MYELOID-RELATED PROTEIN 8/14

A MARKER OF PLAQUE AND PATIENT VULNERABILITY

Gabriela Mihaela Raicu

ISBN: 978-90-393-55060
Cover design: Iulian Stoica, www.iulianstoica.blogspot.com
Lay-out: www.wenziD.nl
Printed by: Gildeprint Enschede

MYELOID-RELATED PROTEIN 8/14

A MARKER OF PLAQUE AND PATIENT VULNERABILITY

MYELOID - GERELATEERD EIWIT 8/14
EEN MARKER VOOR PLAQUE EN PATIENT VULNERABILITEIT
(met een samenvatting in het Nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 17 februari 2011 des middags te 2.30 uur

door

GABRIELA MIHAELA RAICU

geboren op 26 mei 1981 te Buzau, Roemenie

Promotoren: **Prof. dr. D.P.V. de Kleijn**
Prof. dr. G. Pasterkamp
Co-promotor: **Dr. A. Vink**

This work was financed by the grant from the European Community's Sixth Framework Program contract LSHMCT-2006-037400 (IMMUNATH).

“If I have seen further it is only by standing on the shoulders of giants”
Isaac Newton

Dedicated to my family

Contents

CHAPTER I
Introduction and study design

ATHEROSCLEROSIS, A DISEASE AS OLD AS MANKIND…

For decades, morbidity and mortality of the aging population due to cardiovascular disease is a burden for the health system. Along with a boost of hypertension, type II diabetes and obesity in the young generation, the incidence of cardiovascular disease and implicit atherosclerosis is rapidly increasing, with heart disease being the major cause of death in the modern world. The pathogenesis of atherosclerosis is heterogeneous; infection and inflammation[1, 2] and even autoimmunity[3] are implicated.

The term "*atherosclerosis*" has ancient Greek roots. It derives from "*athero*", the Greek word for gruel (symbolized by the lipid core of the plaque) and "*sclerosis*", the Greek word for hardening (referring to the fibrous cap covering the lipid core). One of the first descriptions of atherosclerosis belongs to Leonardo da Vinci (1452-1519), who admirably described it as "d*ebility through lack of blood and deficiency of the artery which nourishes the heart and other lower members*". In 1904, Felix Merchand of Leipzig proposed the term "*atherosclerosis*" to describe those lesions in the arterial wall with increased lipid content. "*Few human diseases have a longer incubation period than atherosclerosis*" (Peter Libby), atherosclerosis begins to affect arteries of individuals from the second and third decades of life.

THE RISK FACTORS

The prevalence and severity of atherosclerosis among individuals are related to risk factors. These risk factors have been identified by several major studies, among which the Framingham Heart Study and Atherosclerosis Risk in Communities Study[4, 5]. The risk factors for atherosclerosis are commonly classified in constitutional (therefore uncontrollable) and acquired (potentially modifiable). Age, gender and genetics form the group of constitutional risk factors; age has a great influence, between the ages 40 and 60 the incidence of myocardial infarction increases tremendously (up to fivefold). Women (premenopausal) seem to be protected against atherosclerosis and its clinical manifestations when compared with the age-matched males. Family history is considered the most significant independent risk factor.

Hyperlipidemia, hypertension, cigarette smoking and diabetes represent the acquired (modifiable) risk factors. Hyperlipidemia, in particular hypercholesterolemia alone can induce lesion development; high levels of low density lipoprotein (LDL) cholesterol (the "bad cholesterol") associate with higher risk while high levels of high density lipoprotein (HDL) cholesterol (the "good cholesterol") relates to lower risk. Approaches (dietary as well as pharmacological) to lower LDL and to raise HDL could restore a healthy cholesterol balance and reduce the complications of atherosclerosis. Hypertension increases the risk of atherosclerotic manifestations by approximately 60%; therefore therapy-controlled blood pressure is of great importance. Prolonged smoking (years) doubles the risk of death

due to atherosclerosis while smoking cessation significantly reduces the risk. Diabetes mellitus increases the risk by inducing hypercholesterolemia. Additional risk factors, which found their utility in daily clinical practice, are: inflammation (assessed by e.g. C-reactive protein); hyperhomocysteinemia; metabolic syndrome (central obesity, hypertension, and glucose tolerance); lipoprotein A (an altered form of LDL); and others.

INFLAMMATION IN ATHEROSCLEROSIS

Atherosclerosis is an inflammatory disease

As long as 200 years ago, in 1815 Joseph Hodgson was claiming that atherosclerosis is an inflammatory disease. Short after that, Rudolf Virchow (1821 – 1902) considered atherosclerosis an inflammatory condition and proposed that atheroma was a result of an inflammatory process within the vessel wall. He also noticed that cholesterol was present in the atheroma but he considered it secondary. A few decades after that, the theory of inflammation as a cause for atherosclerosis was forgotten. Only in 1976, the theory was revived due to the extensive studies and publications of Russel Ross (1929–1999) who introduced the "response-to-injury" concept. Since 1999, atherosclerosis is seen as "a chronic inflammatory and healing response of the arterial wall to endothelial injury; lesion progression occurs through the interaction of modified lipoproteins, monocyte-derived macrophages, and T lymphocytes with the normal cellular constituents of the arterial wall" (Russel Ross).

The initiation of atherosclerosis is secondary to endothelial damage presumably caused by conditions such as hypertension, hypercholesterolemia, diabetes mellitus, smoking and others. The endothelial-cell damage promotes the adhesion of circulating leukocytes and platelets, which in turn leads to increased vessel permeability for lipids, such as LDL. Lipids begin to accumulate within the vessel intima and this attracts leukocytes, mostly monocytes. The infiltrated monocytes uptake the modified lipids (e.g. oxLDL) and transform into lipid-loaded macrophages. Lipid-loaded macrophages accumulate in the vessel intima and give rise to the fatty streaks (the first signs of atherosclerotic transformation of the vessel wall). Fatty streaks develop into mature atherosclerotic plaques due to a continuous infiltration of different subsets of leukocytes (i.e. monocytes, neutrophils, and lymphocytes) and lipids.

A mature plaque consists of a lipid (or necrotic) core surrounded by a cap of smooth muscle cells and a thick collagen matrix. Such a plaque growing in the vessel intima may provoke gradual chronic stenosis or might cause acute stenosis of the artery when the integrity of the cap is disrupted. Disintegration of the cap (complete rupture or erosion) exposes the lipid core to the blood and that triggers the coagulation cascade and the formation of a thrombus; the thrombus can occlude the artery and induce downstream tissue ischemia (i.e. acute ischemic heart or cerebral attacks).

Inflammatory cells in atherosclerosis

Leukocyte subsets accumulate into an atherosclerotic plaque at various stages of lesion progression and play a role in the atherogenesis as well as in the further development. Substantial evidence supports the involvement and the role of monocytes/macrophages and T-lymphocytes in the pathobiology of a plaque. Recently, a role for neutrophils and mast cells was discovered.

Monocytes/macrophages are the dominant cell type in the plaque and they were extensively investigated and discussed in the field of atherosclerosis. Many pages can be written on the role of these cells in atherosclerosis, from which a short summary is given here. Monocytes are attracted into the lesion by a large palette of chemokines, such as CCL2 (also known as MCP-1) and CCL5 (also known as RANTES). Infiltrated monocytes give rise to macrophages which accumulate lipids and develope into foam cells (a hallmark of the disease). Recently, the considerable heterogeneity of the circulating monocyte has found its implementation also in atherosclerosis. Two distinct monocyte subsets were first identified in mice: an inflammatory subset (CX3CR1lowCCR2+Ly6Chi) that is recruited to inflamed tissues and a resident subset (CX3CR1hiCCR2-Ly6Clow) recruited to non-inflamed tissues. Based on CX3CR1 and CCR2 expression, the two subsets identified in mice might have the following human homologues: CD14+CD16- and CD14lowCD16+, respectively. However, the contribution of both these monocyte subsets to atherogenesis (in mice or humans) remains to be elucidated. Plaque macrophages engulf lipoproteins, a phenomenon which stimulates these cells to produce and secrete cytokines (e.g. TNF-alpha). This further increases leukocyte adhesion and production of chemokines, creating a stimulus for recruitment of additional leukocytes. Activated macrophages also secrete reactive oxygen species that induce LDL oxidation and produce growth factors that drive SMCs proliferation.

Lymphocytes. Although T cells are less numerous than macrophages in plaques, they are of great importance in mediating the immune response associated to atherosclerotic lesions. T cells might have a pro-atherogenic effect in mild hypercholesterolemic mice*6*, but data supporting a pro-atherogenic role of these cells in human atherogenesis are missing. Different T cell subsets coexist in atherosclerotic plaques: Th1 (secrete interferon (IFN) γ and may promote atherosclerosis), Th2 (secrete interleukin (IL) 4, 5 and 10 and may suppress inflammation and atherosclerosis), CD8+ (unknown, may induce death of SMCs), CD4+ (produce TGFβ and therefore may have anti-inflammatory, anti-atherogenic functions)[7].

