Circulating Osteoglycin and NGAL/MMP9 Complex Concentrations Predict 1-Year Major Adverse Cardiovascular Events After Coronary Angiography


Objective—Previous proteomics experiments have demonstrated that several proteins are differentially expressed in vulnerable human carotid plaques compared with stable plaques. This study aims to investigate the prognostic value of 13 such circulating biomarkers in patients with coronary artery disease.

Approach and Results—Between 2008 and 2011, 768 patients who underwent coronary angiography for acute coronary syndrome or stable angina pectoris were included in a prospective biomarker study. Plasma concentrations of 13 biomarkers were measured in 88 patients who experienced a major adverse cardiovascular event (MACE) within 1 year and 176 control patients without MACE who were matched on age, sex, and number of diseased coronary vessels. MACE comprised all-cause mortality, acute coronary syndrome, unplanned coronary revascularization, and stroke. After adjustment for established cardiovascular risk factors, osteoglycin (OGN; odds ratio per SD increase in ln-transformed OGN, 1.53; 95% confidence interval, 1.11–2.11; \( P = 0.010 \)) and neutrophil gelatinase–associated lipocalin/matrix metalloproteinase 9 (NGAL/MMP9; odds ratio per SD increase in ln-transformed NGAL/MMP9, 1.37; 95% confidence interval, 1.01–1.85; \( P = 0.042 \)) complex were independently associated with MACE during follow-up. These associations were independent of C-reactive protein levels. Adding OGN or NGAL/MMP9 to a model containing conventional risk factors did not significantly improve discriminatory power (OGN: area under receiver operating characteristic curve, 0.75 versus 0.67; NGAL/MMP9: 0.73 versus 0.67) but did significantly improve risk reclassification (OGN: net reclassification index=0.29; 95% confidence interval, 0.05–0.53; \( P = 0.019 \); NGAL/MMP9: net reclassification index=0.44; 95% confidence interval, 0.20–0.69; \( P < 0.001 \)).

Conclusions—Circulating OGN and NGAL/MMP9 complex are promising biomarkers that are expressed in vulnerable atherosclerotic plaques and may have incremental value for prediction of MACE within 1 year after coronary angiography. (Arterioscler Thromb Vasc Biol. 2014;34:1078-1084.)

Key Words: atherosclerosis ■ biological markers ■ OGN protein, human ■ prognosis

Atherosclerosis and plaque destabilization leading to coronary thrombosis and acute coronary syndrome are the result of a heterogeneous process, involving vascular inflammation, endothelial dysfunction, and hypercoagulability.1,2 Blood biomarkers may reflect these pathophysiological constituents of coronary artery disease (CAD). Consequently, blood biomarker level may be associated with severity of CAD and thus predict occurrence of adverse cardiovascular events in CAD patients. Such associations have been demonstrated already for several biomarkers, such as C-reactive protein (CRP).3–5 The main challenge in investigating which biomarkers are suitable for prediction of adverse cardiovascular events is that plasma contains >900,000 proteins.6 A disadvantage of traditional research is that it tests each of the plasma proteins individually. Proteomics-based research has the potential to offer insight into the full complexity of atherosclerosis with its various components and may reveal novel biomarkers that have the ability to improve prediction of adverse events.7 In a previous proteomic study, we have found several proteins to be differentially released by vulnerable hemorrhagic
human carotid plaques when compared with stable fibrotic plaques.8 The majority of these proteins, including aciculin, oncogene DJ1 (DJ1), microfibril-associated glycoprotein 4, osteoglycin (OGN), procollagen C proteinase enhancer 1, phosphatidylethanolamine-binding protein 1, and peroxiredoxin 2, have not yet been investigated as prognostic biomarkers in CAD patients. In addition, evidence exists that neutrophil gelatinase-associated lipocalin (NGAL) and its NGAL/matrix metalloproteinase 9 (NGAL/MMP9) complex display increased expression in (unstable) atherosclerotic plaques.9,10 These proteins have also not yet been investigated as prognostic biomarkers in CAD patients.

