High Levels of Myeloid-Related Protein 14 in Human Atherosclerotic Plaques Correlate With the Characteristics of Rupture-Prone Lesions

Mihaela G. Ionita, Aryan Vink, I. Esmé Dijke, Jon D. Laman, Wouter Peeters, Petra Homoet van der Kraak, Frans L. Moll, Jean-Paul P.M. de Vries, Gerard Pasterkamp, Dominique P.V. de Kleijn

Objective—Atherosclerotic plaque rupture can lead to severe complications such as myocardial infarction and stroke. Myeloid related protein (Mrp)-14, Mrp-8, and Mrp-8/14 complex are inflammatory markers associated with myocardial infarction. It is, however, unknown whether Mrps are associated with a rupture-prone plaque phenotype. In this study, we determined the association between Mrp-14, -8, -8/14 plaque levels and plaque characteristics.

Methods and Results—In 186 human carotid plaques, levels of Mrp-14, -8, and -8/14 were quantified using ELISA. High levels of Mrp-14 were found in lesions with a large lipid core, high macrophage staining, and low smooth muscle cell and collagen amount. Plaques with high levels of Mrp-14 contained high interleukin (IL)-6, IL-8, matrix metalloproteinase (MMP)-8, MMP-9, and low MMP-2 concentrations. Mrp-8 and Mrp-8/14 showed a similar trend. Within plaques, a subset of nonfoam macrophages expressed Mrp-8 and Mrp-14 and the percentage of Mrp-positive macrophages was higher in rupture-prone lesions compared to stable ones. In vitro, this subset of macrophages does not acquire a foamy phenotype when fed oxLDL.

Conclusion—Mrp-14 is strongly associated with the histopathologic features and the inflammatory status of rupture-prone atherosclerotic lesions, identifying Mrp-14 as a local marker for these plaques. (Arterioscler Thromb Vasc Biol. 2009;29:00-00.)

Key Words: ●●●

Rupture of an atherosclerotic plaque and subsequent thrombosis is the underlying cause of the majority of acute coronary syndromes (ACS) and strokes.1 The major determinants of a rupture-prone plaque are the size of the lipid core, the thickness of the fibrous cap covering the core, and ongoing inflammation and repair within the cap.2 Macrophages can weaken the fibrous cap by secreting matrix degrading proteases and inflammatory proteins, leading to plaque disruption and subsequent thrombosis. Although macrophages are considered a hallmark of the rupture-prone plaque, it is unknown which proteins expressed by these macrophages can be used to identify rupture-prone plaques.

Myeloid related proteins (Mrp)-14 and -8 (also named S100A9 and S100A8 or calgranulin B and A) are 2 calcium-binding proteins mainly expressed in cells of myeloid origin, particularly in monocytes and neutrophils.3 Both proteins are secreted by activated monocytes and neutrophils and have proinflammatory effects.4,5 Mrp-14 and Mrp-8 are expressed by subsets of macrophages during inflammation in different tissues,6 as well as in atherosclerotic lesions.7 On cell activation, the 2 proteins can form a complex, Mrp-8/14, that translocates to the cytoskeleton and plasma membrane where it is secreted.8 Intracellularly, Mrp-8 and Mrp-14 essentially regulate phagocyte (monocytes and neutrophils) migration by integrating the calcium and mitogen-activated protein kinase (MAPK) transduction pathways, thereby controlling reorganization of the phagocyte microtubular system.9 The secreted Mrp-8/14 complex exerts antimicrobial activity,10 stimulates IL-8 production by airway epithelial cells,11 and transports arachidonic acid to endothelial cell (EC) targets affecting pathological responses in inflammation and atherosclerosis.10 The receptor for advanced glycation endproducts (RAGE)12 and toll like receptor (TLR)–413 are 2 putative receptors for Mrp-8, Mrp-14, and Mrp-8/14 complex on phagocytes. The Mrp-8/14 complex is emerging as a new blood biomarker that can discriminate between patients with ACS and those with stable coronary heart disease.14 Systemically as well as at the site of coronary occlusion, the Mrp-8/14 complex is a novel,
early, and sensitive marker of ACS and is elevated before necrotic factors such as myoglobin, CK-MB, and troponin. A recent study of the platelet transcriptome led to the identification of Mrp-14 as a biomarker that can predict future cardio-vascular events in healthy individuals.

Taken together, these results suggest that Mrp-8, Mrp-14, and Mrp-8/14 reflect biological events in plaque progression toward rupture leading to the hypothesis that Mrp plaque levels correlate with the characteristics of high-risk rupture-prone atherosclerotic lesions. Until now, it is unknown whether these proteins are associated with the rupture-prone plaque phenotype. To address this issue, we determined Mrp-8, Mrp-14, and Mrp-8/14 levels in a large cohort of human atherosclerotic specimens and assessed the association with plaque characteristics and the presence of clinically manifest atherosclerotic disease. We found high levels of Mrp-14 in the ruptured-prone lesions which make this protein a suitable candidate for the imaging of high-risk rupture-prone plaques in humans. A subset of nonfoam macrophages expressing Mrp-8 and -14 was predominant in the rupture-prone lesions; in vitro, the Mrp-macrophage subset did not acquire a foamy phenotype when fed with human oxidized low density lipoprotein (oxLDL).

