree.

The study employed an extensive panel of molecular markers for comparison purposes with the MP markers. As the immunoassay techniques to quantify these molecular biomarkers are better established and more widely available than the flow cytometry techniques used to enumerate MPs, MPs need to clearly add to risk prediction. CRP and sVCAM-1 featured in different multivariable models to predict carotid disease cases, but the combination of molecular markers did not displace annexin V^+ MP from either model. None of the proteolytic, vascular inflammatory, or angiogenic markers analysed predicted plaque instability. Contrary to previous studies,[20,21] the MMP enzymes analysed were not different between plaque groups, even though a full MMP panel was assessed. Of note, no cytokines were quantified. We cannot therefore be certain that MPs would emerge as independent predictors had our models included cytokines and chemokines that have demonstrated potential in identifying unstable plaques such as CCL5[22] and TNF-α.[23]

A clear limitation of the study, indeed of many case—control biomarker studies, is the potential confounding effect of prescribed medications on biomarker concentrations. Anti-lipaemic, anti-platelet, and anti-hypertensive medication usage was considerably greater in the group of cases. Studies generally show a reduction in MPs after treatment with statins,[24] clopidogrel,[25] and cilostazol[26] but not aspirin.[27] This potential confounding effect was examined by undertaking the multiple logistic regression analysis with and without prescribed medications as confounding factors. Annexin V^+ MP was significant in both models though the odds ratio was considerably greater when prescribed medications were entered. Soluble VCAM-1 was significant in the first model but displaced by CRP when prescribed medications were entered. Prediction models that do not include biomarker-lowering medications as covariates potentially underestimate biomarker predictive values. There are dilemmas however in adjusting for multiple confounders in small datasets, especially as nearly all cases were using aspirin, anti-lipid, and anti-hypertensive agents. Adjusted ORs can be inflated in these circumstances, and the problems caused by further adjustment can begin to outweigh the confounding controlled.[28] In addition, biomarkers need to be able to predict increased risk of disease or disease stage even in heavily medicated populations for them to have clinical utility. A conservative approach is sometimes warranted by excluding medications from analyses.

The small sample size, particularly for the stable—unstable comparison, is another limiting factor in this study. Although clear potential has been demonstrated for $CD31^+41^-$ EMP and annexin V^+ MP, there is not sufficient statistical power to demonstrate their superiority over other subsets that were also significantly different in the stable—unstable and case—control comparisons. In addition, we did not regard it as appropriate to identify specific risk cut-offs in a small sample and to determine the discriminative value associated with these cut-offs from this same cohort. To our knowledge, only one other study,[11] employing a similar sample size, demonstrated the potential of one LMP subset to distinguish stable from unstable carotid plaques graded post endarterectomy. These studies should be regarded as complementary, together demonstrating the potential of specific EMP and LMP subsets but not PMP to predict plaque instability. Further, possibly multicentre research studies, with considerably larger samples sizes are justified. The majority of the cases in this study were symptomatic. Thus we do not know if EMPs were released after the development of symptoms or if they are also elevated in asymptomatic individuals with vulnerable plaques. Other research approaches are needed. Two prospective studies have demonstrated prognostic potential of EMPs and for total cardiovascular events in high-risk cohorts.[29,30] A key requirement of future carotid studies will be to examine the prognostic potential of both EMP and LMP in identifying asymptomatic individuals who will develop symptoms or suffer a major cerebrovascular event.

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CONFLICT OF INTEREST

None.

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