We have performed a prospective, nested case-control study in a cohort of 768 patients undergoing coronary angiography to investigate whether plasma levels of the above-described novel protein biomarkers are associated with adverse cardiovascular events. We have also evaluated whether these biomarkers improve discrimination and risk reclassification.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Baseline Characteristics
Mean age of the patients was 64.9 (SD, 10.6) years, 77% were men, and 52% had acute coronary syndrome (Table 1). Percutaneous coronary intervention was performed in 82% of the patients. The group of patients who experienced major adverse cardiovascular event (MACE) during follow-up displayed a higher prevalence of diabetes mellitus (27.3% versus 14.9%; P=0.016) and a tendency toward a higher prevalence of renal insufficiency (11.4% versus 4.6%; P=0.053) compared with the group of patients who did not have MACE. Other baseline characteristics did not display significant differences between cases and controls.

Associations With Cardiovascular Outcome
Plasma NGAL seemed to be normally distributed. Aciculin, DJ1, heat shock protein 27, microfibril-associated glycoprotein 4, NGAL/MMP9, OGN, procollagen C proteinase enhancer 1, phosphatidylethanolamine-binding protein 1, pro-B-type natriuretic protein 1 to 108 (proBNP), peroxiredoxin 2, tissue inhibitor of proteinase 1 (TIMP1), and thrombospondin 2 were not normally distributed and were thus ln-transformed for further analyses. Patients with MACE during follow-up had higher NGAL (cases: median, 114 [interquartile range, 67–167] versus controls: 96 [58–133] ng/mL; P=0.038), OGN (cases: 13.7 [8.0–18.4] versus controls: 9.6 [7.2–13.7] ng/mL; P=0.011), and TIMP1 (cases: 147 [113–175] versus controls: 123 [94–158] ng/mL; P=0.020) levels at the time of coronary angiography compared with patients without MACE during follow-up (Table 2). Furthermore, patients with MACE during follow-up also tended to have higher thrombospondin 2 (cases: 37.4 [30.4–48.7] versus controls: 34.8 [28.9–42.4] ng/mL; P=0.066), proBNP (cases: 63 [16–170] versus controls: 50 [15–123] ng/mL; P=0.088), and NGAL/MMP9 (cases: 0.43 [0.27–0.61] versus controls: 0.35 [0.26–0.56]; P=0.070) levels than patients without MACE.

After adjustment for conventional cardiovascular risk factors in multivariable conditional logistic regression analyses, higher OGN (odds ratio [OR] per SD increase in ln-transformed OGN, 1.53; 95% confidence interval [CI], 1.11–2.11; P=0.010) and NGAL/MMP9 (OR per SD increase in ln-transformed NGAL/MMP9, 1.37; 95% CI, 1.01–1.85; P=0.042) levels were independently associated with incident MACE during follow-up (Table 3; Figure 1). OGN (OR per SD increase in ln-transformed OGN, 1.50; 95% CI, 1.08–2.08; P=0.015) and NGAL/MMP9 (OR per SD increase in ln-transformed NGAL/MMP9, 1.38; 95% CI, 1.01–1.89; P=0.043) levels remained independently associated with MACE after additional adjustment for baseline CRP levels.

Discrimination
First, we evaluated a model containing conventional cardiovascular risk factors, including diabetes mellitus, hypertension, history of myocardial infarction, renal impairment, and indication for coronary angiography. This model displayed an area under the receiver operating characteristic curve of 0.67 (Figure 2). When we added CRP to this model, it displayed an area under the receiver operating characteristic curve of 0.71 (P=0.41). Although not statistically significant, adding OGN (area under the receiver operating characteristic curve = 0.75; P=0.12 compared with model with conventional factors; P=0.47 compared with model with conventional factors+CRP) or NGAL/MMP9 (area under the receiver operating characteristic curve = 0.73; P=0.19 compared with model with conventional factors; P=0.64 compared with model with conventional factors+CRP) slightly improved discriminatory ability of the model.

