ORIGINAL RESEARCH

Markers of Basement Membrane Remodeling Are Associated With Higher Mortality in Patients With Known Atherosclerosis

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***Background*—**Patients with atherosclerosis have a high risk of cardiovascular events and death. Atherosclerosis is characterized by accumulation of lipids, cells and extracellular matrix proteins in the intima. We hypothesized that dysregulated remodeling of the basement membrane proteins may be associated with clinical outcomes in patients with atherosclerosis.

***Methods and Results*—**Neoepitope fragments of collagen type IV (C4M) and laminin (LG1M) were assessed by ELISAs in serum from 787 endarterectomy patients. Matrix metalloproteinases were measured using proximity extension assay and correlated to C4M and LG1M levels using Spearman correlations. A total of 473 patients were followed up for 6 years using national registers, medical charts, and telephone interviews. The incidence of cardiovascular events, cardiovascular mortality, and all-cause mortality were associated to levels of C4M and LG1M using Kaplan–Meier curves and Cox regression analyses. A total of 101 patients had cardiovascular events, 39 died of cardiovascular mortality, and 64 patients died from all-cause mortality. C4M levels were increased in patients with symptomatic carotid atherosclerotic disease before surgery (P=0.048). High C4M and LG1M levels were associated with increased risk of all-cause mortality (P=0.020 and 0.031, respectively) and predicted all-cause death together with glomerular filtration rate and diabetes mellitus.

***Conclusions*—**High LG1M and C4M levels were associated with all-cause mortality, together with glomerular filtration rate and diabetes mellitus. These novel biomarkers need further evaluation but might be tools to identify high-risk patients. **(*J Am Heart Assoc*. 2018;7:e009193. DOI: 10.1161/JAHA.118.009193.)**

Key Words: atherosclerosis • biomarkers • extracellular matrix • inflammation

Cardiovascular disease is, despite advancement in treatments, still the major cause of mortality and morbidity worldwide.[1,2] One of the most important underlying causes of cardiovascular disease is atherosclerosis. Atherosclerosis is a systemic disease characterized by development of plaques containing lipids, inflammatory cells, apoptotic cells, calcium, and extracellular matrix proteins in the arterial wall. Thrombus formation on top of ruptured atherosclerotic plaques is a major cause of acute cardiovascular events. Plaques that are prone to rupture, vulnerable plaques, have larger lipid-rich cores, more inflammatory cells, and thinner fibrous caps that are degraded by proteases.[3,4] However, there is accumulating evidence that lifestyle changes in combination with a more frequent use of cholesterol-lowering therapies have changed the characteristics of the disease and that traditional vulnerable plaques are becoming less common.[5–7] Instead, recent studies reveal that today as much as 30% to 40% of all cardiovascular events are caused by thrombus formation on the surface of plaques with an intact and thick fibrous cap. These events are considered to be caused by endothelial erosion, but the pathophysiological mechanisms responsible for development of these remain to be fully understood.[8,9]

The endothelial cells covering the luminal side of arteries rest on top of an extracellular matrix structure known as the

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Accompanying Tables S1 through S3 and Figure S1 are available at https://www.ahajournals.org/doi/suppl/10.1161/JAHA.118.009193

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This work was presented at the European Society of Cardiology Congress, August 25 to 29, 2018, in Munich, Germany.

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Received June 15, 2018; accepted August 27, 2018.

Clinical Perspective

What Is New?

- In this study, we found 2 serologic biomarkers measuring basement membrane remodeling, as possible tools to identify patients with a high risk of a second cardiovascular event and/or death.
- These biomarkers measure matrix metalloproteinase–generated epitopes of collagen type IV (C4M) and laminin (LG1M), which are structural components of the basement membrane.
- This is, to our knowledge, the first study describing neoepitope-specific basement membrane biomarkers as prognostic biomarkers in patients with known atherosclerosis.

What Are the Clinical Implications?

- These markers may provide valuable information detecting subjects in need of intensified strategies for secondary prevention.

basement membrane. The vascular basement membrane is a thin, sheet-like structure mainly composed of laminin and collagen type IV.[10,11] Similar basement membranes also surround smooth muscle cells in the media of the artery wall. In a healthy artery, matrix metalloproteinases (MMPs) are able to degrade collagen type IV and laminin and induce de novo synthesis of new proteins and maintain tissue homeostasis of the basement membrane.[12] In atherosclerosis, MMP activity is increased, causing accelerated basement membrane degradation.[12,13] Circulating fragments of basement membrane proteins in serum could thus reflect the accelerated remodeling of the arterial basement membrane and function as a marker of degraded basement membrane, vascular tissue remodeling, and possibly also endothelial erosions.

Here, we evaluated newly developed immunoassays for the detection of the neoepitope fragments C4M (MMP-2, -9 and -12-mediated degradation of collagen type IV) and LG1M (MMP-9-mediated degradation of the laminin-γ1 chain) in serum of patients with atherosclerosis. Our aim was to study whether the circulating levels of these markers were associated with (1) cardiovascular events, (2) cardiovascular death, and (3) all-cause mortality in patients with advanced atherosclerosis.