Methods

Athero-Express Biobank

Athero-Express is an ongoing longitudinal cohort study, initiated in 2002 by 2 Dutch hospitals: the University Medical Center Utrecht and the St Antonius Hospital in Nieuwegein. The study has been approved by the institutional boards of both hospitals, and written informed consent was obtained from all participants. The study is designed to investigate the expression of atherosclerotic tissue-derived biological markers in relation to plaque phenotype of patients undergoing carotid endarterectomy (CEA) and adverse cardiovascular events during follow-up. Patients who undergo carotid endarterectomy (CEA) fill in an extensive questionnaire, and diagnostic examinations are performed.

Patient Inclusion

In this study a random set of 186 plaques from symptomatic (n = 154) and asymptomatic (n = 32) patients undergoing carotid endarterectomy (CEA) were included. The indication for CEA for asymptomatic patients was based on the recommendations published by the Asymptomatic Carotid Surgery Trial (ACST) and for symptomatic patients was based on recommendations based on the European Carotid Surgery Trial (ECST) and the North American Symptomatic Carotid Endarterectomy Trial (NASCET). All patients were reviewed by the vascular surgeon or neurologist before CEA to assess the nature and timing of clinical symptoms.

Plaque Processing

All carotid plaques were carefully dissected from the carotid arteries and immediately transferred to the laboratory for further processing as described previously and in detail in the supplemental materials (available online at http://atvb.ahajournals.org). In short, in the laboratory the atherosclerotic fragments were dissected by a dedicated technician into 0.5-cm-thick cross-sectional segments along the longitudinal axis of the vessel. The plaque segment showing the largest plaque burden was called the culprit lesion and was used for histological analysis to determine plaque morphology. The definitions of each staining category (H&E, Elastin von Gieson, picrosirius red, -actin, and CD68) have been described previously.

Levels of interleukin (IL)-6 and IL-8 were measured by a multiplex suspension array system according to the manufacturer’s protocol (Bender Med Systems). Matrix metalloproteinase (MMP)-2, MMP-8, and MMP-9 activities were measured using the Biotrak activity assays RPN 2631, RPN 2635, and RPN 2634 (Amersham Biosciences), respectively.

Immunoadays for Mrp-8, Mrp-14, and Mrp-8/14

Concentrations of Mrp-8, Mrp-14, and Mrp-8/14 were measured with a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) using commercially available kits (BMA Biomedicals AG) according to the manufacturer’s protocols. The detection limits for Mrp-8 homodimers, Mrp-14 homodimers, and Mrp-8/14 heterodimers were 0.69, 0.31, and 4.69 ng/mL, respectively. Each ELISA kit was specific for the target Mrp-protein, and the cross-reactivity was minimal (according to the manufacturer). All concentrations were corrected (normalized) for the amount of protein in each sample.

Immunohistochemistry for Mrp-8 and Mrp-14

To determine the cellular source of Mrp-8 and Mrp-14, a random set of 80 plaques, from the total 186 plaques included in the present study, was selected for immunohistological analysis. Sections were processed with EDTA and stained with mouse antihuman Mrp-8 (mouse IgG2h, dilution 1:750; Santa-Cruz Biotechnologies) monoclonal antibody. Consecutive sections were boiled in citrate buffer (M = 294.1 g/mol, pH 6.0, 20 minutes.) and stained with a monoclonal antihuman Mrp-14 antibody (mouse IgG1, dilution 1:200; Santa-Cruz Biotechnologies). Powerversion poly HRP-antimouse IgG (Immunologuc) was used as secondary antibody. Mouse IgG of the same isotype and same subclass as the primary antibody was used as negative control. The signal was visualized using diaminobenzidine.

Sections were counterstained with hematoxylin.

The CD68 staining and the Mrp-8 staining were quantified using image-analyzing software (Soft Imaging Systems). Expression of Mrp-8 and Mrp-14 was detected in nonfoam CD68-positive macrophages. We therefore decided to select only the nonfoam CD68-positive macrophage areas for quantitative analysis. Areas rich in macrophage foam cells were excluded from the analysis. CD68 positive macrophage foam cells were identified by their classical morphology (increased cell size, lipid droplets in the cytoplasm, and nucleus pushed to the membrane side of the cytoplasm).