Reclassification
We examined whether adding OGN and NGAL/MMP9 to the conditional logistic regression model consisting of conventional cardiovascular risk factors and CRP level (as described above and in Table 3) results in correct reclassification of risk of MACE during follow-up (Table 4). OGN (net reclassification index = 0.29; 95% CI, 0.05–0.53; P=0.019) and NGAL/MMP9 (net reclassification index = 0.44; 95% CI, 0.20–0.69; P<0.001) each significantly improved classification. Adding both OGN and NGAL/MMP9 resulted in a net reclassification index of 0.40 (95% CI, 0.17–0.64; P<0.001).
This study investigated the associations between circulating plasma biomarkers, which were previously identified by proteomics or immunohistochemistry experiments in human carotid plaques and adverse cardiovascular outcome in patients undergoing coronary angiography. The prognostic value of the majority of these proteins, including OGN and NGAL/MMP9 complex, for MACE had not yet been investigated. Higher circulating OGN and NGAL/MMP9 complex levels were associated with incident MACE during the first year of follow-up, independently of conventional cardiovascular risk factors. Adding OGN or NGAL/MMP9 to a model containing conventional cardiovascular risk factors improved risk classification and discriminatory ability, although the later was not statistically significant. These associations with incident MACE and improvements in predictive ability were independent of CRP.

In previous proteomic experiments, we have identified a series of novel potential markers of vulnerable atherosclerotic plaque. We used human carotid atherosclerotic plaques, obtained from patients who underwent endarterectomy (n=80), which were classified as fibrotic plaques or hemorrhagic plaques according to the AHA classification and hemoglobin content. We performed protein enrichment and mass spectrometry analysis and subsequently validation by Western blotting. We found that several tissue proteins within human atherosclerotic plaques were able to differentiate between vulnerable, hemorrhagic and stable, fibrolipidic lesions. These proteins are known to be involved in various pathophysiological pathways, such as inflammation, cell integrity and arterial matrix remodeling (microfibril-associated glycoprotein 4, thrombospondin 2, OGN, procollagen C proteinase enhancer 1, TIMP1, aciculin, OGN), oxidative stress (DJ1, peroxiredoxin 2), and cell stress (heat shock protein 27). In the present study, we a priori hypothesized that circulating levels of these proteins may reflect presence of vulnerable plaques and may therefore also have prognostic value in patients with CAD.

OGN is a bone-associated glycoprotein but is also found to be a basic component of the vascular extracellular matrix. A previous study compared OGN expression in atherosclerotic plaque.
lesions with normal vasculature in rabbits.\textsuperscript{13} In atherosclerotic lesions, OGN was upregulated in activated endothelium and thick neointima and in the front edge of migrating smooth muscle cells. Another study demonstrated that OGN was substantially increased in the adventitia and neointima after balloon injury, implying a role for this protein in vessel matrix remodeling.\textsuperscript{14} In line with these findings, close homologues of OGN, such as chicken proteoglycan Lb, were found to contribute to ordering of the matrix by interacting with collagens.\textsuperscript{14,15} Furthermore, OGN has the ability to bind transforming growth factor $\beta$, which has been shown to have significant effects on vascular smooth muscle cell behavior in vascular

<table>
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<th>Table 2. Difference in Biomarker Levels Between Cases and Controls</th>
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<td><strong>Cases</strong> (n=88)</td>
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<td>Aciculin, ng/mL</td>
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<td>DJI, ng/mL</td>
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<td>HSP27, ng/mL</td>
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<td>MFA4, ng/mL</td>
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<td>NGAL, ng/mL</td>
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<td>NGAL/MMP9 complex, AU</td>
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<td>OGN, ng/mL</td>
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<td>PCPE1, ng/mL</td>
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<td>PEBP1, ng/mL</td>
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<td>ProBNP, pg/mL</td>
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<td>PRX2, ng/mL</td>
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<td>TIMP1, ng/mL</td>
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<td>TSP2, ng/mL</td>
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Data are presented as median (interquartile range). $P$ values are obtained by using conditional logistic regression analyses. AU indicates arbitrary unit; CI, confidence interval; DJI, oncogene DJI; HSP27, heat shock protein 27; MFA4, microfibril-associated glycoprotein 4; NGAL/MMP9, neutrophil gelatinase–associated lipocalin/matrix metalloproteinase 9; OGN, osteoglycin; OR, odds ratio; PCPE1, procollagen C proteinase enhancer 1; PEBP1, phosphatidylethanol-amine-binding protein 1; proBNP, pro-B-type natriuretic protein 1 to 108; PRX2, peroxiredoxin 2; TIMP1, tissue inhibitor of proteinase 1; and TSP2, thrombospondin 2.