Materials and Methods

Transparency and Openness

The data, analytic methods, and study materials will not be made available to other researchers for purposes of reproducing the results or replicating the procedure. The human material is from a biobank cohort, and the samples are limited and cannot leave the Experimental Cardiovascular Research Unit at Lund University, Malmö, Sweden, because of regulatory and ethical permission issues.

Study Population

A total of 787 human serum samples were collected on the day before carotid endarterectomy. The indications for surgery were plaques associated with ipsilateral symptoms (transient ischemic attack, stroke, or amaurosis fugax) and stenosis measured by duplex >70% (n=610) or plaques not associated with symptoms and stenosis >80% (n=177). All patients were preoperatively examined by a neurologist. Clinical data regarding cardiovascular risk factors, namely, hypertension (systolic blood pressure >140 mm Hg), diabetes mellitus, smoking, levels of fasting lipoproteins (total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and triglycerides), as well as the use of statins and antihypertensive drugs, were registered the day before surgery. Serum samples were processed immediately after collection according to predefined standard operating procedures and stored at −80°C until analysis. Informed consent was obtained from the study participants, and the study was approved by the local ethical committee.

Follow-Up

The primary outcomes comprised: (1) cardiovascular events, (2) cardiovascular mortality, (3) all-cause mortality, all within 6 years. The cardiovascular events studied longitudinally included myocardial infarction, transient ischemic attacks, amaurosis fugax, and vascular interventions not planned at the time of the operation such as carotid endarterectomy, carotid artery stenting, coronary artery bypass grafting, or percutaneous coronary artery intervention, and all deaths with an underlying cardiovascular cause of death. Cardiovascular events were located by analyzing the Swedish National Patient Register for all hospital discharge codes for all the patients included in the study. Patients were identified through their personal identification number. The Swedish National patient register has a high coverage with 99% of all somatic (including surgery) and psychiatric hospital discharges registered.[14] The following *International Classification of Diseases, Tenth Revision* (*ICD-10*) codes were used to identify cardiovascular events: G45.9, G45.3, G46, I63.1 to 5, I63.8 to 9, I64.5, I21 to 22, I24.8 to 9, I25.1 to 2, I25.5 to 6, and I25.8.

All events were verified by patient medical charts and by telephone interviews. Events occurring within 72 hours after carotid endarterectomy were considered procedure related

and were not included in the analysis. For patients having multiple events, only the first one was taken into account in the survival analysis.

All deaths were extracted from the Swedish cause-of-death register.[15] In this study, the following underlying cause of death *ICD-10* codes were used to define cardiovascular mortality: I21.9, I25.1, I25.8 to 9, I48, I50.9, I60.9, I61.9, I63.2, I64, I69.4, I71.0, I73.9, I74.9, and I99.

ELISA Measurement of C4M and LG1M

The 2 markers measured in this study included markers of collagen type IV and laminin degradation, which were measured by competitive ELISAs developed by Nordic Bioscience (Herlev, Denmark).[16] Briefly, a 96-well ELISA plate purchased coated with streptavidin (cat. 11940279, Roche, Hvidovre, Denmark) was coated with the synthetic peptide and incubated at 20°C for 30 minutes with constant shaking at 300 rpm. The plate was then washed 5 times in washing buffer (20 mmol/L Tris, 50 mmol/L NaCl, pH 7.2). Thereafter, 20 μL of the standard peptide or sample diluted according to the protocol were added, followed by 100 μL of peroxidase conjugated antihuman monoclonal antibodies. The plate was incubated for 1 hour at 20°C or for 3 hours at 4°C with constant shaking at 300 rpm (according to the specifications for the individual assay). Afterwards, the plate was washed 5 times in washing buffer, 100 μL 3,3′,5,5′ tetramethyl-benzidine (Kem-En-Tec, Taastrup, Denmark) was added, and the plate was incubated for 15 minutes in the dark with constant shaking at 300 rpm. The reaction was stopped by the addition of 100 μL stop solution (1% H_2SO_4), and the plate was analyzed on an ELISA reader at 450 nm with 650 nm as the reference wavelength. All samples were measured within the detection range of the assay. Samples below the lower limit of quantification were assigned the value of LLOQ, while samples above upper limit of quantification were assigned the value of ULOQ.

MMP-1, -3, -7, -10 and 12 Measurement by Proximity Extension Assay

Circulating levels of plasma levels of MMP-1, -3, -7, -10 and -12 were assessed in 551 patients using the proximity extension assay technique using the Proseek Multiplex CVD96×96 reagents kit (Olink Bioscience, Uppsala, Sweden) at the Clinical Biomarkers Facility, Science for Life Laboratory, Uppsala, as previously described.[17]

Statistical Analysis

Baseline characteristics are described as number (frequency) and percentage for categorical variables, and as median (interquartile range) for continuous variables, as presented variables were non-normally distributed. Spearman correlations were used because the levels of C4M and LG1M were not normally distributed.