Neutrophils are found in small numbers in plaques. Despite this, neutrophils seem to be pro-atherogenic[8, 9] and may also be of importance in plaque development and destabilization

of an advance lesion. Specific neutrophil products, such as alarmins, human neutrophil peptides, elastase, cathepsin G and proteinase-3 are present in human plaques[10]. All these products might induce destruction of the collagen matrix and might facilitate macrophage penetration in plaques. Further studies are required to elucidate the role of these cells in atherosclerosis.

Mast cells are rare cells in plaque, found in human lesions at sites of cap rupture, erosion or in areas with hemorrhage. Mast cells products are implicated in lesion progression and in lipid accumulation, since they impair cholesterol efflux and degrade HDL[11].

Innate immunity in atherosclerosis

Atherosclerosis is nowadays reckoned as a complex inflammatory process that involves both arms of immunity: innate and adaptive[12]. Immunity mediates the initiation, progression and eventual thrombotic complications of atherosclerosis[13]. The innate immune response rises quickly after a danger signal and combats foreign pathogens but also self-structures released under cell stress. It demands no prior "education" of the immune cells and can recognize hundreds of different structures, therefore being labeled as "fast and blunt". Cells of the innate immune system, namely monocytes/macrophages and neutrophils are present within atherosclerotic lesions and express various pattern-recognition receptors among which, toll-like receptors (TLR*s*). TLR*s* are occurring in atherosclerotic lesions[14] where they are triggered by both exogenous (bacterial and viral pathogens) and endogenous (tissue-damage associated) molecules[15].

Endogenous Toll-like receptor activators in Atherosclerosis

Several endogenous ligands for Tlrs are expressed during inflammation in atherosclerotic plaques where they might play a role in disease pathogenesis and progression. These are discussed in chapter 2 of the current thesis. *Myeloid-related protein (Mrp) 8 and 14*, also known as S100A8 and S100A9, were recently identified as activators of Tlr-4 and they will be further discussed in detail.

MYELOID RELATED PROTEIN 8 AND 14

Myeloid-related protein (Mrp) are two calcium binding proteins mainly expressed in cells of myeloid origin, particularly in monocytes and neutrophils[16]. Both proteins are secreted by activated monocytes and neutrophils and have pro-inflammatory effects[17, 18]. Upon cell activation, the two proteins form a complex, Mrp-8/14, that translocates to the cytoskeleton and plasma membrane where it is secreted[19]. Intracellular, 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[20]. The secreted Mrp-8/14 complex exerts antimicrobial activity[21], stimulates IL-8 production by airway epithelial cells[22], and transports arachidonic acid to endothelial cell (EC) targets affecting pathological responses in inflammation and atherosclerosis[23]. Mrp-8, -14 and -8/14 are expressed in both mouse[24] and human atherosclerotic plaques[25]. Mrp-14 and Mrp-8 are expressed by subsets of macrophages during inflammation in different tissues26, as well as in atherosclerotic lesions[27, 28]. Taking advantage of mice lacking functional Mrp-8/14 complexes, Vogl et al. have identified the endogenous Mrp-8 and Mrp-14 as activators of TLR-4[29]. Their study shows that although leukocytes of the Mrp-14 knockout mice have normal TLR-4 and CD14 (TLR-4 co-receptor for LPS) cell-surface expression, they have an impaired response toward LPS stimulation with less tumor necrosis factor (TNF)-α production and secretion when compared to wild type leukocyte's LPS response; addition of extracellular Mrp-8/14 compensates for the reduced response in TNF-α of Mrp-14 knockout leukocytes. Mrp-8 alone stimulates TNF-α RNA and protein secretion and has an additive effect on LPS-induced TNF-α secretion. Moreover, the same study has shown that leukocytes from mice with nonfunctional TLR-4 show no response to Mrp-8. Direct binding of Mrp-8 to Tlr-4/MD2 complex is confirmed by surface plasmon resonance studies[30]. These findings suggest that Mrp-8/14 acts as an endogenous TLR-4 activator and is involved in amplification of LPS effects on leukocytes. Recently was shown that Mrp-8/14 is critical for the biological response to vascular injury. Using mice lacking functional Mrp-8/14 complexes, the authors demonstrate reduced atherosclerotic lesion area and macrophage accumulation within the lesions[31] in Mrp-14 knockout mice, supporting a role for Mrp in plaque formation and development. Systemic Mrp-8/14 complexes relate to cardiac ischemic events: plasma Mrp-8/14 levels are elevated in patients with acute coronary syndromes (ACS) compared to patients with stable coronary artery disease (CAD) or with normal coronary arteries[32]. Moreover, levels of systemic Mrp-8/14 are elevated prior to markers of myocardial necrosis (myoglobin, creatine kinase - MB and troponin) and associate with increased risk of recurrent cardiovascular events compared to patients with lower Mrp-8/14 levels[33]. Plasma Mrp-8/14 concentrations are higher in patients with ST-segment-elevation myocardial infarction compared to patients with stable CAD[34] and the risk of the first cardiovascular event increases with each quartile of plasma Mrp-8/14. For detailed information regarding the Mrp-8 and Mrp-14 genes, the reader is referred to the OMIM database.

THE VULNERABLE PLAQUE AND THE VULNERABLE PATIENT

Initial atherosclerotic transformations of the vessel wall occur early in life and develop over decades, during which the affected individual often has no symptoms. Eventually,

the plaque built within the vessel wall becomes so big that it limits the blood flow through that artery. Arterial stenosis greater than 60% can cause flow limitations under conditions of increased demand; this commonly provokes chronic stable angina pectoris or intermittent claudication. However, the obstructive atherosclerotic plaques are outnumbered by multiple non-obstructive ones that are diffuse along the vasculature. These non-obstructive plaques are hidden in positively remodeled vessels and remain clinically silent until rupture with thrombus formation. These lesions, called the *vulnerable plaques*, are prone to rupture and therefore responsible for the vast majority of acute coronary syndromes and strokes. A vulnerable plaque has specific morphological characteristics: a macrophage infiltrated thin fibrous cap, a large lipid (or necrotic) core, reduced number of SMCs and reduced amount of collagen. Although the aforementioned morphological features are present in most of the lesions, the dynamic changes that a vulnerable, high-risk plaque undergoes over time are not defined in humans. The technological development in the field of plaque imaging is making remarkable progress in the detection of different structures of the vulnerable plaques. However, we are far from being capable to prevent major cardiovascular events by detecting the high-risk plaque before it ruptures. Ultimately, the detection of such plaques should be followed by systemic or local therapies to prevent plaque rupture.
The term cardiovascular "*vulnerable patient*" defines the patient at high risk of developing an adverse cardiovascular event (e.g. myocardial infarction) based on plaque, blood and myocardial vulnerability.

STUDY DESIGN

Using a proteomics screening approach, Myeloid related protein -8 and Mrp-14 were identified as highly expressed in atherosclerotic plaques of patients with recurrent cardiovascular events during three years follow-up after their carotid endarterectomy when compared with patients free of events (age and gender matched). The focus of this thesis is the association between Mrp-8 and -14 with the occurrence of adverse cardiovascular events (latest and recurrent) in patients undergoing carotid endarterectomy and with the features of the vulnerable plaque.
For this purpose, the *Athero-Express* (Differential ATHEROsclerotic plaque EXPRESSion of mRNA and protein in relation to cardiovascular events and patient characteristics) biobank was used. *Athero-Express* is a large longitudinal biobank comprising different types of vascular surgery: carotid and femoral endarterectomy specimens. The study was initiated in 2002 by two Dutch hospitals, UMC Utrecht and St. Antonius Hospital Nieuwegein. Two features make the *Athero-Express* an unique biobank: first, the volume of patients included (at this moment, aprox. 2500 patients) and new patients are being included each week and second, the link between plaque composition clinical parameters

and clinical and duplex follow-up. The patients sign a written inform consent and complete an extensive questionnaire. All clinical data (e.g. symptoms prior to operation, the degree of luminal narrowing) are collected from the medical records.
In this thesis, only carotid endarterectomy specimens from *Athero-Express* were used. Immediately after the surgical excision, the plaque is transferred to the laboratory where it is processed according to standardized protocols. The plaque is cut in 5 mm segments along the longitudinal axis of the vessel and the segment with the highest plaque burden is called the culprit segment and it is used for histology. The other segments are snap frozen in liquid nitrogen and are stored at -800C. The segments adjacent to the culprit segment are used for protein, RNA and DNA isolation. Next to plaques, the biobank also contains blood samples, withdrawn prior to surgical incision.
Every post-operative year, patients receive at home a questionnaire they have to fill in with information regarding any new complaints or hospitalization that happened in the past year. This information is verified by the researchers. The primary outcome of *Athero-Express* is defined as a composite of events including: any death of vascular origin (fatal stroke, fatal myocardial infarction, sudden death, and other vascular death), non-fatal stroke, non-fatal myocardial infarction, and any arterial vascular intervention that had not already been planned at the time of inclusion (i.e. carotid surgery or angioplasty, coronary artery bypass, percutaneous coronary artery intervention, peripheral vascular surgery or angioplasty).