*Cases: patients who experienced a major adverse cardiovascular event, including all-cause mortality, acute coronary syndrome, unplanned coronary revascularization, and stroke, within the first year of follow-up.

†Control patients were matched on age, sex, and number of diseased coronary vessels.

‡Odds ratio per SD increase in (ln-transformed) biomarker concentration.

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<th>Table 3. Associations Between Biomarkers and Major Adverse Cardiovascular Events in Multivariable Analysis</th>
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<tr>
<td><strong>OR (95% CI) Adjusted for Conventional Cardiovascular Risk Factors</strong></td>
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<td>Aciculin</td>
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CI indicates confidence interval; CRP, C-reactive protein; DJI, oncogene DJI; HSP27, heat shock protein 27; MFA4, microfibril-associated glycoprotein 4; NGAL/MMP9, neutrophil gelatinase–associated lipocalin/matrix metalloproteinase 9; OGN, osteoglycin; OR, odds ratio; PCPE1, procollagen C proteinase enhancer 1; PEBP1, phosphatidylethanol-amine-binding protein 1; proBNP, pro-B-type natriuretic protein 1 to 108; PRX2, peroxiredoxin 2; TIMP1, tissue inhibitor of proteinase 1; and TSP2, thrombospondin 2.

*Odds ratio per SD increase in (ln-transformed) biomarker concentration. Conventional cardiovascular risk factors: diabetes mellitus, hypertension, smoking, dyslipidemia, history of myocardial infarction, renal impairment, and indication for coronary angiography.
In our previous proteomics experiments, however, OGN was found to be downregulated in hemorrhagic plaques compared with fibrotic plaques. The lower OGN levels suggested an altered strength and integrity of the vascular wall, which may make hemorrhagic plaques more prone to rupture. Its role as a prognostic circulating biomarker has also not been investigated yet. To our best knowledge, our study is the first to demonstrate that higher circulating OGN concentrations have prognostic value in patients with CAD. The seemingly contradictory results between the carotid proteomics study and the circulating levels in the present study may be because of global endothelial activation in acute conditions leading to the production of OGN, reflected by increased plasma levels. Another explanation for the decreased OGN in hemorrhagic plaques is that it may be caused by elution of OGN into the circulation. Also, differences in carotid versus coronary atherothrombotic plaque biology may provide an explanation for this discrepancy. However, evidence that may support these hypotheses is currently lacking. Again, these results underscore that the precise role of (circulating) OGN requires further investigation.

NGAL is a protein that is expressed in neutrophils, in the heart, in aorta tissue, and in low levels in the kidney. It is proposed to be a scavenger of bacterial products at sites of inflammation and a modulator of inflammation. Although NGAL is well known as a biomarker of kidney injury, it has been shown that NGAL could also have a role in atherosclerotic disease. NGAL is able to form a stable, biologically active complex with MMP9, preventing its degradation and thereby prolonging MMP9 activity. MMP9 is a protease of the MMP family that is capable of degrading a broad spectrum of extracellular matrix components and is held responsible for vascular remodeling and breakdown of the fibrous cap of atherosclerotic lesions leading to plaque vulnerability. Several clinical studies have investigated the role of NGAL and MMP9 in cardiovascular disease, and associations of these markers with cardiovascular outcome have been found. Our results with regard to NGAL concur with these findings. However, although NGAL was univariately associated with adverse cardiovascular events in our study, this association was not independent of conventional cardiovascular risk factors. Renal insufficiency played a part in confounding of this association (post hoc analysis resulted in an OR adjusted for renal impairment only of 1.28 [0.97–1.68]; P = 0.08). The contribution of this factor was probably enhanced by the tendency toward a higher prevalence of renal insufficiency in the patients that experienced MACE.