Kaplan–Meier survival analyses were performed for C4M and LG1M, divided according to the median value of each marker (high versus low) for the 3 primary outcomes. Significant differences between the groups were assessed by a log-rank test. Cox proportional hazard regression analysis (hazard ratios [HRs] with 95% confidence interval [CI]) was used to analyze the time-to-event association between baseline characteristics and risk of cardiovascular events, cardiovascular mortality, and all-cause mortality. Multivariate models were created, correcting for known cardiovascular risk factors: age, sex, hypertension, diabetes mellitus, current smoking, total cholesterol, and estimated glomerular filtration rate (eGFR). These parameters were chosen based on the Framingham risk score[18] and on the fact that patients with diabetes mellitus have per se a 2- to 4-fold increased risk of cardiovascular disease.[18,19] The proportional hazards assumption was assessed using Cox regression with time-dependent covariate in SPSS. $P<0.05$ was considered statistically significant. Statistical analyses were performed using SPSS, version 24 (IBM Corporation).

Results

Baseline Characteristics of the Study Cohort

The study participants were 63.9% male, median age was 72 years (interquartile range, 66–77) and median body mass index was 26.4 (interquartile range, 24.0–29.3). The median degree of stenosis was 90% (interquartile range, 75–95), and 77.5% of the patients had a symptomatic carotid plaque. Baseline demographics and clinical and laboratory data for the study cohort divided by the median of C4M and LG1M are presented in Tables 1 and 2, respectively.

When dividing the cohort by the median C4M levels, patients above the median had higher levels of total cholesterol, low-density lipoprotein, and C-reactive protein and a lower eGFR and high-density lipoprotein levels. When dividing the cohort by the median levels of LG1M, patients above the median levels of LG1M were more commonly current smokers and had higher levels of C-reactive protein and lower high-density lipoprotein levels compared with patients with LG1M below the median.

C4M levels were associated with decreased kidney function as assessed by eGFR ($r=-0.230$, $P=0.001$). Current smokers had a significantly higher level of LG1M at inclusion (8.5 [8.5–14.39] ng/mL versus 9.45 [8.5–18.05] ng/mL; $P=0.009$). No significant differences in levels

Table 1. Clinical Characteristics of the Study Cohort Divided by the Median of C4M (Cutoff, 20.5 ng/mL)

	Low (n=326)	High (n=461)	*P* Value
Age	71 (66–77)	72 (66–78)	0.536
Male sex, n (%)	223 (68.4)	280 (60.7)	0.027*
Degree of stenosis, %	85 (75–95)	90 (75–95)	0.144
BMI	26.4 (24–29.3)	26.4 (24.1–29.3)	0.687
Current smoker, n (%)	93 (28.5)	162 (35.1)	0.051
Hypertension, n (%)	240 (73.6)	355 (77.0)	0.540
Symptomatic plaque, n (%)	244 (74.8)	366 (79.4)	0.132
Fasting plasma levels of lipids			
Cholesterol, mmol/L	4.2 (3.5–5.1)	4.4 (3.7–5.4)	0.035*
LDL, mmol/L	2.40 (1.7–3.3)	2.7 (2.0–3.5)	0.004*
HDL, mmol/L	1.2 (0.98–1.52)	1.1 (0.90–1.40)	0.003*
Triglycerides, mmol/L	1.26 (0.9–1.7)	1.3 (1.0–1.8)	0.170
Diabetes mellitus, n (%)	83 (25.5)	115 (24.9)	0.870
Coronary artery disease, n (%)	128 (39.3)	167 (36.2)	0.251
CRP, mmol/L	2.0 (0.6–3.0)	4.0 (2.0–9.4)	<0.001*
HbA_{1c}, mmol/mol	44 (38–53.13)	42.34 (38–50.00)	0.274
Medications, n (%)			
Statins	304 (83.3)	400 (56.8)	0.004*
Antihypertensive drugs	255 (78.2)	362 (78.5)	0.919
eGFR mL/min per 1.73 m^2	67.36 (54.67–83.13)	62.41 (48.59–76.58)	0.009*

Nonnormally distributed data variables are presented as median (interquartile range). Categorical values are presented as numbers and percentages. BMI indicates body mass index; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; HbA_{1c}, hemoglobin A_{1c}; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
*Significant difference between low and high levels of C4M.

of C4M and LG1M were found for patients with previous coronary artery disease, hypertension, or diabetes mellitus at inclusion.

Follow-Up Outcomes

A total of 101 patients suffered from cardiovascular events during the 6-year follow-up period. Thirty-nine patients (60.9% of total deaths) died of cardiovascular causes, and 64 patients died of all causes during the 6-year follow-up.