OVERVIEW OF THE THESIS

This thesis describes the association of Mrp -8 and -14 with plaque and patient vulnerability. In **Chapter II**, the roles of TLRs and of their endogenous ligands in cardiovascular disease are reviewed. **Chapter III** describes the correlation between Mrp-14, but also Mrp-8 and Mrp-8/14 complex with the histological characteristics and the inflammatory status of the vulnerable plaque. In addition, the expression of Mrps in subset of non-foam plaque macrophages, predominately present in vulnerable plaques is recorded. *In vitro* studies show that human monocyte-derived macrophages expressing Mrps do not acquire a foamy phenotype when fed human oxLDL while those lacking Mrp do. In **Chapter IV**, Mrp-8/14 expressing macrophages are described as a functional macrophage subset which resembles the anti-inflammatory alternatively activated macrophages side of the M1-M2 spectrum in vivo and in vitro. **Chapter V** describes Mrps expression in plaque neutrophil granulocytes and establishes the association between high plaque neutrophil numbers and the features (histological and molecular) of the vulnerable lesions. **Chapter VI** the association between Mrp-8/14 plaque and plasma levels and the type and time after cerebrovascular ischemic event is described. **Chapter VII** identifies Mrp-8/14 complex levels (plasma and plaque)

as potential diagnostic tools to identify the patients at high risk of developing recurrent cardiovascular events after carotid endarterectomy (CEA). In **Chapter VIII**, all the data presented in this thesis are discussed and in **Chapter IX** a summary is provided. **Chapter X** provides additional information, such as a list of abbreviations, author affiliations, review committee, list of publications and author's curriculum vitae.

Reference List

1 Ben-Haim S, Gacinovic S, Israel O. Cardiovascular infection and inflammation. Semin Nucl Med 2009 March;39(2):103-14.

2 Libby P, Ridker PM, Hansson GK. Inflammation in atherosclerosis: from pathophysiology to practice. J Am Coll Cardiol 2009 December 1;54(23):2129-38.

3 Zhao Q. Inflammation, autoimmunity, and atherosclerosis. Discov Med 2009 June;8(40):7-12.

4 Nambi V, Chambless L, Folsom AR, He M, Hu Y, Mosley T, Volcik K, Boerwinkle E, Ballantyne CM. Carotid intima-media thickness and presence or absence of plaque improves prediction of coronary heart disease risk: the ARIC (Atherosclerosis Risk In Communities) study. J Am Coll Cardiol 2010 April 13;55(15):1600-7.

5 Ridker PM, Stampfer MJ, Rifai N. Novel risk factors for systemic atherosclerosis: a comparison of C-reactive protein, fibrinogen, homocysteine, lipoprotein(a), and standard cholesterol screening as predictors of peripheral arterial disease. JAMA 2001 May 16;285(19):2481-5.

6 Zhou X, Nicoletti A, Elhage R, Hansson GK. Transfer of CD4(+) T cells aggravates atherosclerosis in immunodeficient apolipoprotein E knockout mice. Circulation 2000 December 12;102(24):2919-22.

7 Weber C, Zernecke A, Libby P. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. Nat Rev Immunol 2008 October;8(10):802-15.

8 Zernecke A, Bot I, Djalali-Talab Y, Shagdarsuren E, Bidzhekov K, Meiler S, Krohn R, Schober A, Sperandio M, Soehnlein O, Bornemann J, Tacke F, Biessen EA, Weber C. Protective Role of CXC Receptor 4/CXC Ligand 12 Unveils the Importance of Neutrophils in Atherosclerosis. Circ Res 2008 February 1;102(2):209-17.

9 van LM, Gijbels MJ, Duijvestijn A, Smook M, van de Gaar MJ, Heeringa P, de Winther MP, Tervaert JW. Accumulation of myeloperoxidase-positive neutrophils in atherosclerotic lesions in LDLR-/- mice. Arterioscler Thromb Vasc Biol 2008 January;28(1):84-9.

10 Soehnlein O, Weber C. Myeloid cells in atherosclerosis: initiators and decision shapers. Semin Immunopathol 2009 June;31(1):35-47.

11 Weber C, Zernecke A, Libby P. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. Nat Rev Immunol 2008 October;8(10):802-15.

12 Packard RR, Lichtman AH, Libby P. Innate and adaptive immunity in atherosclerosis. Semin Immunopathol 2009 June;31(1):5-22.

13 Packard RR, Lichtman AH, Libby P. Innate and adaptive immunity in atherosclerosis. Semin Immunopathol 2009 June;31(1):5-22.

14 Edfeldt K, Swedenborg J, Hansson GK, Yan Zq. Expression of Toll-Like Receptors in Human Atherosclerotic Lesions: A Possible Pathway for Plaque Activation. Circulation 2002 March 12;105(10):1158-61.

15 Oude Nijhuis MM, van Keulen JK, Pasterkamp G, Quax PH, de Kleijn DP. Activation of the innate immune system in atherosclerotic disease. Curr Pharm Des 2007;13(10):983-94.

16 Hessian PA, Edgeworth J, Hogg N. MRP-8 and MRP-14, two abundant Ca(2+)-binding proteins of neutrophils and monocytes. J Leukoc Biol 1993 February 1;53(2):197-204.

17 Roth J, Vogl T, Sorg C, Sunderk÷tter C. Phagocyte-specific S100 proteins: a novel group of proinflammatory molecules. Trends in Immunology 2003 April;24(4):155-8.

18 Frosch M, Strey A, Vogl T, Wulffraat NM, Kuis W, Sunderkotter C, Harms E, Sorg C, Roth J. Myeloid-related proteins 8 and 14 are specifically secreted during interaction of phagocytes and activated endothelium and are useful markers for monitoring disease activity in pauciarticular-onset juvenile rheumatoid arthritis. Arthritis Rheum 2000 March;43(3):628-37.

19 Teigelkamp S, Bhardwaj RS, Roth J, Meinardus-Hager G, Karas M, Sorg C. Calcium-dependent complex assembly of the myeloic differentiation proteins MRP-8 and MRP-14. J Biol Chem 1991 July 15;266(20):13462-7.

20 Vogl T, Ludwig S, Goebeler M, Strey A, Thorey IS, Reichelt R, Foell D, Gerke V, Manitz MP, Nacken W, Werner S, Sorg C, Roth J. MRP8 and MRP14 control microtubule reorganization during transendothelial migration of phagocytes. Blood 2004 December 15;104(13):4260-8.

21 Nacken W, Roth J, Sorg C, Kerkhoff C. S100A9/S100A8: Myeloid representatives of the S100 protein family as prominent players in innate immunity. Microsc Res Tech 2003 April 15;60(6):569-80.

22 Ahmad A, Bayley DL, He S, Stockley RA. Myeloid Related Protein-8/14 Stimulates Interleukin-8 Production in Airway Epithelial Cells. Am J Respir Cell Mol Biol 2003 October 1;29(4):523-30.
23 Nacken W, Roth J, Sorg C, Kerkhoff C. S100A9/S100A8: Myeloid representatives of the S100 protein family as prominent players in innate immunity. Microsc Res Tech 2003 April 15;60(6):569-80.
24 Croce K, Gao H, Wang Y, Mooroka T, Sakuma M, Shi C, Sukhova GK, Packard RR, Hogg N, Libby P, Simon DI. Myeloid-related protein-8/14 is critical for the biological response to vascular injury. Circulation 2009 August 4;120(5):427-36.
25 Ionita MG, Vink A, Dijke IE, Laman JD, Peeters W, van der Kraak PH, Moll FL, de Vries JP, Pasterkamp G, de Kleijn DP. High levels of myeloid-related protein 14 in human atherosclerotic plaques correlate with the characteristics of rupture-prone lesions. Arterioscler Thromb Vasc Biol 2009 August;29(8):1220-7.
26 Edgeworth J, Gorman M, Bennett R, Freemont P, Hogg N. Identification of p8,14 as a highly abundant heterodimeric calcium binding protein complex of myeloid cells. J Biol Chem 1991 April 25;266(12):7706-13.
27 McCormick MM, Rahimi F, Bobryshev YV, Gaus K, Zreiqat H, Cai H, Lord RSA, Geczy CL. S100A8 and S100A9 in Human Arterial Wall: IMPLICATIONS FOR ATHEROGENESIS. J Biol Chem 2005 December 16;280(50):41521-9.
28 Ionita MG, Vink A, Dijke IE, Laman JD, Peeters W, van der Kraak PH, Moll FL, de Vries JP, Pasterkamp G, de Kleijn DP. High levels of myeloid-related protein 14 in human atherosclerotic plaques correlate with the characteristics of rupture-prone lesions. Arterioscler Thromb Vasc Biol 2009 August;29(8):1220-7.
29 Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MAD, Nacken W, Foell D, van der Poll T, Sorg C, Roth J. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat Med 2007 September;13(9):1042-9.
30 Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MAD, Nacken W, Foell D, van der Poll T, Sorg C, Roth J. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat Med 2007 September;13(9):1042-9.
31 Croce K, Gao H, Wang Y, Mooroka T, Sakuma M, Shi C, Sukhova GK, Packard RR, Hogg N, Libby P, Simon DI. Myeloid-related protein-8/14 is critical for the biological response to vascular injury. Circulation 2009 August 4;120(5):427-36.
32 Altwegg LA, Neidhart M, Hersberger M, Muller S, Eberli FR, Corti R, Roffi M, Sutsch G, Gay S, von Eckardstein A, Wischnewsky MB, Luscher TF, Maier W. Myeloid-related protein 8/14 complex is released by monocytes and granulocytes at the site of coronary occlusion: a novel, early, and sensitive marker of acute coronary syndromes. Eur Heart J 2007 April 2;28(8):941-8.
33 Morrow DA, Wang Y, Croce K, Sakuma M, Sabatine MS, Gao H, Pradhan AD, Healy AM, Buros J, McCabe CH, Libby P, Cannon CP, Braunwald E, Simon DI. Myeloid-related protein 8/14 and the risk of cardiovascular death or myocardial infarction after an acute coronary syndrome in the Pravastatin or Atorvastatin Evaluation and Infection Theraphy: Thrombolysis in Myocardial Infarction (PROVE IT-TIMI 22) trial. American Heart Journal 2008 January;155(1):49-55.
34 Healy AM, Pickard MD, Pradhan AD, Wang Y, Chen Z, Croce K, Sakuma M, Shi C, Zago AC, Garasic J, Damokosh AI, Dowie TL, Poisson L, Lillie J, Libby P, Ridker PM, Simon DI. Platelet Expression Profiling and Clinical Validation of Myeloid-Related Protein-14 as a Novel Determinant of Cardiovascular Events. Circulation 2006 May 16;113(19):2278-84.