In Vitro Generation of Human oxLDL-Laden Macrophages

Human monocyte-derived macrophages were generated as previously described: monocytes were isolated from anonymous healthy blood donors’ buffy coats using Ficoll and Percoll density gradients (density: 1.077 g/mL and 1.063 g/mL, respectively). Next, the monocytes were differentiated into macrophages by culturing the monocytes under nonadherent conditions in RPMI 1640 medium (BioWhittaker) supplemented with 25 nM Heps, Uletralumatin1, and 5% human AB serum and without further cytokine stimulation. After 7 days, macrophages were sorted based on CD14 and Mrp-8/14 membrane expression as described below. The 2 sorted macrophage populations, namely Mrp-8/14 negative and Mrp-8/14 positive, were plated into 12-well (flow cytometric analysis) or 96-well (oil red O staining) flat-bottom culture plates (Nunc). The macrophages were incubated during 24 hours with 10 μg/mL human oxLDL, isolated and oxidized as described previously or with culture medium as control. OxLDL uptake was detected with oil red O, which stains neutral lipids, as previously described.

Flow Cytometry and Fluorescence-Activated Cell Sorting

Fluorescence-activated cell sorting on a FACS Aria (BD Biosciences) was performed to sort the CD14-positive Mrp-8/14–negative and CD14-positive Mrp-8/14–positive cells. CD14-positive cell sorting was based on forward light scattering (FSC) and sideward light scattering (SSC), and subsequent gating of cells negative for CD3-PerCP (BD Biosciences), CD19-APC (BD Biosciences), and CD56-RPE (Bio-Connect BV). Mrp-8/14 FITC (2E10) (mouse IgG1, Santa Cruz) antibody, recognizing only the
Mrp-8/14 heterodimers, was used to identify the Mrp-8/14–negative and Mrp-8/14–positive cells within the CD14 positive population. For flow cytometry, the following antibodies were used: CD14-PerCP (BD Biosciences); CD68-AFC (R&D Systems); Mrp-8/14FITC (2E10), Mrp-8-FITC, and Mrp-14-FITC (all from Santa Cruz). The samples were measured on a LSR II (BD Biosciences) and analyzed using FACS Diva version 6.1.1. (BD biosciences) and Flow Jo Version 7.2.5 (Tree Star Inc) software.

Data Analysis
Data are expressed as means±SEM. Correlations between different parameters were assessed using Spearman correlation test; the statistical significance of the difference between two groups was determined using Mann–Whitney test; probability values <0.05 were considered significant.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agreed to the manuscript as written.

Results

Mrp Plaque Levels and Atherosclerotic Plaque Histology
The clinical characteristics of the patients and the histological features of the atherosclerotic specimens are shown in the supplemental materials.

Protein levels of Mrp-8, Mrp-14, and Mrp-8/14 were quantified in 186 atherosclerotic specimens and compared with different characteristics of the atherosclerotic plaque: size of the lipid core, the amount of collagen, the frequency of macrophages, and SMCs (Figure 1). Mrp-8, -14, and -8/14 levels were associated with the size of the lipid core (Figure 1); this positive association was statistically significant (P=0.001, P=0.001, P=0.004 for Mrp-8, -14, and -8/14, respectively). Higher Mrp-8, -14, and -8/14 levels were observed in plaques with a lipid core greater than 40% of the plaque area compared to plaques with a lipid core smaller than 10%.

An inverse association was observed between plaque Mrp-14 levels and the amount of collagen and SMC. High Mrp-14 levels correlated with low collagen levels in the plaque (P=0.01; Figure 1B) and with low SMC content in the plaque (P=0.001; Figure 1B). Mrp-8 and -8/14 showed a similar trend, although without reaching statistical significance (Figure 1A and 1C).

A positive correlation was observed between the macrophage content of the plaque and high levels of Mrp-14 (Figure 1B); this correlation was statistically significant (P=0.008). In plaques with heavy macrophage staining, the concentrations of Mrp-14 were higher compared to plaques with minor macrophage staining. High Mrp-8 levels were significantly associated with heavy macrophage content (P=0.001; Figure 1A), whereas for Mrp-8/14 a similar but nonsignificant trend was observed (Figure 1C). The absolute levels of Mrp-8/14 were higher compared to Mrp-8 and Mrp-14 levels, suggesting that Mrp-8/14 is more abundant in plaques.

In the lesions of patients with clinical symptoms (n=154) we detected higher levels of Mrp-14 and Mrp-8/14 compared to the asymptomatic lesions (n=32; supplemental Figure 1).

Mrp Plaque Levels in Relation to MMPs and Cytokines
To investigate the relationship between plaque Mrp levels and matrix degradation and inflammation as features of the rupture-prone plaque, we determined the levels of the proteases MMP-2, MMP-8, MMP-9, and the proinflammatory molecules IL-6 and IL-8 in the lesion (Table). Mrp-14 showed significant positive correlations with IL-6, IL-8, MMP-8, and MMP-9 and a significant negative correlation with MMP-2. Mrp-8 was associated with IL-6, IL-8, and MMP-8 and was not associated with MMP-2 and MMP-9.

Mrp-8/14 was associated with IL-8, MMP-8, and MMP-9 plaque levels.