C4M and Cardiovascular Events

C4M levels were not significantly associated with cardiovascular events even though a trend was seen for high levels of C4M and an increased risk for cardiovascular events (log-rank test, *P*=0.053; Figure 1A). The univariate Cox regression showed an HR for C4M of 1.51; 95% CI, 0.99–2.29; and *P*=0.056. When correcting for known cardiovascular risk factors (age, sex, hypertension, diabetes mellitus, current smoking, total cholesterol, and eGFR) with a multivariate Cox analysis, C4M was not an independent predictor of future cardiovascular events (HR for C4M, 1.48; 95% CI, 0.95–2.32; *P*=0.083; Table 3, Table S1).

C4M and Cardiovascular Mortality

With regard to C4M and cardiovascular mortality, the Kaplan–Meier curves are shown in Figure S1A. For C4M, statistical significance was not obtained in the log-rank test, the univariate Cox regression, or the multivariate Cox regression analysis (HR for C4M, 1.65; 95% CI, 0.77–3.52; *P*=0.164), when adjusting for cardiovascular risk factors (age, sex, hypertension, diabetes mellitus, current smoking, total cholesterol, and eGFR) (Table 3, Table S1).

C4M and All-Cause Mortality

Patients with high C4M had increased risk of all-cause mortality (log-rank test, *P*=0.011; Figure 2A). In the univariate Cox regression, C4M predicted all-cause mortality (HR, 2.05; 95% CI, 1.16–3.61; *P*=0.013). In the multivariate Cox regression analysis, C4M (HR, 2.08; 95% CI, 1.12–3.87; *P*=0.02), diabetes mellitus (HR, 1.94; 95% CI, 1.12–3.36;

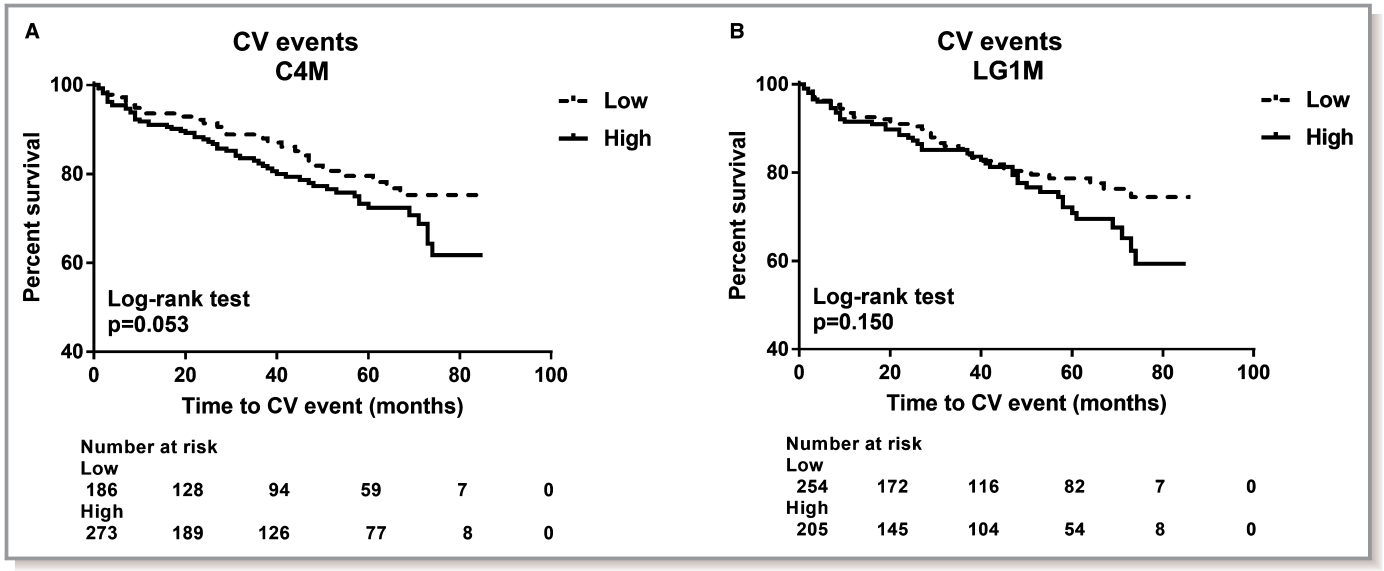

Figure 1. Associations of C4M and LG1M with cardiovascular events. A total of 101 patients suffered from cardiovascular events during the 6-year follow-up period. A, Kaplan–Meier curves for cardiovascular events were performed for C4M levels above and below the median. There were no significant differences (P=0.053). B, Kaplan–Meier curves for cardiovascular (CV) events were performed for LG1M levels above and below the median. There were no significant differences (P=0.150).

P=0.018) and eGFR (HR, 0.97; 95% CI, 0.96–0.99; P=0.004) predicted all-cause mortality, whereas the other covariates were not significant in this model (Table 3, Table S1).

C4M and Preoperative Symptoms

Patients who suffered from preoperative cerebrovascular symptoms had higher levels of C4M compared with asymptomatic patients (20.82 [17.31–26.23] ng/mL versus 19.78 [16.95–24.84] ng/mL; P=0.048; Figure 3A).