CHAPTER II

Endogenous inflammatory molecules engage toll-like receptors in cardiovascular disease

Journal of Innate Imunity. 2010 April

Mihaela G. Ionita, Fatih Arslan, Dominique De Kleijn And Gerard Pasterkamp

Innate immunity is important in the pathogenesis and progression of cardiovascular disease. Innate immune cells express various pattern-recognition receptors among which, toll-like receptors (Tlrs). Tlrs are occurring in atherosclerotic lesions where they are triggered by both exogenous (bacterial and viral pathogens) and endogenous (tissue-damage associated) molecules. Several endogenous Tlr activators are described in relation to atherosclerotic disease or ischemia induced-cardiac injury. Experimental animal models have proved the role of Tlr endogenous activators in disease initiation and further development. Researchers aim to unravel the mechanisms downstream of this interaction and to discover new potential Tlr activators, released during pathological conditions such as atherosclerosis and related ischemic manifestations. This review provides and overview of the currently known endogenous molecules which trigger innate immunity via toll-like receptors in cardiovascular disease.

Introduction

For decades, morbidity and mortality of the aging population due to cardiovascular disease (CVD) is a burden for the health system. Along with a boost of hypertension, type II diabetes and obesity in the young generation, the incidence of CVD is rapidly increasing, with heart disease being the major cause of death in the modern world. The pathogenesis of cardiovascular diseases is heterogeneous; infection and inflammation[33, 34] and even autoimmunity[35] are implicated. For most clinical presentations of CVD (e.g. stroke, acute coronary syndromes), atherosclerosis is the underlining pathological cause. Long considered a passive lipid deposition on the vessel wall, atherosclerosis is nowadays reckoned as a complex inflammatory process that involves both arms of immunity: innate and adaptive[36]. Immunity mediates the initiation, progression and eventual thrombotic complications of atherosclerosis[37]. The innate immune response rises quickly after a danger signal and combats foreign pathogens but also self-structures released under cell stress. It demands no prior "education" of the immune cells and can recognize hundreds of different structures, therefore being labeled as "fast and blunt". Fundamental cells of the innate immune system, namely monocytes and macrophages, are present within atherosclerotic lesions and express various pattern-recognition receptors among which, toll-like receptors (Tlrs). Tlrs are occurring in atherosclerotic lesions[38] where they are triggered by both exogenous (bacterial and viral pathogens) and endogenous (tissue-damage associated) molecules[13]. Several endogenous ligands for Tlrs are express during inflammation in atherosclerotic plaques or ischemic heart where they might play a role in disease pathogenesis and progression. With the use of experimental animal studies, researchers have gained insight into the mechanisms behind endogenous ligand induced Tlr-activation. This review provides an overview of the endogenous molecules, released under cell stress during inflammation, which can trigger innate immunity via toll-like receptors in cardiovascular diseases.

The role of Toll-like receptors in cardiovascular disease

Toll-like receptors, especially Tlr-2 and Tlr-4, are extensively studied in cardiovascular disease and implicit in atherosclerosis. Toll-like receptors are important components of innate immunity that bridge inflammation, infection and atherosclerosis[39]. Atherosclerosis is a chronic inflammatory process affecting the vasculature at predetermined sites. Lipid accumulation within the vessel intima leads, in time, to an accelerated inflammatory response that encompasses both innate and adaptive immune systems. Previous epidemiological studies have suggested that exogenous bacterial ligands can induce an inflammatory response in the arterial wall and initiate atherosclerosis[40] and therefore a link between atherosclerosis and Tlrs have been established. Experimental data have shown that lipopolysaccharide (LPS) and bacterial peptidoglycan (PGN), both potent Tlr

activators, can accelerate plaque formation in atherosclerotic mice13. These results point to a potential role of Tlr-2 and Tlr-4 in the pathogenesis of atherosclerotic disease. Indeed, Tlr-2 knockout mice crossed with low density lipoprotein receptor (Ldlr) show a reduction in the degree of atherosclerosis[41]. Tlr-4 knockout mice crossed with the apolipoprotein E (apoE) knockout mice have reduced atherosclerosis compared to apoE knockout controls[42]. Tlr-4 deficient mice also reveal inappropriate arterial remodeling, namely reduced outward remodeling[43]. ApoE-deficient mice lacking Tlr-4 but also a signal protein downstream the Tlr pathway, namely myeloid differentiation factor 8 (MyD88), have reduced aortic atherosclerosis that is associated with reductions in circulating levels of pro-inflammatory cytokine interleukin -12 or monocyte chemoattractant protein 1, plaque lipid content, numbers of macrophage, and cyclooxygenase-2 immunoreactivity in their plaques[44]. Another group has reported reduced atherosclerosis through a decrease in chemokine levels and in macrophages infiltrating into plaques, in apoE/MyD88 null mice[45]. The relation between Tlr-4 and atherosclerosis is strengthening by genetic analyses of polymorphisms in genes encoding Tlr-4. However, large clinical studies bring contradictory findings: Tlr-4 polymorphisms is related to a reduction[46] or no effect[47] on atherosclerosis and in intima-media thickness; to an increased risk of ischemic stroke[48] and MI[49] or an decreased risk of acute coronary syndromes[50]. Others have reported no association between Tlr-4 polymorphisms and the risk of acute ischemic events[51, 52]. Frantz et al. are among the first to study the role of Tlrs in the inflammatory response due to cardiac ischemia and they have trigged extensive research in the field of Tlrs and cardiac ischemia-related pathological conditions. Their observation that Tlr-4 is upregulated in heart failure[53], has been confirmed in Tlr-4 knockout mice, which are relatively protected against adverse ventricular remodeling after infarction[54]. The association of Tlr-4 signaling with myocardial/ischemia/reperfusion injury is documented in various studies [55, 56;57;58] and, moreover, the pharmacological inhibition of Tlr-4 signaling reduces infarct size[59]. In humans, circulating Tlr-4 positive cells and Tlr-4 signaling are increased in patients with unstable angina and myocardial infarction[60].

Similar experimental proofs are present for Tlr-2, as a critical modulator of post-infarct injury and repair. Tlr-2 knockout mice have higher survival rates and less expansive remodeling after infarction[61]. In a murine myocardial ischemia/reperfusion injury model, we and others have showed that lethal reperfusion injury was mediated by circulating Tlr-2. Administration of a monoclonal anti-Tlr2 antibody at a clinically applicable time point reduces infarct size with approximately 50% and improves cardiac function[62;63]. These data, together with the fact that the post-infarct period and Tlr-2 and -4 signaling share similar immunological characteristics[64], suggest that Tlr-2 and -4 are promising therapeutic targets in cardiac ischemia. It is expected that in the near future these promising compounds will enter clinical trials for their efficacy and feasibility.