Mrp-8 and Mrp-14 Expression in a Subset of Plaque Macrophages
Mrp-8 and Mrp-14 were expressed by the same cells within atherosclerotic plaques (Figure 2a and 2b). Mrp-8 and Mrp-14 expression was observed in a subset of CD68 positive macrophages (Figure 2c through 2f). The Mrp-positive macrophages did not exhibit a foamy phenotype as CD68-foam macrophages stain negative for Mrp (Figure 2e and 2f). Within nonfoam CD68-positive macrophage areas, the percentage of Mrp-positive macrophages is significantly higher in rupture-prone (n=154, mean 33.57±3.74%) than in stable lesions (n=25, mean 9.56±3.5%; P=0.003; Figure 3).

In addition to macrophages, Mrp expression was observed in a small number of neutrophilic granulocytes (supplemental Figure II).

Mrp and In Vitro Development of Monocytes Into Foamy Macrophages
Because Mrp-8 and Mrp-14 were expressed in nonfoam CD68 plaque macrophages, we asked whether human oxLDL will induce foam morphology in Mrp-expressing macrophages. We monitored Mrp-expression during the development of healthy human monocytes via monocyte-derived macrophages into oxLDL-loaded foamy macrophages using an in vitro system (Figure 4a). Monocytes were identified based on their CD14 positivity. These monocytes lacked Mrp-8/14 membrane expression and intracellular CD68 expression but all showed intracellular Mrp-8, -14, and -8/14 positivity (Figure 4b). After 7 days of culture under nonadherent conditions and without further cytokine stimulation as expected, the CD14–monocyte population developed into monocyte-derived macrophages, without losing CD14 membrane expression (Figure 4c). These primary macrophages were now also CD68-positive. All macrophages showed intracellular Mrp-8, -14, and -8/14 expression, but only half of these cells expressed Mrp-8/14 on the membrane (Figure 4c). Subsequent sorting based on FSC, SSC, and Mrp-8/14 surface expression resulted in 2 human primary macrophage populations, namely Mrp-8/14–positive and -negative. Both populations were fed for 24 hours with 10 μg/mL oxLDL, and changes in the morphology were determined by light microscopy and by oil red O staining to detect intracellular neutral lipids. Macrophages expressing membrane-bound Mrp-8/14 did not acquire a foamy phenotype, whereas the vast majority of the macrophages lacking Mrp-8/14 membrane expression acquired a foamy phenotype and contained lipid droplets in the cytoplasm (Figure 5). The Mrp-8/14 membrane expressing macrophages maintained intracellular Mrp-8, Mrp-14, and Mrp-8/14 expression (Figure 4d). In
contrast, almost all macrophages that developed a foamy morphology lacked intracellular and membrane Mrp-8/14 as well as intracellular Mrp-14, and approximately half of these cells lacked intracellular Mrp-8 expression (Figure 4d).

**Discussion**

Rupture of an atherosclerotic plaque and subsequent thrombosis is the most common cause of acute coronary syndromes and stroke, and markers are needed to identify these dangerous lesions.

The present study identifies Mrp-14 as a marker of the high-risk prone-to-rupture plaque. We report that high levels of Mrp-14 are associated with atherosclerotic lesions displaying features of a rupture-prone plaque. Levels of Mrp-8 and Mrp-8/14 complex showed similar trends but did not always reach statistical significance.
High Mrp-14 plaque levels are significantly associated with high levels of IL-6, IL-8, MMP-8, and MMP-9 and low levels of MMP-2. Mrp-8 showed no significant correlation with MMP-2 and MMP-9, whereas Mrp-8/14 was not correlated with IL-6 and MMP-2. Within the atherosclerotic plaque, MMP-2 is a molecule associated with a stable plaque phenotype.25 Proinflammatory cytokines IL-6 and IL-8 are associated with active plaque inflammation. The levels of proteases, such as MMP-8 and MMP-9, are elevated in the rupture-prone plaques and these MMPs are very active in the most vulnerable regions of the plaque: cap and shoulder.26 The cellular source of these cytokines (IL-6, IL-8) and proteases (MMP-2, -8, -9) in atherosclerotic lesions is heterogeneous; IL-6 is secreted by active plaque macrophages, smooth muscle cells, endothelial cells, and T-cells whereas IL-8 is secreted by active macrophages, endothelial cells, and T-cells.27 MMP-2 and MMP-9 colocalize with activated smooth muscle cells and macrophages within atherosclerotic plaques.26 MMP-8 is an extremely efficient type I collagenolytic enzyme in humans28 and is traditionally considered a neutrophil product, a cell type not commonly observed in atheroma29; however, within human atherosclerotic lesions also vascular endothelial cells, smooth muscle cells, and macrophages express MMP-8.30

Mrp-8 and Mrp-14 are expressed in healthy human blood monocytes and neutrophils and in subpopulation of macrophages in inflammatory tissues.31 In our study, Mrp-8 and Mrp-14 were detected in a subset of macrophages in atherosclerotic plaques. This observation is in accordance with a previous report of subsets of Mrp-positive macrophages,7 suggesting differential activation of plaque macrophages and underlining existence of subsets of macrophages in atherosclerotic lesions. Expression of plaque Mrp-8 and -14 was only observed in nonfoamy macrophages with a higher