Conversely, limited data are available on NGAL/MMP9 complex in renal insufficiency and atherosclerosis. A previous study investigated NGAL/MMP9 complex in human atherosclerotic plaques and found that increased levels of NGAL/MMP9 complex were associated with high lipid content, high number of macrophages, high interleukin-6 and interleukin-8 levels, and low smooth muscle cell content in 122 human atherosclerotic lesions. These results suggest that NGAL/MMP9 complex plays a part in the inflammation cascade leading to plaque instability. To our best knowledge, our study is the first to investigate circulating NGAL/MMP9 complex in renal insufficiency and atherosclerosis. A previous study investigated NGAL/MMP9 complex in human atherosclerotic plaques and found that increased levels of NGAL/MMP9 complex were associated with high lipid content, high number of macrophages, high interleukin-6 and interleukin-8 levels, and low smooth muscle cell content in 122 human atherosclerotic lesions. These results suggest that NGAL/MMP9 complex plays a part in the inflammation cascade leading to plaque instability. To our best knowledge, our study is the first to investigate circulating NGAL/MMP9 complex in renal insufficiency and atherosclerosis.
levels in a large clinical setting and to demonstrate that circulating NGAL/MMP9 levels have prognostic value in patients with CAD. Although NGAL/MMP9 was not significantly associated with MACE in univariate analysis \((P=0.070)\), the association became statistically significant after adjustment for conventional risk factors (including renal insufficiency; \(P=0.042\)). Although lack of a univariable association may complicate interpretation of these findings, we think that it does not compromise clinical use. For the purpose of prognostication, biomarker information is traditionally evaluated in combination with the patient’s clinical characteristics, and our study shows that NGAL/MMP9 significantly adds information to clinical characteristics, as demonstrated by an improvement of the net reclassification index. MMP9 is inhibited by TIMP1, a tissue inhibitor of metalloproteinases. TIMP1 was previously found to be an independent predictor of mortality and myocardial infarction in patients with CAD.23 Both markers were previously found to be differentially associated with CAD and patients with acute coronary syndrome.3–5 CRP is produced in the liver and reflects the overall inflammatory status of the patient1,3–5 and thus does not distinguish particular causes of inflammation. Our results imply that OGN and NGAL/MMP9 may provide incremental value for prediction of cardiovascular risk on top of CRP.

The remaining biomarkers in this study did not show significant associations with MACE. Although natriuretic peptides are well known as markers for heart failure, it has been shown that natriuretic peptides also have prognostic information in patients with acute coronary syndrome.24 In our study, proBNP tended to be associated with MACE in univariable analyses. We may have lacked power to detect a significant association between proBNP and MACE. With regard to heat shock protein 27, previous studies have rendered inconsistent results. With regard to thrombospondin 2, research was mainly focussed on single-nucleotide polymorphisms. The other proteins (aciculin, DJ1, microfibril-associated glycoprotein 4, procollagen C proteinase enhancer 1, phosphatidylethanol-amine-binding protein 1, and peroxiredoxin 2) have not been investigated as prognostic biomarkers in CAD patients before. An issue that warrants consideration in our study is the fact that we examined a total of 13 biomarkers, and if we were to account for multiple testing, our findings would lose statistical significance. However, our study was not data driven but hypothesis driven; the choice of biomarkers we investigated was based on our previous proteomics experiment. Therefore, accounting for multiple testing may not be fully justified. In any case, our findings should be considered as indicative of a potential association and merit validation in other, larger, studies.

In conclusion, OGN and NGAL/MMP9 are novel and promising prognostic biomarkers in patients with CAD. Both markers were previously found to be differentially expressed in vulnerable atherosclerotic plaques and are suggested to have important roles in arterial matrix remodeling and endothelial activation. Higher circulating OGN and NGAL/MMP9 levels were independently predictive for occurrence of MACE within 1 year after coronary angiography and displayed incremental value over conventional cardiovascular risk factors and CRP in terms of risk reclassification. Further studies are required to determine the precise pathophysiological role of OGN and NGAL/MMP9 in atherosclerosis and to confirm their roles as prognostic biomarkers of CAD.