Potential Endogenous activators of Toll-like receptors: expression and role in cardiovascular disease

Toll-like receptors were initially described as recognizing proteins for bacterial and viral antigens. Bacterial lipopolysaccharide (LPS)[65], peptidoglycans (PGN)[66], different bacterial lipoproteins (triacyl[67], diacyl[68]) and single[69] or double-stranded viral RNA[70], signal via Tlrs. *Chlamydia pneumoniae*, an infectious agent which happen to be a potent Tlr activator, is found in different atherosclerotic lesions[71]. In animal models *Chlamydia pneumoniae* can stimulate atherogenesis[72] and therefore is considered a therapeutic target for atherosclerosis[73]. Peptidoglycan, a Tlr-2 ligand, is present in the bacterial flora at all mucosal sites from where it can travel along the bloodstream via monocytes. Peptidoglycan is an exogenous microbial derived Tlr ligand and activator that is widely present in the gut of each individual. PG-loaded monocytes adhere to the activated endothelium at sites of inflammation and are found in atherosclerotic plaques with features of unstable/rupture-prone lesions[74]. Along with the classical bacterial and viral antigen recognition, Tlrs are able to detect and respond to endogenous molecules released during tissue injury[75, 76]. Below we summarize (see also figure) and discuss the role of potential Tlr endogenous activators in the progression of cardiovascular diseases.

Heat-shock proteins (Hsp) are present in most cells, serving as molecular chaperones, and they play a role in cell protection from damage in response to stress stimuli[77]. Tlr-2 and Tlr-4 are able to recognize and respond to the soluble forms of Hsp60[78], Hsp 70[79, 80] and the endoplasmic reticulum Hsp90[81] (gp96). Several groups have reported that in human mononuclear cells, human soluble Hsp60 binds to Tlr-4/Cd14, leading to p38 mitogen-activated protein kinase activation[82], whereas in smooth muscle and epithelial cells chlamydial and human soluble Hsp60 stimulates extracellular signal regulated kinase 42/44 activation[83]. In macrophages and endothelial cells, the binding of soluble Hsp60 to the Tlr-4/Cd14 complex leads to the activation of MyD88–NF- B pathways; the same Tlr-4 mediated signal pathway is described for soluble Hsp70[84]. Hsps have been found to be highly expressed in cardiovascular tissues and to induce inflammatory responses. These proteins are expressed during the development of atherosclerosis and elicit an immune response as autoantigens 85. Hsp60 is observed in the cell mitochondria (endothelial, smooth muscle cells and mononuclear cells of the carotid and aortic plaque specimens[86]) and is transported to the cell membrane in response to stress stimuli. Auto antibodies against Hsp60 (demonstrated in patients with heart attacks and strokes) bind to cell-membrane exposed Hsp60 and therefore induce damage of the cell[87]. Hsps are secreted under stress stimuli and the soluble forms have proinflammatory activities[88]; Chen et al have demonstrated that autologous Hsp60 serves as a danger signal to the innate immune system, which results in proinflammatory responses, including the production of tumor necrosis factor (TNF)-α, interleukin (IL)-12, and IL-15[89]. The soluble Hsp60 is found in

high concentrations in the serum of subjects with prevalent/incident carotid atherosclerosis[90]. The intensity of Hsp60 expression in human plaque specimens positively correlates with the atherosclerotic severity[91, 92]. Furthermore, the expressions of Hsp60 and the stress-inducible form of Hsp70 correlate with the development of atherosclerotic lesions in the aortic tree of apoE-deficient mice[93]. Both Hsps are present on the aortic root and endothelium at lesion-prone sites of apoE knockout mice, before infiltration of mononuclear cells. The same study has shown a temporary expression of Hsp60 and Hsp70 on all major cell types in lesion-predisposed areas during atherogenesis[53]. In the failing heart, Hsp60 associates with increased apoptosis of the myocardium and induces cardiomyocyte death via Tlr-4 signaling[94;95].Taken together, these results demonstrate that Hsp60 and Hsp70 are involved in both initiation as well as progression of atherosclerosis.

High-mobility group box-1 (HMGB-1) is described as a non-histone, chromatin-associated nuclear protein[96]. HMGB-1 appears to have two distinct functions in cellular systems. First, it is an intracellular regulator of transcription, and, second, HMGB-1 can occupy an extracellular role in which it promotes tumor metastasis and inflammation[97, 98]. Extracellular HMGB-1 participates in inflammatory processes and stimulates the secretion of TNF-α and different proinflammatory cytokines[99-101]. HMGB-1 binds to Tlr-2 and Tlr-4[102] and induces activation of endothelial cells and macrophages/monocytes with subsequent release of proinflammatory cytokines, chemokines and adhesion molecules. In human atherosclerotic plaques, HMGB-1 is highest in fibrofatty lesions where it is expressed and secreted by macrophages[103]. Along with an increase in HMGB-1 levels, also a concomitant decrease in HMGB-1 inhibitory proteins such as the anticoagulant protein thrombomodulin, which binds and sequesters HMGB-1[104] is documented. In addition, the receptors for HMGB-1, namely Tlr-2, Tlr-4 and receptor of advanced end glycation products (RAGE) are upregulated in atherosclerotic lesions[105, 106]. Thus, HMGB-1 might be an important molecule linking innate immunity and inflammation in the pathogenesis of atherosclerosis. HMBG-1 may also elicit an inflammatory response following myocardial ischemia. Disruption of an atherosclerotic plaque results in thrombus formation at the site of rupture that causes a sudden disruption of the blood flow with subsequent ischemic injury and injury-related inflammation. HMGB-1 plays a pathogenic role in ischemic injury, because its circulating levels are increased shortly after ischemia/reperfusion, and its inhibition is protective against liver ischemia/reperfusion injury[107]. Moreover, the expression levels of Tlr-2, Tlr-4 and RAGE are increased after cardiac injury[108], which might facilitate HMGB-1 to mediate an inflammatory response. In a murine myocardial ischemia/reperfusion injury model, HMGB1 administration increases infarct size, while its inhibition significantly reduces infarct size via RAGE[109]. Several other HMGB-1 inhibiting agents (e.g. ethyl pyruvate, green tea and adrenomedulin) preserve cardiac function after myocardial ischemia[110-112], consistent with a pathogenic role of HMGB-1 in cardiovascular diseases.

Extra domain A of fibronectin (EDA), generated by regulated fibronectin RNA splicing[113], is absent from healthy plasma and extracellular matrix and yielded only during pathological tissue alterations. EDA is expressed after tissue injury due to inflammation and stimulates production of various cytokines[114] via Tlr-4 through NF-kB pathway[115]. In atherosclerotic plaques, fibronectin abundantly contains EDA whereas in the adjacent unaffected vessel wall, fibronectin lacks EDA. In a model of atherogenetic predisposed mouse (apoE knockout), systemic as well as local (plaque) EDA levels are elevated while mice lacking both apoE and EDA have decreased atherosclerosis (as much as 67%)[116]. The observed effect is justified by EDA involvement in plasma lipoprotein metabolism and in macrophage foam cell formation. These observations imply a pro-atherogenic role for EDA in the pathogenesis of atherosclerosis. The findings in mice are strengthened by those in humans: our group has previously reported, in human carotid atherosclerotic plaques, high levels of fibronectin containing EDA in fibrous (more stable) and low levels in highly-inflamed (unstable) lesions[117]. Additionally, patients with carotid atherosclerotic stenosis (> 75%) and no clinical associated complains, reveal high EDA plaque levels contrary to patients with clinical manifestations of carotid stenosis which have low EDA plaque levels. Although the exact role of EDA in the pathogenesis of atherosclerosis remains to be elucidated, it is no doubt that EDA is a molecule linking innate immunity and inflammation in atherosclerosis.

Myeloid-related protein (Mrp) 8 and 14, also known as S100A8 and S100A9, are two calcium binding proteins mainly expressed in cells of myeloid origin, particularly in monocytes and neutrophils[118]. Both proteins are secreted by activated monocytes and neutrophils and have pro-inflammatory effects[119, 120]. Upon cell activation, the two proteins form a complex, Mrp-8/14, that translocates to the cytoskeleton and plasma membrane where it is secreted[121]. Intracellular, 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[122]. The secreted Mrp-8/14 complex exerts antimicrobial activity[123], stimulates IL-8 production by airway epithelial cells[124], and transports arachidonic acid to endothelial cell (EC) targets affecting pathological responses in inflammation and atherosclerosis[125]. Mrp-8, -14 and -8/14 are expressed in both mouse[126] and human atherosclerotic plaques[127]. Mrp-14 and Mrp-8 are expressed by subsets of macrophages during inflammation in different tissues[128], as well as in atherosclerotic lesions [129, 130].Taking advantage of mice lacking functional Mrp-8/14 complexes, Vogl et al. have identified the endogenous Mrp-8 and Mrp-14 as activators of Tlr-4[131]. Their study shows that even thou leukocytes of the Mrp-14 knockout mice have a normal Tlr-4 and Cd14 (Tlr-4 co-receptor for LPS) cell-surface expression, they have an impaired response toward LPS stimulation with less tumor necrosis factor (TNF)-α production and secretion when