### Table. Correlations Between Mrp Plaque Levels and Inflammatory Cytokines and Proteases

<table>
<thead>
<tr>
<th>Cytokines/chemokines</th>
<th>Mrp-8</th>
<th>Mrp-14</th>
<th>Mrp-8/14</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>P=0.048*</td>
<td>P=0.001*</td>
<td>P=0.114</td>
</tr>
<tr>
<td>IL-8</td>
<td>P=0.001*</td>
<td>P=0.001*</td>
<td>P=0.001</td>
</tr>
<tr>
<td>MMP-2</td>
<td>R(+) 0.587</td>
<td>R(+) 0.718</td>
<td>R(+) 0.528</td>
</tr>
<tr>
<td>MMP-9</td>
<td>P=0.102</td>
<td>P=0.001*</td>
<td>P=0.005*</td>
</tr>
</tbody>
</table>

Spearman correlation (R=correlation coefficient); significance at *P<0.05; IL indicates interleukin; MMP, matrix metalloproteinase.

Figure 2. Expression of Mrps in carotid atherosclerotic plaque. Mrp-8 (a) and Mrp-14 (b) colocalize in consecutive sections. c through f, Mrp-8 expression in CD68-positive macrophages. c and d, Consecutive sections showing Mrp-8 staining (d, brown) in a small subset of CD68-positive macrophages (c, red) in a stable plaque. e and f, Consecutive sections showing Mrp-8 staining (f) in a larger subset of CD68-positive macrophages (e) in a rupture-prone plaque. CD68-positive macrophage foam cells (e, arrows) are negative for Mrp-8 (f, arrows). Bars indicate 50 μm.

Figure 3. Quantitative assessment of the Mrp-8 area within the nonfoam CD68 area in stable (n=25) and rupture-prone (n=55) atherosclerotic lesions. The percentage of Mrp-8-positive macrophages was significantly (P=0.003) higher in rupture-prone plaques (mean=34.78) compared to stable ones (mean=12.63). Lines represent medians; statistics Mann–Whitney test.
percentage of Mrp-8– and 14–positive area of the nonfoam CD68 area in the rupture-prone lesions compared to stable ones. This points to Mrps as markers for macrophages that do not develop into foam cells. In vitro data confirmed this observation; showing that Mrp-8/14 membrane bound expressing macrophages did not acquire a foamy phenotype when oxLDL was added to the culture. In contrast, the majority of macrophages lacking Mrp-8/14 membrane expression accumulated lipid droplets in the cytoplasm when fed with oxLDL. A variety of intracellular functions have been implied for Mrp-8, -14, and -8/14 in phagocyte physiology, however nothing is documented regarding involvement of these proteins in phagocytosis. A mouse study suggested that secreted CP-10 (58% amino acid identity with human Mrp-8) has chemotactic properties for monocytes and enhances scavenger receptor expression and uptake of modified LDL by these attracted macrophages. The Mrp-positive macrophage subset is associated with a high plaque inflammatory status as reflected by the levels of proinflammatory interleukins and proteases, suggesting that this subset might be involved in plaque destabilization (see supplemental Table V). Involvement of Mrp in determining foam cell development or inflammatory active macrophages, however, remains to be determined.

In addition, Mrp-8 and -14 staining was observed in a small number of neutrophilic granulocytes within the plaque area (supplemental Figure II). This observation coupled with the positive correlation between IL-8 and Mrp-8, -14, -8/14 plaque levels might suggest a possible role for neutrophils in plaque associated inflammation. A recent study demonstrated that the infiltrated neutrophils within atherectomy specimens of patients with unstable angina were Mrp-8/14–positive. Although the focus of the present study was on the Mrp-subset of nonfoam plaque macrophages, it does not exclude the importance of other possible Mrp-cellular sources within plaque (eg, the neutrophils).

We found high levels of Mrp-14 and Mrp-8/14 in clinically symptomatic atherosclerotic plaques. This observation is in accordance with previous studies showing that symptomatic plaques often exhibit a rupture-prone phenotype. Altwegg et al recently identified Mrp-8/14 as a new biomarker that can discriminate between patients with ACS and patients with stable coronary heart disease. They report that Mrp-8/14 is markedly elevated in ACS culprit lesions (thrombus and plaque material) compared with systemic levels, suggesting that Mrp-8/14 is locally expressed at the site of coronary occlusion because of plaque rupture or erosion. Our study clearly demonstrates that high levels of Mrp-14 but probably also Mrp-8 and Mrp-8/14 plaque are associated with rupture-prone atherosclerotic plaques. This has important implications for possible noninvasive imaging techniques to detect in vivo, high-risk, hidden plaque destabilization and...
rupture before this leads to dangerous cardiovascular complications and therefore adds to patient stratification for therapy.