Correlations Between C4M and MMP-1, -3, -7, -10 and -12

Because the C4M neoepitope fragments are formed after MMP cleavage of collagen type IV, we examined whether circulating levels of MMPs were associated with C4M.

C4M correlated with MMP-1 (r=0.088; P=0.039), MMP-7 (r=0.133; P=0.002), MMP-10 (r=0.118; P=0.006) and MMP-12 (r=0.191; P<0.001), but not with MMP-3 (r=0.049; P=0.338).

LG1M and Cardiovascular Events

With regard to LG1M and its association with cardiovascular events, statistical significance was not obtained in the log-rank test (Figure 1B), the univariate, or the multivariate Cox regression analysis adjusting for cardiovascular risk factors (age, sex, hypertension, diabetes mellitus, current smoking, total cholesterol, and eGFR (Table 3, Table S2). Only eGFR was significant in the model with an HR of 0.97; 95% CI, 0.96 to 0.99; and P=0.028. When analyzing LG1M as a continuous variable (instead of using the cutoff of median for LG1M high and low) in a univariate Cox analysis, LG1M predicted cardiovascular events (HR, 1.01; 95% CI, 1.0–1.03; P=0.033), whereas it did not remain significant in the multivariate analyses (Table S3).

LG1M and Cardiovascular Mortality

Patients with high LG1M levels (above median) had an increased risk of cardiovascular mortality (log-rank test, P=0.044; Figure S1B). In a univariate Cox regression analysis, a trend was observed for LG1M and increased cardiovascular mortality (HR for LG1M,:1.91; 95% CI, 1.00–3.64; P=0.050). When performing a multivariate Cox regression analysis adjusting for the previously described covariates, LG1M did not predict cardiovascular mortality (Table 3, Table S2). When analyzing LG1M as a continuous variable (instead of using the cutoff of median for LG1M high and low) in a univariate Cox analysis, LG1M predicted cardiovascular mortality (HR, 1.02; 95% CI, 1.01–1.04; P=0.013), whereas it did not remain significant in the multivariate analyses (Table S3).

LG1M and All-Cause Mortality

Kaplan–Meier curves for all-cause mortality of LG1M is shown in Figure 2B. Patients with high levels of LG1M also had an increased all-cause mortality (log-rank test, 0.006). The increased risk for all-cause mortality was also confirmed in the univariate Cox regression analysis (HR, 2.01; 95% CI, 1.21–3.34; P=0.007). Adjusting for the covariates described above,

Table 2. Clinical Characteristics of the Study Cohort Divided by the Median of LG1M (Cutoff, 8.5 ng/mL)

	Low (n=411)	High (n=375)	*P* Value
Age	71 (66–77)	72 (66–78)	0.244
Male sex, n (%)	272 (66.2)	230 (61.3)	0.158
Degree of stenosis, %	85 (75–95)	90 (75–95)	0.010*
BMI	26.2 (24–29.2)	26.55 (24.00–29.30)	0.820
Current smoker, n (%)	116 (28.2)	139 (37.1)	0.008*
Hypertension, n (%)	109 (26.5)	78 (20.8)	0.105
Symptomatic plaque, n (%)	307 (74.7)	302 (80.5)	0.050
Fasting plasma levels of lipids			
Cholesterol, mmol/L	4.3 (3.6–5.2)	4.4 (3.6–5.3)	0.424
LDL, mmol/L	2.5 (1.9–3.3)	2.6 (1.9–3.5)	0.384
HDL, mmol/L	1.2 (0.94–1.50)	1.1 (0.91–1.40)	0.039*
Triglycerides, mmol/L	1.2 (0.9–1.7)	1.3 (1.0–1.8)	0.345
Diabetes mellitus, n (%)	98 (23.8)	100 (26.7)	0.363
Coronary artery disease, n (%)	150 (36.5)	145 (18.4)	0.735
CRP, mmol/L	2.0 (0.73–4.00)	4.55 (1.93–9.93)	<0.001*
HbA_{1c}, mmol/mol	2.84 (38.00–51.04)	43.0 (38.0–53.6)	0.715
Medications, n (%)			
Statins	372 (90.5)	331 (88.3)	0.306
Antihypertensive drugs	315 (76.6)	301 (80.3)	0.218
eGFR mL/min per 1.73 m^2	65.08 (53.32–80.14)	64.66 (48.09–78.40)	0.248

Nonnormally distributed data variables are presented as median (interquartile range). Categorical values are presented as numbers and percentages. BMI indicates body mass index; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; HbA_{1c}, hemoglobin A_{1c}; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
*Significant difference between low and high levels of LG1M.

in a multivariate Cox regression analysis, LG1M, diabetes mellitus, and eGFR predicted all-cause mortality (Table 3, Table S2). When analyzing LG1M as a continuous variable (instead of using the cutoff of median for LG1M high and low) in a univariate Cox analysis, LG1M predicted all-cause mortality (HR, 1.03; 95% CI, 1.02–1.05; $P<0.001$). Additionally, in the multivariate analyses, it also predicted all-cause mortality (HR, 1.02; 95% CI, 1.01–1.03; $P=0.007$) together with eGFR (HR, 0.97; 95% CI, 0.96–0.99; $P=0.004$) (Table S3).