compared to wild type leukocyte's LPS response; addition of extracellular Mrp-8/14 compensates for the reduced response in TNF-α of Mrp-14 knockout leukocytes. Further analysis has shown that Mrp-8 alone stimulates TNF-α RNA and protein secretion and has an additive effect on LPS-induced TNF-α secretion. Moreover, the same study has shown that leukocytes from mice with nonfunctional Tlr-4 show no response to Mrp-8. Direct binding of Mrp-8 to Tlr-4/MD2 complex is confirmed by surface Plasmon resonance studies[132]. These findings prove that Mrp-8/14 acts as an endogenous Tlr-4 activator and is involved in amplification of LPS effects on leukocytes. Libby et al. have recently shown that Mrp-8/14 is critical for the biological response to vascular injury. Using mice lacking functional Mrp-8/14 complexes, the authors demonstrate reduced atherosclerotic lesion area and macrophage accumulation within the lesions[133] in Mrp-14 knockout mice, supporting a role for Mrp in plaque formation and development. In human carotid endarterectomy (CEA) specimens, we have previously described an association between high Mrp-8, -14 and -8/14 levels and the features of rupture-prone lesions[134], suggesting a role for Mrp - proteins in plaque destabilization and disruption. Additionally, we identified Mrp-proteins expression in a subset of non-foam plaque macrophages and *in vitro*, human monocyte-derived macrophages expressing Mrp-proteins do not acquire a foamy-phenotype when fed human oxidized LDL. This indicates Mrp-proteins as potential regulators of macrophage lipid-induced phagocyte resources, although additive proof is required. Systemic Mrp-8/14 complexes relate to cardiac ischemic events: plasma Mrp-8/14 levels are elevated in patients with acute coronary syndromes (ACS) compared to patients with stable coronary artery disease (CAD) or with normal coronary arteries[30]. Moreover, levels of systemic Mrp-8/14 are elevated prior to markers of myocardial necrosis (myoglobin, creatine kinase - MB and troponin) and associate with increased risk of recurrent cardiovascular events compared to patients with lower Mrp-8/14 levels[31]. Plasma Mrp-8/14 concentrations are higher in patients with ST-segment-elevation MI compared to patients with stable CAD[32] and the risk of the first cardiovascular event increases with each quartile of plasma Mrp-8/14. Acknowledging all these findings, we conclude that Mrp-proteins are endogenous activators of Tlr-4 occurring during atherosclerosis or acute coronary syndromes, therefore exacerbating the inflammatory response associated to those diseases.

Minimally modified low density lipoprotein (mmLDL) represents a primitive form of oxidized LDL that is present in atherosclerotic plaques where it binds CD14 and Tlr-4[135] on macrophages and induces Tlr-4 dependent cytokine expression[136]. In addition to the Tlr-4 cytokine response, mmLDL induces Tlr-4/MD-2 dependent actin polymerization and spreading of macrophages[137]. Recently, Choi et al. have shown that mmLDL, via Tlr-4, enhances macrophage macropinocytosis, which results in lipid accumulation in macrophages and foam cell formation. The authors further demonstrate the in vivo

relevance of these observations by injecting mmLDL into mice and proving its uptake by the circulating monocytes, phenomenon modulated via Tlr-4. The active component in mmLDL, responsible for most of the observed effects is lypoxigenase (15LO) - cholesteryl esters (CE). Atherosclerotic prone mice (ApoE or LDLR knockout) lacking 12/15 LO (mouse homologue of human 15LO) have reduced atherosclerosis[138]; furthermore enhanced expression and activity of 12/15LO has been reported in macrophages and endothelial cells in atherosclerotic plaques in mice[139].

Free fatty acids (FFAs) promote insulin resistance and atherosclerosis in mice[102,103] and are proposed as endogenous activators of Tlr-2 and Tlr-4 in these diseases. A saturated fatty acid, namely lauric acid, can activate Tlr-2 in 293T cells and Tlr-4 in RAW264.7 cells[104]. Similarly, an oleate/palmitate mixture of fatty acids induces Tlr-4 signaling in 293T cells and causes inflammation in wild-type but not in Tlr-4-deficient adipocytes[105]. Palmitate causes Tlr-4-dependent IkBα degradation in elicited peritoneal macrophages [102]. However, recent publications suggest that fatty acids do not directly stimulate Tlr-4 and Tlr-2, and that the observed effects might be due to sample contamination with Tlr-4-stimulating bacterial LPSs (endotoxin), Tlr-2-stimulating lipopeptide products or with an unidentified Tlr endogenous ligand[106, 107]. Therefore, the mechanisms linking high-fat diets with Tlr-associated pathologies such as atherosclerosis and insulin resistance remain to be discovered.

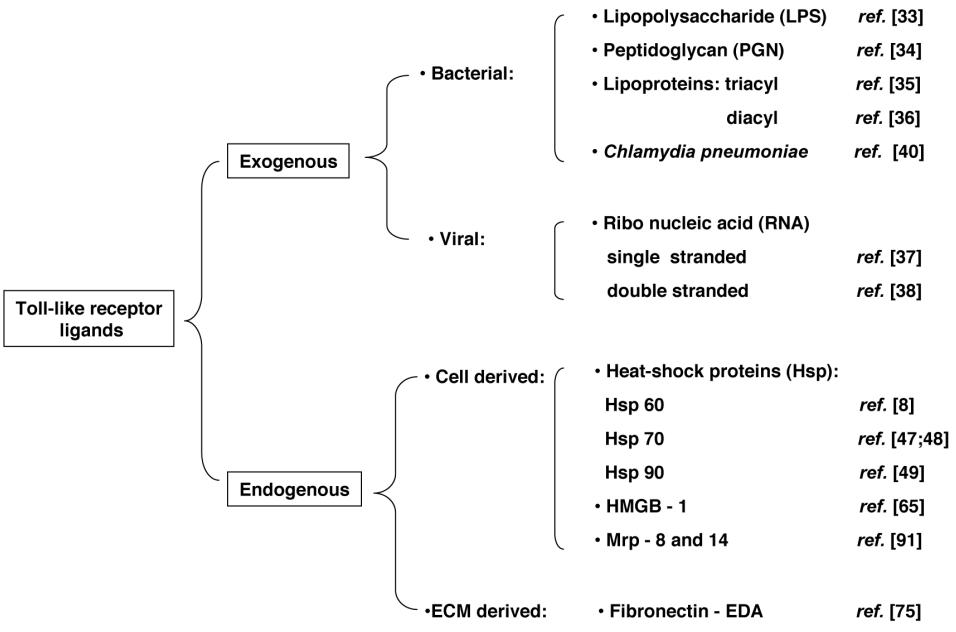


Figure 1. Schematic representation of potential toll-like receptor ligands (endogenous and exogenous).

Summary

Cardiovascular disease is an inflammation driven malignancy which involves innate immune responses partially due to toll-like receptor signaling. Tlrs are emerging as promising therapeutic targets for atherosclerosis and cardiac ischemia due to their involvement in disease pathogenesis. Tlrs are triggered by classical bacterial and viral particles (e.g. *Chlamydia pneumoniae*, peptidoglycan) but recent data suggest that endogenous molecules (e.g. heat-shock proteins, high-mobility group box-1, extra domain A of fibronectin, myeloid-related proteins) released from cells undergoing damage, can also bind and stimulate these receptors. Exogenous and endogenous Tlr activators have proved to be important contributors to the progression of atherosclerosis and related thrombotic complications. Tlr related research has gained much attention since Tlr discovery in humans and continues to be a dynamic field. In the near future, studies will reveal new potential endogenous ligands and provide more mechanistic insight into their biological functions. Especially endogenous activators are appealing for both researchers and clinicians, since they are yielded only during pathological conditions and therefore might represent useful diagnostic tools and therapeutic targets. In addition, pharmacological interventions antagonizing these endogenous molecules will likely cause fewer side effects.