In summary, we show that high levels of Mrp-14 and to a lesser extent also Mrp-8 and Mrp-8/14, expressed by a subset of nonfoam macrophages in human plaques, are strongly associated with both histopathologic features and the inflammatory status of rupture prone lesions. These results identify Mrps as possible imaging markers to detect the hidden rupture-prone plaque.

Limitations
The nonsignificant results obtained for Mrp-8 and -8/14 might be attributable to limitations of the commercial ELISA kits used for the detection of those proteins. The Mrp-8 levels might be underestimated because of lower sensitivity of the Mrp-8 ELISA kit compared to the Mrp-14 ELISA kit. The detection of the Mrp-8/14 heterodimer could be influenced by different conditions used when performing the assays; we performed all assays under the same conditions, and little variation between tests was observed. It is important to note that the present study is purely observational and no implications regarding causality can be drawn. However, considering the facts that Mrp-positive cells are absent in the normal vessel wall, are differentially expressed in stable versus rupture-prone plaques, with high levels in the rupture-prone plaque, it is more than reasonable to speculate that these proteins play a role in plaque destabilization. The exact function of Mrps in atherosclerotic lesions, however, remains unclear at this point.

Figure 5. Macrophages expressing surface Mrp-8/14 complex did not acquire foam cell morphology on oxLDL ingestion, whereas the macrophages lacking Mrp-8/14 surface expression did acquire foam morphology. Bars indicate 50 μm; bottom photos represent magnifications of the above black boxes.

Acknowledgments
The authors acknowledge Evelyn Velema, Santusha S. Karia, and E.F.E. de Haas for technical assistance. We thank Prof Johan Kuiper (Leiden University) for providing human oxLDL.

Sources of Funding
This work was supported by the grant from the European Community’s Sixth Framework Program contract LSHMCT-2006-037400 (IMMUNATH). Work in the laboratory of J.D.L. is supported by the Dutch MS Research Foundation.

Disclosures
The authors report that Dominique P.V. de Kleijn, Frans Moll, and Gerard Pasterkamp are cofounders of Cavadis, a biomarker company. The other authors report no significant conflicts of interest.

References


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Arterioscler Thromb Vasc Biol. published online June 11, 2009;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272
Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the
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Figure 2. Consecutive sections of a rupture-prone atherosclerotic plaque showing Mrp-8 (b) and MPO (c) co-localization in CD66b-positive neutrophils (a). Bars represent 25 µm.
### Supplemental Material

### Methods

#### I. Plaque processing

All carotid plaques were carefully dissected from the carotid arteries and immediately transferred to the laboratory for further processing as described previously\(^1\). In the laboratory the atherosclerotic fragments were dissected by a dedicated technician into 0.5cm-thick cross-sectional segments along the longitudinal axis of the vessel. The plaque segment showing the largest plaque burden as determined by visual assessment of plaque macroscopy was defined as the culprit lesion. This segment was fixed in 4% formaldehyde and paraffin embedded. The adjacent segments (0.5cm thickness) were grinded in liquid nitrogen and dissolved in Tripure™ Isolation Reagent Boehringer Mannheim to separate RNA from protein samples. Samples were carefully washed several times to remove the Tripure and to subsequent dissolve in 1% Sodium-Dodecyl-Sulfate. Prior to storage at -80\(^\circ\)C, the total protein concentration of each sample was quantified. Expressions were corrected for total amount of protein.

The paraffin-embedded culprit lesion was cut into sections of 5 microns and used for histological stainings with hematoxilin and eosin (H&E), elastin von Gieson, picrosirius red, \(\alpha\)-actin and CD68. Sections were studied semiquantitatively for macrophages (CD68), smooth muscle cells (\(\alpha\)-actin), collagen (picrosirius red) and the lipid-core size and were subsequently classified as minor, moderate and heavy. The definitions of each staining category have been described previously\(^1\). The criteria for classification were defined as follows: macrophages (1) minor CD68 staining with a few scattered cells; (2) moderate and (3) heavy CD68 staining, clusters of cells with >10 cells present; smooth muscle cells (1) minor \(\alpha\)-actin staining over the entire circumference with absent staining at parts of the circumference of the arterial wall; (2)
moderate and (3) heavy α-actin staining positive cells along the circumference of the luminal border; collagen: (1) minor staining along part of the luminal border of the plaque; (2) moderate and (3) heavy staining along the entire luminal border; lipid-core size: (1) less than 10% of plaque area; (2) between 10 and 40% of plaque area; (3) more than 40% of plaque area. The overall phenotype was established according to the estimation of the percentage of lipid core size of total plaque area and of collagen, smooth muscle cells and macrophage content: the rupture-prone plaque contains a large lipid core, low collagen and smooth muscle cell content with high macrophage infiltration; the stable plaque contains a small or absent lipid core (less then 10% of plaque area), high collagen and smooth muscle cell content with low macrophage number. Recently, we demonstrated that the segments adjacent to the culprit lesion showed good correlations with histological characteristics, that the histological analyses were well reproducible and revealed an acceptable inter observer agreement².