Acknowledgments

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Disclosures
Delphine Merle, Dominique Piquer, Emilie du Paty, Pascale Galéa, and Jeannette Fareh are employed by Bio-Rad Laboratories, Marnes La Coquette, France. The other authors report no conflicts.

References

Significance
Blood biomarkers may improve prediction of cardiovascular outcome in patients with coronary artery disease. The main challenge in the search for suitable biomarkers is that plasma contains >900,000 proteins. This study investigated the associations between circulating plasma biomarkers that were previously identified by proteomics or immunohistochemistry experiments in human carotid plaques and adverse cardiovascular outcome in patients undergoing coronary angiography. The prognostic value of the majority of these proteins, including osteoglycin and neutrophil gelatinase–associated lipocalin/matrix metalloproteinase 9 complex, had not yet been investigated. Higher circulating osteoglycin and neutrophil gelatinase–associated lipocalin/matrix metalloproteinase 9 levels were independently predictive for occurrence of major adverse cardiovascular events within 1 year after coronary angiography and displayed incremental value over conventional cardiovascular risk factors and C-reactive protein in terms of risk reclassification. Further studies are required to determine the precise pathophysiological role of osteoglycin and neutrophil gelatinase–associated lipocalin/matrix metalloproteinase 9 in atherosclerosis and to confirm their roles as prognostic biomarkers of coronary artery disease.
Circulating Osteoglycin and NGAL/MMP9 Complex Concentrations Predict 1-Year Major Adverse Cardiovascular Events After Coronary Angiography

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The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/34/5/1078
MATERIALS AND METHODS

Study population and baseline data collection
Between November 2008 and January 2011, a cohort of 768 patients who underwent coronary angiography for acute coronary syndrome or stable angina pectoris in Erasmus MC, Rotterdam, the Netherlands were included in a prospective biomarker study. The current nested case-control study included a total of 88 patients that experienced a major adverse cardiovascular event (MACE) within 1 year after the initial coronary angiography (cases), and 176 control patients who did not experience MACE and were matched on age, sex and number of diseased coronary vessels. MACE was defined as all-cause mortality, acute coronary syndrome, unplanned coronary revascularization and stroke. This study was approved by the medical ethics committee of Erasmus MC. The study was performed in accordance with the criteria described in the declaration of Helsinki. Written informed consent was obtained from all patients.

Baseline characteristics of all patients were collected prospectively by trained research physicians. These physicians reviewed the medical charts of the patients at the time of inclusion in the study, and extracted variables regarding demographics, medical history, cardiovascular risk factors and procedural characteristics. Medical history and cardiovascular risk factors are a routine part of clinical patient assessment at the department of Cardiology. Thus, presence of diabetes mellitus, hypertension, hypercholesterolemia, history of renal insufficiency and history of heart failure were defined as a clinical diagnosis of these conditions as reported by the treating physician in the medical chart. Smoking was defined as current smoking, reported by the patient. Procedural characteristics were prospectively extracted from the catheterization report.

Biomarkers
Biomarkers were chosen based on a previous discovery proteomics study that identified several proteins to be differentially released by vulnerable hemorrhagic human carotid plaques when compared to stable fibrotic plaques. In the current study, ELISA assays were used to measure the biomarker concentrations.

Blood samples were drawn from the arterial sheath prior to the coronary angiography procedure. The blood samples were transported to the clinical laboratory of Erasmus MC for further processing and storage at a temperature of -80°C within 2 hours after blood collection.

CRP was measured in serum samples using a immunoturbidimetric high sensitivity assay (Roche Diagnostics Ltd., Rotkreuz, Switzerland) on the Cobas 8000 modular analyzer platform (Roche Diagnostics Ltd., Rotkreuz, Switzerland). The diagnostic range of this assay is 0.3-350 mg/L with a coefficient of variation of 1.3% at a mean value of 2.63 mg/L.