Reference List

1 Ben-Haim S, Gacinovic S, Israel O: Cardiovascular infection and inflammation. Semin Nucl Med 2009;39:103-114.
2 Libby P, Ridker PM, Hansson GK: Inflammation in atherosclerosis: from pathophysiology to practice. J Am Coll Cardiol 12-1-2009;54:2129-2138.
3 Zhao Q: Inflammation, autoimmunity, and atherosclerosis. Discov Med 2009;8:7-12.
4 Packard RR, Lichtman AH, Libby P: Innate and adaptive immunity in atherosclerosis. Semin Immunopathol 2009;31:5-22.
5 Edfeldt K, Swedenborg J, Hansson GK, Yan Zq: Expression of Toll-Like Receptors in Human Atherosclerotic Lesions: A Possible Pathway for Plaque Activation. Circulation 3-12-2002;105:1158-1161.
6 Oude Nijhuis MM, van Keulen JK, Pasterkamp G, Quax PH, de Kleijn DP: Activation of the innate immune system in atherosclerotic disease. Curr Pharm Des 2007;13:983-994.
7 Balogh S, Kiss I, Csaszar A: Toll-like receptors: link between "danger" ligands and plaque instability. Curr Drug Targets 2009;10:513-518.
8 Vabulas RM, hmad-Nejad P, da CC, Miethke T, Kirschning CJ, Hacker H, Wagner H: Endocytosed HSP60s use toll-like receptor 2 (TLR2) and TLR4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells. J Biol Chem 8-17-2001;276:31332-31339.
9 Mullick AE, Tobias PS, Curtiss LK: Modulation of atherosclerosis in mice by Toll-like receptor 2. J Clin Invest 2005;115:3149-3156.
10 Michelsen KS, Wong MH, Shah PK, Zhang W, Yano J, Doherty TM, Akira S, Rajavashisth TB, Arditi M: Lack of Toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in apolipoprotein E. Proc Natl Acad Sci U S A 7-20-2004;101:10679-10684.
11 Hollestelle SC, De Vries MR, van Keulen JK, Schoneveld AH, Vink A, Strijder CF, Van Middelaar BJ, Pasterkamp G, Quax PH, de Kleijn DP: Toll-like receptor 4 is involved in outward arterial remodeling. Circulation 1-27-2004;109:393-398.
12 Michelsen KS, Wong MH, Shah PK, Zhang W, Yano J, Doherty TM, Akira S, Rajavashisth TB, Arditi M: Lack of Toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in apolipoprotein E. Proc Natl Acad Sci U S A 7-20-2004;101:10679-10684.
13 Bjorkbacka H, Kunjathoor VV, Moore KJ, Koehn S, Ordija CM, Lee MA, Means T, Halmen K, Luster AD, Golenbock DT, Freeman MW: Reduced atherosclerosis in MyD88-null mice links elevated serum cholesterol levels to activation of innate immunity signaling pathways. Nat Med 2004;10:416-421.
14 Kiechl S, Lorenz E, Reindl M, Wiedermann CJ, Oberhollenzer F, Bonora E, Willeit J, Schwartz DA: Toll-like receptor 4 polymorphisms and atherogenesis. N Engl J Med 7-18-2002;347:185-192.
15 Netea MG, Hijmans A, van WS, Smilde TJ, Trip MD, Kullberg BJ, de BT, Van der Meer JW, Kastelein JJ, Stalenhoef AF: Toll-like receptor-4 Asp299Gly polymorphism does not influence progression of atherosclerosis in patients with familial hypercholesterolaemia. Eur J Clin Invest 2004;34:94-99.
16 Lin YC, Chang YM, Yu JM, Yen JH, Chang JG, Hu CJ: Toll-like receptor 4 gene C119A but not Asp299Gly polymorphism is associated with ischemic stroke among ethnic Chinese in Taiwan. Atherosclerosis 2005;180:305-309.
17 Edfeldt K, Bennet AM, Eriksson P, Frostegard J, Wiman B, Hamsten A, Hansson GK, de FU, Yan ZQ: Association of hypo-responsive toll-like receptor 4 variants with risk of myocardial infarction. Eur Heart J 2004;25:1447-1453.
18 Ameziane N, Beillat T, Verpillat P, Chollet-Martin S, Aumont MC, Seknadji P, Lamotte M, Lebret D, Ollivier V, de PD: Association of the Toll-like receptor 4 gene Asp299Gly polymorphism with acute coronary events. Arterioscler Thromb Vasc Biol 2003;23:e61-e64.
19 Zee RY, Hegener HH, Gould J, Ridker PM: Toll-like receptor 4 Asp299Gly gene polymorphism and risk of atherothrombosis. Stroke 2005;36:154-157.
20 Koch W, Hoppmann P, Pfeufer A, Schomig A, Kastrati A: Toll-like receptor 4 gene polymorphisms and myocardial infarction: no association in a Caucasian population. Eur Heart J 2006;27:2524-2529.
21 Frantz S, Kobzik L, Kim YD, Fukazawa R, Medzhitov R, Lee RT, Kelly RA: Toll4 (TLR4) expression in cardiac myocytes in normal and failing myocardium. J Clin Invest 1999;104:271-280.

22 Timmers L, Sluijter JP, van Keulen JK, Hoefer IE, Nederhoff MG, Goumans MJ, Doevendans PA, van Echteld CJ, Joles JA, Quax PH, Piek JJ, Pasterkamp G, de Kleijn DP: Toll-like receptor 4 mediates maladaptive left ventricular remodeling and impairs cardiac function after myocardial infarction. Circ Res 2-1-2008;102:257-264.
23 Oyama J, Blais C, Jr., Liu X, Pu M, Kobzik L, Kelly RA, Bourcier T: Reduced myocardial ischemia-reperfusion injury in toll-like receptor 4-deficient mice. Circulation 2-17-2004;109:784-789.
24 Chong AJ, Shimamoto A, Hampton CR, Takayama H, Spring DJ, Rothnie CL, Yada M, Pohlman TH, Verrier ED: Toll-like receptor 4 mediates ischemia/reperfusion injury of the heart. J Thorac Cardiovasc Surg 2004;128:170-179.
25 Stapel H, Kim SC, Osterkamp S, Knuefermann P, Hoeft A, Meyer R, Grohe C, Baumgarten G: Toll-like receptor 4 modulates myocardial ischaemia-reperfusion injury: Role of matrix metalloproteinases. Eur J Heart Fail 2006;8:665-672.
26 Hua F, Ha T, Ma J, Li Y, Kelley J, Gao X, Browder IW, Kao RL, Williams DL, Li C: Protection against myocardial ischemia/reperfusion injury in TLR4-deficient mice is mediated through a phosphoinositide 3-kinase-dependent mechanism. J Immunol 6-1-2007;178:7317-7324.
27 Shimamoto A, Chong AJ, Yada M, Shomura S, Takayama H, Fleisig AJ, Agnew ML, Hampton CR, Rothnie CL, Spring DJ, Pohlman TH, Shimpo H, Verrier ED: Inhibition of Toll-like receptor 4 with eritoran attenuates myocardial ischemia-reperfusion injury. Circulation 7-4-2006;114:I270-I274.
28 Methe H, Kim JO, Kofler S, Weis M, Nabauer M, Koglin J: Expansion of circulating Toll-like receptor 4-positive monocytes in patients with acute coronary syndrome. Circulation 5-24-2005;111:2654-2661.
29 Shishido T, Nozaki N, Yamaguchi S, Shibata Y, Nitobe J, Miyamoto T, Takahashi H, Arimoto T, Maeda K, Yamakawa M, Takeuchi O, Akira S, Takeishi Y, Kubota I: Toll-like receptor-2 modulates ventricular remodeling after myocardial infarction. Circulation 12-9-2003;108:2905-2910.
30 Sakata Y, Dong JW, Vallejo JG, Huang CH, Baker JS, Tracey KJ, Tacheuchi O, Akira S, Mann DL: Toll-like receptor 2 modulates left ventricular function following ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 2007;292:H503-H509.
31 Arslan F, Smeets MB, O'Neill LA, Keogh B, McGuirk P, Timmers L, Tersteeg C, Hoefer IE, Doevendans PA, Pasterkamp G, de Kleijn DP: Myocardial ischemia/reperfusion injury is mediated by leukocytic toll-like receptor-2 and reduced by systemic administration of a novel anti-toll-like receptor-2 antibody. Circulation 1-5-2010;121:80-90.
32 Arslan F, de Kleijn DP, Timmers L, Doevendans PA, Pasterkamp G: Bridging innate immunity and myocardial ischemia/reperfusion injury: the search for therapeutic targets. Curr Pharm Des 2008;14:1205-1216.
33 Poltorak A, He X, Smirnova I, Liu MY, Van HC, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B: Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 12-11-1998;282:2085-2088.
34 Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K, Akira S: Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 1999;11:443-451.
35 Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, Modlin RL, Akira S: Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J Immunol 7-1-2002;169:10-14.
36 Takeuchi O, Kawai T, Muhlradt PF, Morr M, Radolf JD, Zychlinsky A, Takeda K, Akira S: Discrimination of bacterial lipoproteins by Toll-like receptor 6. Int Immunol 2001;13:933-940.
37 Lund JM, Alexopoulou L, Sato A, Karow M, Adams NC, Gale NW, Iwasaki A, Flavell RA: Recognition of single-stranded RNA viruses by Toll-like receptor 7. Proc Natl Acad Sci U S A 4-13-2004;101:5598-5603.
38 Alexopoulou L, Holt AC, Medzhitov R, Flavell RA: Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 10-18-2001;413:732-738.
39 Libby P: Inflammation in atherosclerosis. Nature 12-19-2002;420:868-874.
40 Moazed TC, Campbell LA, Rosenfeld ME, Grayston JT, Kuo CC: Chlamydia pneumoniae infection accelerates the progression of atherosclerosis in apolipoprotein E-deficient mice. J Infect Dis 1999;180:238-241.
41 Saikku P, Leinonen M, Mattila K, Ekman MR, Nieminen MS, Makela PH, Huttunen JK, Valtonen V: Serological evidence of an association of a novel Chlamydia, TWAR, with chronic coronary heart disease and acute myocardial infarction. Lancet 10-29-1988;2:983-986.