For assessing changes in plaque composition at protein level, expression of proteins playing a role in inflammatory pathways (cytokines) or plaque destabilization (matrix metalloproteinases) were measured in all plaque specimens from the Athero-Express study.

Levels of interleukin (IL)-6 and IL-8 were measured by a multiplex suspension array system according to the manufacturer’s protocol (Bender Med Systems, Vienna, Austria). Matrix metalloproteinase (MMP)-2, MMP-8 and MMP-9 activities were measured using the Biotrak activity assays RPN 2631, RPN 2635 and RPN 2634 (Amersham Biosciences, Buckinghamshire, UK), respectively.

II. Immunohistochemistry for Mrp-8, CD66b and MPO

To determine the expression of Mrp-8 in neutrophils within atherosclerotic plaques, consecutive sections of fifty carotid atherosclerotic specimens were selected for
immunohistological analysis. Sections were pretreated with EDTA and stained with mouse anti-human Mrp-8 (mouse IgG2b, dilution 1:750; Santa-Cruz Biotechnologies, Santa-Cruz, CA) monoclonal antibody. Consecutive sections were boiled in citrate buffer (M = 294.1 g/Mol, pH 6.0, 20 min.) and stained with a monoclonal anti-human CD66b antibody (80H3, mouse IgG1, dilution 1:150, Abcam, MA, USA) and a monoclonal anti-human MPO antibody (mouse IgG1, dilution 1:100, Abcam, MA, USA). Powervision poly HRP-anti-mouse IgG (Immunologic, Duiven, the Netherlands) was used as secondary antibody. Mouse IgG of the same isotype and same subclass as the primary antibody was used as negative control. The signal was visualized using diaminobenzidine. Sections were counterstained with hematoxylin.

Results

Figure 1. Distribution of Mrp-8, Mrp-14 and Mrp-8/14 levels in carotid plaques from symptomatic (n=154) and asymptomatic patients (n=32). Lines represent medians; statistics Mann-Whitney test, significance at \( p<0.05 \), ns = not significant.

Figure 2. Consecutive sections of a rupture-prone atherosclerotic plaques showing Mrp-8 (b) and MPO (c) co-localization in CD66b-positive neutrophils (a). Bars represent 25 µm.

Table I Clinical and histological plaque characteristics of the study population

<table>
<thead>
<tr>
<th></th>
<th>Number (N)</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical characteristics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, mean</td>
<td>73 (48 - 93)</td>
<td>8.6 %</td>
</tr>
</tbody>
</table>
Male gender 146 79 %
Current smoker 49 26 %
Diabetes 37 20 %
Hypercholesterolemia 39 21 %
Hypertension 50 27 %
Symptomatic stenosis 154 83 %

**Medication use**

Statin use 111 60 %
Aspirin use 61 33 %
ACE inhibitor 61 33 %
Oral anticoagulants 28 15 %
Corticosteroids 10 5 %

**Plaque characteristics**

Large lipid core (>40% of plaque area) 64 35 %
Heavy macrophage staining 32 17 %
Heavy collagen staining 42 23 %
Heavy smooth muscle cell staining 43 23 %

---

**Table II** Clinical and histological plaque characteristics of symptomatic and asymptomatic patients
N=186  

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years (mean)</td>
<td>71 (55-84)</td>
<td>74 (48-93)</td>
</tr>
<tr>
<td>Male gender</td>
<td>75 % (116)</td>
<td>65 % (21)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>24 % (37)</td>
<td>21 % (7)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>17 % (27)</td>
<td>19 % (6)</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>56 % (86)</td>
<td>53 % (17)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>64 % (98)</td>
<td>44 % (14)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Medication use</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Statin use</td>
<td>62 % (96)</td>
<td>47 % (15)</td>
</tr>
<tr>
<td>Aspirin use</td>
<td>33 % (51)</td>
<td>31 % (10)</td>
</tr>
<tr>
<td>ACE inhibitor</td>
<td>36 % (55)</td>
<td>19 % (6)</td>
</tr>
<tr>
<td>Oral anticoagulants</td>
<td>16 % (25)</td>
<td>9 % (3)</td>
</tr>
<tr>
<td>Corticosteroids</td>
<td>7 % (10)</td>
<td>0 % (0)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plaque characteristics</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Large lipid core (&gt;40% of plaque area)</td>
<td>33 % (50)</td>
<td>28 % (9)</td>
</tr>
<tr>
<td>Heavy macrophage staining</td>
<td>16 % (24)</td>
<td>16 % (5)</td>
</tr>
<tr>
<td>Heavy collagen staining</td>
<td>20 % (31)</td>
<td>19 % (6)</td>
</tr>
<tr>
<td>Heavy smooth muscle cell staining</td>
<td>18 % (28)</td>
<td>16 % (5)</td>
</tr>
<tr>
<td>Rupture-prone (overall phenotype)</td>
<td>59 % (92)</td>
<td>59 % (19)</td>
</tr>
</tbody>
</table>
Table III Patient distribution per medication use (No or Yes) and Mrp plaque levels (Low versus High)