Frozen EDTA-plasma samples were transported under controlled conditions (at a temperature of -80°C) to Sysdiag Laboratory at Montpellier, where the concentrations of the 13 novel biomarkers were measured in a blinded manner. Laboratory investigators had no access to the clinical data. Using commercially available enzyme-linked immunosorbent assays (ELISA), PCPE1 (USCN Life Science, Wuhan, Hubei, China), DJ1 (Circulex Human DJ-1/PARK7 ELISA Kit, MBL International Corporation, Woburn, Massachusetts, USA), heat shock protein 27 (HSP27; Merck, Whitehouse Station, New Jersey, USA), OGN (USCN Life Science, Wuhan, Hubei, China), PRX2 (R&D Systems, Minneapolis, Minnesota, USA), metalloproteinase inhibitor 1 (TIMP-1; R&D Systems, Minneapolis, Minnesota, USA) and thrombospondin-2 (TSP2; R&D Systems, Minneapolis, Minnesota, USA) were measured according to manufacturer’s instructions. Pro-B-type natriuretic protein 1-108 (proBNP), aciculin, MFAP4, NGAL and NGAL/MMP9 were measured by home-made ELISA assays using specific and sensitive monoclonal antibodies that were developed by the HTMaB platform (Sysdiag Laboratory, Montpellier, France). Details of the assay performances have been described elsewhere.1, 2

Clinical follow-up
Clinical follow-up started at inclusion and lasted 1 year. Post-discharge survival status was obtained from municipal civil registries. Post-discharge rehospitalizations were prospectively assessed during follow-up. Questionnaires focusing on the occurrence of MACE were sent to all living patients. Response rate of the questionnaires was 99.6%. Subsequently, hospital discharge letters were obtained and treating physicians and institutions were contacted for additional information whenever necessary. ACS was defined as the clinical diagnosis of ST segment elevation myocardial infarction (STEMI), non-STEMI or unstable angina pectoris in accordance with the guidelines of the European Society of Cardiology. Unplanned coronary revascularization was defined as unplanned repeat PCI (either culprit or non-culprit coronary artery) or coronary artery bypass grafting (CABG). Stroke was defined according to the guidelines of the European Stroke Organization. The endpoints were adjudicated by a clinical event committee that had no knowledge of the biomarker data.

**Statistical analysis**

The distributions of continuous baseline characteristics, as well as the biomarker levels, were tested for normality using the Kolmogorov-Smirnov test. Variables that were not normally distributed were ln-transformed for further analyses. We used conditional logistic regression analyses to compare baseline characteristics between cases and controls. Conditional logistic regression analyses were also performed to evaluate the associations between biomarker levels and incident MACE. In multivariable analyses, the following clinical covariates were additionally entered into the model: diabetes mellitus, hypertension, smoking, dyslipidemia, history of myocardial infarction, history of renal impairment and indication for coronary angiography. Subsequently, CRP was also entered into the model to evaluate whether the associations between biomarkers and MACE were independent of CRP concentration. The final results are presented as crude and adjusted odds ratios (OR) with 95% confidence interval (95% CI).

For biomarkers that were independently associated with incident MACE, receiver operating characteristic (ROC) curves were constructed to evaluate the supplemental value of these biomarkers for discrimination between cases and controls over conventional cardiovascular risk factors. Furthermore, continuous net reclassification improvement indices (NRI) were calculated to evaluate improvement in risk classification by the new biomarkers over conventional cardiovascular risk factors. In brief, NRI is calculated by examining cases and controls separately. It is desirable to increase the predicted probabilities of event for those who experience events, and hence any upward reclassification among events is considered beneficial. For control patients, the reasoning is reversed: downward movement in categories is considered beneficial. The total NRI is calculated as the sum of the two. All data were analyzed with SPSS software (SPSS 20.0, IBM corp., Armonk, NY, USA). ROC curves were compared using the method that was described by Hanley et al. All statistical tests were two-tailed and p-values <0.05 were considered statistically significant.
REFERENCES