LG1M and Preoperative Symptoms

A trend toward increased levels of LG1M was also found in patients who had preoperative symptoms compared with those who had not (16.17 [8.5–28–22] ng/mL versus 8.5 [8.5–14–41] ng/mL; $P=0.081$; Figure 3B).

Correlations Between LG1M and MMP-1, -3, -7, -10 and -12

LG1M correlated with MMP-7 ($r=0.143$; $P=0.001$), MMP-10 ($r=0.208$; $P<0.001$), and MMP-12 ($r=0.177$; $P<0.001$), but not with MMP-1 ($r=0.031$; $P=0.46$) and MMP-3 ($r=0.075$, $P=0.137$).

Taken together, this implies that there might be an association between MMP-induced breakdown of the basement membrane and the levels of circulating neoepitopes.

Discussion

In the present study, we evaluated 2 novel immunoassays targeting MMP-generated neoepitope fragments of 2 of the most important components of the vascular basement membrane, C4M of collagen type IV and LG1M of laminin, as potential prognostic markers of cardiovascular events, cardiovascular mortality, and all-cause mortality in patients with known atherosclerosis.

Our main findings were that C4M and LG1M predicted all-cause mortality together with diabetes mellitus and eGFR.

Collagen type IV and laminin are the 2 major constituents of the arterial basement membrane.[20,21] These proteins contribute to the network required to maintain the tissue integrity of the basement membrane.[22,23]

Table 3. Uni- and -Multivariate Cox Proportional Hazard Regression Model for Cardiovascular Events and Cardiovascular- and All-Cause Mortality for C4M and LG1M

	Cardiovascular Events		Cardiovascular Mortality		All-Cause Mortality	
Variable	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
Univariate Cox analysis						
C4M*	1.51 (0.99–2.29)	0.056	1.72 (0.86–3.46)	0.128	2.05 (1.16–3.61)	0.013†
Multivariate Cox analysis						
C4M*	1.48 (0.95–2.32)	0.083	1.65 (0.77–3.52)	0.164	2.08 (1.12–3.87)	0.020†
Age	0.99 (0.96–1.03)	0.705	1.00 (0.94–1.07)	0.928	1.03 (0.98–1.08)	0.297
Sex	1.39 (0.87–2.22)	0.164	1.61 (0.75–3.43)	0.219	1.26 (0.72–2.21)	0.425
Hypertension	0.81 (0.50–1.29)	0.365	0.91 (0.40–2.08)	0.818	0.97 (0.51–1.85)	0.936
Diabetes mellitus	1.53 (0.97–2.42)	0.069	2.06 (1.01–4.19)	0.046†	1.94 (1.12–3.36)	0.018†
Smoking	0.86 (0.52–1.43)	0.568	0.53 (0.19–1.51)	0.235	1.05 (0.53–2.07)	0.892
Cholesterol	1.04 (0.87–1.24)	0.686	1.21 (0.91–1.62)	0.189	1.02 (0.80–1.29)	0.888
eGFR	0.99 (0.97–1.00)	0.037†	0.96 (0.94–0.99)	0.004†	0.97 (0.96–0.99)	0.004†
Variable	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
Univariate Cox analysis						
LG1M*	1.33 (0.90–1.96)	0.157	1.91 (1.00–3.64)	0.050	2.01 (1.21–3.34)	0.007†
Multivariate Cox analysis						
LG1M*	1.035 (0.89–2.05)	0.163	1.48 (0.74–2.98)	0.269	1.81 (1.05–3.10)	0.031†
Age	0.99 (0.96–1.03)	0.716	1.00 (0.94–1.06)	0.954	1.02 (0.98–1.07)	0.361
Sex	1.35 (0.85–2.15)	0.202	1.50 (0.71–3.18)	0.287	1.17 (0.67–2.04)	0.587
Hypertension	0.81 (0.51–1.30)	0.376	0.89 (0.39–2.05)	0.780	0.96 (0.51–1.83)	0.906
Diabetes mellitus	1.48 (0.93–2.34)	0.097	1.98 (0.97–4.06)	0.061	1.88 (1.08–3.25)	0.025†
Smoking	0.88 (0.53–1.46)	0.611	0.55 (0.20–1.55)	0.260	1.07 (0.55–2.11	0.837
Cholesterol	1.03 (0.86–1.23)	0.779	1.19 (0.90–1.58)	0.221	1.01 (0.80–1.27)	0.948
eGFR	0.99 (0.97–1.00)	0.028†	0.96 (0.94–0.99)	0.004†	0.97 (0.95–0.99)	0.002†

Known cardiovascular risk factors, age, sex, hypertension, diabetes mellitus, current smoking, total cholesterol, and eGFR were included in the model. Data are presented as hazard ratio (95% CI). CI indicates confidence interval; eGFR, estimated glomerular filtration rate; HR, hazard ratio.