<table>
<thead>
<tr>
<th></th>
<th>Mrp-8</th>
<th>Mrp-14</th>
<th>Mrp-8/14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Mrp-8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>29/52(56)</td>
<td>28/56(50)</td>
<td>28/56(50)</td>
</tr>
<tr>
<td>Mrp-14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mrp-8/14</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Statin use

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>23/52(44)</td>
<td>28/56(50)</td>
<td>30/56(54)</td>
<td>26/56(46)</td>
</tr>
<tr>
<td>Yes</td>
<td>68/129(53)</td>
<td>61/129(47)</td>
<td>64/133(48)</td>
<td>69/133(52)</td>
</tr>
</tbody>
</table>

Aspirin use

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>52/112(47)</td>
<td>52/111(47)</td>
<td>52/117(45)</td>
<td>65/117(55)</td>
</tr>
<tr>
<td>Yes</td>
<td>39/69(57)</td>
<td>40/71(56)</td>
<td>42/72(58)</td>
<td>30/72(42)</td>
</tr>
</tbody>
</table>

Corticosteroids use

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>87/170(51)</td>
<td>87/172(51)</td>
<td>90/178(51)</td>
<td>88/178(49)</td>
</tr>
<tr>
<td>Yes</td>
<td>4/11(36)</td>
<td>5/10(50)</td>
<td>4/11(36)</td>
<td>7/11(64)</td>
</tr>
</tbody>
</table>

Table IV Medication influence on Mrp plaque levels

<table>
<thead>
<tr>
<th></th>
<th>Mrp-8</th>
<th>Mrp-14</th>
<th>Mrp-8/14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean; 95%CI</td>
<td>Mean; 95%CI</td>
<td>Mean; 95%CI</td>
</tr>
<tr>
<td>Statin use</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>135; 100-171</td>
<td>758; 166-1315</td>
<td>2689; 1364-4013</td>
</tr>
<tr>
<td>Yes</td>
<td>138; 112-165</td>
<td>610; 338-881</td>
<td>3149; 2165-4133</td>
</tr>
</tbody>
</table>
\begin{align*}
P & = 0.8 && P = 0.888 && P = 0.555 \\
\textbf{Aspirin use}&&&&&&\nonumber \\
\text{No}&&141; \ 113-169 && 652; \ 309-995 && 3161; \ 2183-4139 \\
\text{Yes}&&131; \ 98-165 && 661; \ 259-1063 && 2771; \ 1410-4133 \\
\text{P} & = 0.366 && P = 0.183 && P = 0.013 \\
\textbf{Corticosteroid use}&&&&&&\nonumber \\
\text{No}&&139; \ 117-162 && 666; \ 393-938 && 3066; \ 2232-3900 \\
\text{Yes}&&107; \ 42-172 && 476; \ -133-1087 && 2140; \ 452-3828 \\
\text{P} & = 0.719 && P = 0.47 && P = 0.341 \\
\end{align*}

Significance at \(P<0.05\), Mann-Whitney U test
Table V Plaque interleukin and protease levels in relation with Mrp levels (low vs high) and macrophage number (low vs high)

<table>
<thead>
<tr>
<th></th>
<th>Mrp-8 Low</th>
<th>Mrp-8 High</th>
<th>Mrp-14 Low</th>
<th>Mrp-14 High</th>
<th>Mrp-8/14 Low</th>
<th>Mrp-8/14 High</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-8</td>
<td>5.2 [2.5–8]</td>
<td>5.2 [3.3-7]</td>
<td>5.8 [2.8-8.8]</td>
<td>6.7 [4.4-8.9]</td>
<td>4.4 [3-5.6]</td>
<td>8.2 [4-12]</td>
</tr>
<tr>
<td>MMP-9</td>
<td>0.62 [0.4–0.8]</td>
<td>0.67 [0.5–0.8]</td>
<td>0.6 [0.4-0.8]</td>
<td>0.7 [0.6-0.9]</td>
<td>0.6 [0.4-0.8]</td>
<td>0.7 [0.5-0.8]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Mrp-8 Low</th>
<th>Mrp-8 High</th>
<th>Mrp-14 Low</th>
<th>Mrp-14 High</th>
<th>Mrp-8/14 Low</th>
<th>Mrp-8/14 High</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9</td>
<td>0.6 [0.5-0.8]</td>
<td>0.8 [0.6-1]</td>
<td>0.7 [0.4-0.9]</td>
<td>0.8 [0.6-1]</td>
<td>0.6 [0.4-0.8]</td>
<td>0.9 [0.7-1]</td>
</tr>
</tbody>
</table>

Data shown as mean [95% confidence interval].
Reference List