*Above vs below median.

†Statistical significant.

The C4M and LG1M neoepitopes are generated by some of the most expressed MMPs in human atherosclerosis through their degradation of collagen type IV and laminin.[21,24,25] Plaque rupture is considered to be a potential consequence of excessive extracellular matrix degradation caused by increased MMP activity, especially MMP-12 activity.[26] Herein, markers measuring active extracellular matrix remodeling could potentially be used to monitor plaque remodeling and to identify patients at increased risk of a cardiovascular event.[27]

We observed increased serum levels of C4M in patients who had preoperative cerebrovascular symptoms before surgery. In line with these findings, significant correlations between C4M and LG1M and several MMPs were identified. It is noteworthy that MMP-12, important in plaque vulnerability and collagen type IV degradation, correlated to both C4M and LG1M levels.[26] However, the correlations of C4M and LG1M to MMPs should be interpreted with caution because of the rather low *r* values and thereby weak correlations. Nevertheless, our finding in the present study do not provide any evidence for the role of C4M or LG1M as markers of basement membrane degradation in the vessel wall. Neither can we rule out that the increased levels of these components contribute to the development of symptoms or if they are a consequence of the event of a plaque rupture. However, no correlation between C4M levels (1 day before surgery) and the time between surgery and symptoms/plaque rupture was found (r=−0.001, P=0.9). Taking this into consideration, it is more likely that C4M levels are associated with the underlying ongoing pathology, rather than a result from the plaque rupture.

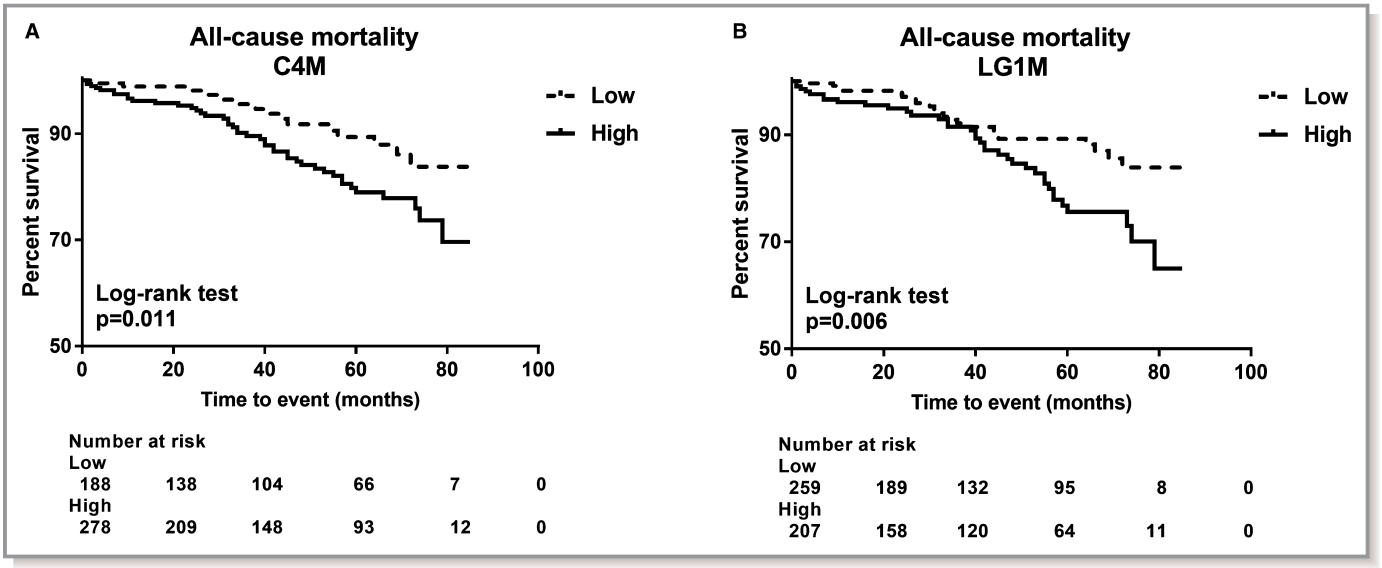

Figure 2. Associations of C4M and LG1M with all-cause mortality. Sixty-four patients died during the 6-year follow-up period. A, Kaplan–Meier curves for all-cause mortality were performed for C4M levels above and below the median. Patients with circulating C4M levels higher than the median were significantly more likely to die (*P*=0.011). B, Kaplan–Meier curves for all-cause mortality were performed for LG1M levels above and below the median. Patients with circulating LG1M levels higher than the median were significantly more likely to die (*P*=0.006).

As the basement membrane plays an important role in maintaining endothelial integrity and function, degradation of the basement membrane might be one possible cause of endothelial erosions.[7] An alternative explanation would be that the association between serum levels of basement membrane neoepitopes and future death, as well as a trend for cardiovascular events, reflects an increased leakage of the neoepitopes into the circulation and not per se an increased degradation of the basement membrane. A dysfunctional endothelium is also associated with a higher risk of future cardiovascular events.[28] A defective endothelium could likely also be involved in the development of a rupture-prone plaque with or without an increased degradation of the fibrous cap or the basement membrane.

Finally, we observed that serum levels of C4M and LG1M were associated with all-cause mortality, together with diabetes mellitus and eGFR. This might suggest that neoepitopes may not only reflect cardiovascular pathology but also other biological processes that affect the basement membrane such as aging, kidney failure, or diseases with high tissue turnover, such as cancer.[29,30] However, the possible role of these marker in noncardiovascular processes is beyond the scope of this study and warrant new, dedicated investigations.

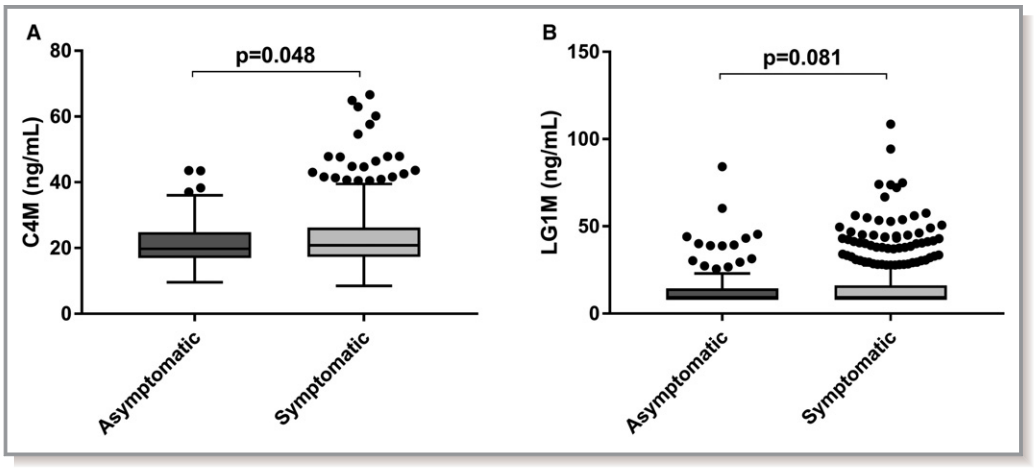

Figure 3. Levels of (A) C4M and (B) LG1M in patients with an asymptomatic vs symptomatic plaque. Differences were calculated by a nonparametric Wilcoxon-Mann–Whitney t test. Data are presented as Tukey boxplots.

The strength of this study is the use of a large prospective cohort of patients with advanced atherosclerosis to explore the association of 2 novel biomarkers of basement membrane remodeling with relevant clinical end points during a large follow-up period. The limitations include the lack of mechanistic explanations for the associations between the biomarkers and the clinical outcomes and the fact that the laminin γ1 chain and collagen type IV α1 chain are expressed in the majority of the basement membranes throughout the body. Therefore, the biomarkers cannot be fully qualified as vascular specific. Despite the size of the cohort, it did not have enough events to allow a separated analysis between the different types of cardiovascular events (eg, coronary versus cerebrovascular). Because we saw that eGFR was a strong predictor in the regression analysis, we cannot exclude a contribution from the kidney extracellular matrix to the fragments of LG1M and C4M measured in serum. Finally, we can also not extrapolate the findings to healthy populations or populations with less advanced atherosclerosis, as this will demand more studies in different cohorts.

Conclusions

In conclusion, increased levels of the neoepitope marker of collagen type IV degradation, C4M, and laminin γ1 chain, LG1M, predicted all-cause mortality, together with diabetes mellitus and eGFR in patients with advanced atherosclerosis. These novel serum markers of basement membrane remodeling might be potential clinical tools to identify, among patients with known advanced atherosclerosis, those who are at an increased risk for death, demanding tighter or more intensive treatment strategies.

Acknowledgments

We are grateful to Ana Persson, Lena Sundius, and Mihaela Nitulescu.

Sources of Funding

This work was supported by the Danish Research Foundation, Swedish Research Council, and the Swedish Heart and Lung Foundation, Skånes University Hospital; Southern Sweden Regional Research Funding and Sparbanks Färs and Frosta Foundation, Swedish Society for Medical Research, Hjelt Foundation, Bundy Academy, Emil and Wera Cornell Foundation.

Disclosures

Drs Holm Nielsen, Karsdal, and Genovese are full-time employees at Nordic Bioscience A/S. Nordic Bioscience is a privately owned, small-to-medium-sized enterprise (SME) partly focused on the development of biomarkers. The remaining authors have no disclosures to report. None of the authors received fees, bonuses, or other benefits for the work described in the manuscript. Dr Karsdal holds stocks in Nordic Bioscience A/S.

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