42 Laman JD, Schoneveld AH, Moll FL, van Meurs M, Pasterkamp G: Significance of peptidoglycan, a proinflammatory bacterial antigen in atherosclerotic arteries and its association with vulnerable plaques. The American Journal of Cardiology 7-15-2002;90:119-123.
43 Beg AA: Endogenous ligands of Toll-like receptors: implications for regulating inflammatory and immune responses. Trends in Immunology 11-1-2002;23:509-512.
44 Tsan MF, Gao B: Endogenous ligands of Toll-like receptors. J Leukoc Biol 2004;76:514-519.
45 Xu Q: Role of heat shock proteins in atherosclerosis. Arterioscler Thromb Vasc Biol 10-1-2002;22:1547-1559.
46 Vabulas RM, hmad-Nejad P, Ghose S, Kirschning CJ, Issels RD, Wagner H: HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway. J Biol Chem 4-26-2002;277:15107-15112.
47 Dybdahl B, Wahba A, Lien E, Flo TH, Waage A, Qureshi N, Sellevold OFM, Espevik T, Sundan A: Inflammatory Response After Open Heart Surgery: Release of Heat-Shock Protein 70 and Signaling Through Toll-Like Receptor-4. Circulation 2-12-2002;105:685-690.
48 Vabulas RM, Braedel S, Hilf N, Singh-Jasuja H, Herter S, hmad-Nejad P, Kirschning CJ, da CC, Rammensee HG, Wagner H, Schild H: The endoplasmic reticulum-resident heat shock protein Gp96 activates dendritic cells via the Toll-like receptor 2/4 pathway. J Biol Chem 6-7-2002;277:20847-20853.
49 Kol A, Lichtman AH, Finberg RW, Libby P, Kurt-Jones EA: Cutting Edge: Heat Shock Protein (HSP) 60 Activates the Innate Immune Response: CD14 Is an Essential Receptor for HSP60 Activation of Mononuclear Cells. J Immunol 1-1-2000;164:13-17.
50 Sasu S, LaVerda D, Qureshi N, Golenbock DT, Beasley D: Chlamydia pneumoniae and Chlamydial Heat Shock Protein 60 Stimulate Proliferation of Human Vascular Smooth Muscle Cells via Toll-Like Receptor 4 and p44/p42 Mitogen-Activated Protein Kinase Activation. Circ Res 8-3-2001;89:244-250.
51 Vabulas RM, hmad-Nejad P, Ghose S, Kirschning CJ, Issels RD, Wagner H: HSP70 as Endogenous Stimulus of the Toll/Interleukin-1 Receptor Signal Pathway. Journal of Biological Chemistry 4-26-2002;277:15107-15112.
52 Pockley AG: Heat Shock Proteins, Inflammation, and Cardiovascular Disease. Circulation 2-26-2002;105:1012-1017.
53 Kleindienst R, Xu Q, Willeit J, Waldenberger FR, Weimann S, Wick G: Immunology of atherosclerosis. Demonstration of heat shock protein 60 expression and T lymphocytes bearing alpha/beta or gamma/delta receptor in human atherosclerotic lesions. Am J Pathol 6-1-1993;142:1927-1937.
54 Foteinos G, Xu Q: Immune-mediated mechanisms of endothelial damage in atherosclerosis. Autoimmunity 2009;42:627-633.
55 Xu Q, Schett G, Perschinka H, Mayr M, Egger G, Oberhollenzer F, Willeit J, Kiechl S, Wick G: Serum Soluble Heat Shock Protein 60 Is Elevated in Subjects With Atherosclerosis in a General Population. Circulation 7-4-2000;102:14-20.
56 Chen W, Syldath U, Bellmann K, Burkart V, Kolb H: Human 60-kDa Heat-Shock Protein: A Danger Signal to the Innate Immune System. J Immunol 3-15-1999;162:3212-3219.
57 Hammerer-Lercher A, Mair J, Bonatti J, Watzka SBC, Puschendorf B, Dirnhofer S: Hypoxia induces heat shock protein expression in human coronary artery bypass grafts. Cardiovasc Res 4-1-2001;50:115-124.
58 Kanwar RK, Kanwar JR, Wang D, Ormrod DJ, Krissansen GW: Temporal Expression of Heat Shock Proteins 60 and 70 at Lesion-Prone Sites During Atherogenesis in ApoE-Deficient Mice. Arterioscler Thromb Vasc Biol 12-1-2001;21:1991-1997.
59 Lin L, Kim SC, Wang Y, Gupta S, Davis B, Simon SI, Torre-Amione G, Knowlton AA: HSP60 in heart failure: abnormal distribution and role in cardiac myocyte apoptosis. Am J Physiol Heart Circ Physiol 2007;293:H2238-H2247.
60 Kim SC, Stice JP, Chen L, Jung JS, Gupta S, Wang Y, Baumgarten G, Trial J, Knowlton AA: Extracellular heat shock protein 60, cardiac myocytes, and apoptosis. Circ Res 12-4-2009;105:1186-1195.
61 Andersson U, Erlandsson-Harris H, Yang H, Tracey KJ: HMGB1 as a DNA-binding cytokine. J Leukoc Biol 12-1-2002;72:1084-1091.
62 Andersson U, Wang H, Palmblad K, Aveberger AC, Bloom O, Erlandsson-Harris H, Janson A, Kokkola R, Zhang M, Yang H, Tracey KJ: High Mobility Group 1 Protein (Hmg-1) Stimulates Proinflammatory Cytokine Synthesis in Human Monocytes. J Exp Med 8-21-2000;192:565-570.
63 WANG HAIC, YANG HUAN, CZURA CJ, SAMA AE, Tracey KJ: HMGB1 as a Late Mediator of Lethal Systemic Inflammation. Am J Respir Crit Care Med 11-15-2001;164:1768-1773.

64 Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, Manogue KR, Faist E, Abraham E, Andersson J, Andersson U, Molina PE, Abumrad NN, Sama A, Tracey KJ: HMG-1 as a Late Mediator of Endotoxin Lethality in Mice. Science 7-9-1999;285:248-251.
65 Park JS, Svetkauskaite D, He Q, Kim JY, Strassheim D, Ishizaka A, Abraham E: Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. J Biol Chem 2-27-2004;279:7370-7377.
66 Kalinina N, Agrotis A, Antropova Y, DiVitto G, Kanellakis P, Kostolias G, Ilyinskaya O, Tararak E, Bobik A: Increased Expression of the DNA-Binding Cytokine HMGB1 in Human Atherosclerotic Lesions: Role of Activated Macrophages and Cytokines. Arterioscler Thromb Vasc Biol 12-1-2004;24:2320-2325.
67 Abeyama K, Stern DM, Ito Y, Kawahara K, Yoshimoto Y, Tanaka M, Uchimura T, Ida N, Yamazaki Y, Yamada S, Yamamoto Y, Yamamoto H, Iino S, Taniguchi N, Maruyama I: The N-terminal domain of thrombomodulin sequesters high-mobility group-B1 protein, a novel antiinflammatory mechanism. J Clin Invest 2005;115:1267-1274.
68 Cipollone F, Iezzi A, Fazia M, Zucchelli M, Pini B, Cuccurullo C, De CD, De BG, Muraro R, Bei R, Chiarelli F, Schmidt AM, Cuccurullo F, Mezzetti A: The receptor RAGE as a progression factor amplifying arachidonate-dependent inflammatory and proteolytic response in human atherosclerotic plaques: role of glycemic control. Circulation 9-2-2003;108:1070-1077.
69 Hansson GK, Libby P, Schonbeck U, Yan ZQ: Innate and adaptive immunity in the pathogenesis of atherosclerosis. Circ Res 8-23-2002;91:281-291.
70 Tsung A, Sahai R, Tanaka H, Nakao A, Fink MP, Lotze MT, Yang H, Li J, Tracey KJ, Geller DA, Billiar TR: The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. J Exp Med 4-4-2005;201:1135-1143.
71 Dybdahl B, Wahba A, Lien E, Flo TH, Waage A, Qureshi N, Sellevold OF, Espevik T, Sundan A: Inflammatory response after open heart surgery: release of heat-shock protein 70 and signaling through toll-like receptor-4. Circulation 2-12-2002;105:685-690.
72 Andrassy M, Volz HC, Igwe JC, Funke B, Eichberger SN, Kaya Z, Buss S, Autschbach F, Pleger ST, Lukic IK, Bea F, Hardt SE, Humpert PM, Bianchi ME, Mairbaurl H, Nawroth PP, Remppis A, Katus HA, Bierhaus A: High-mobility group box-1 in ischemia-reperfusion injury of the heart. Circulation 6-24-2008;117:3216-3226.
73 Woo YJ, Taylor MD, Cohen JE, Jayasankar V, Bish LT, Burdick J, Pirolli TJ, Berry MF, Hsu V, Grand T: Ethyl pyruvate preserves cardiac function and attenuates oxidative injury after prolonged myocardial ischemia. J Thorac Cardiovasc Surg 2004;127:1262-1269.
74 Wang P: Andrenomedullin and cardiovascular responses in sepsis. Peptides 2001;22:1835-1840.
75 Schwarzbauer JE: Alternative splicing of fibronectin: three variants, three functions. Bioessays 1991;13:527-533.
76 Saito S, Yamaji N, Yasunaga K, Saito T, Matsumoto S, Katoh M, Kobayashi S, Masuho Y: The fibronectin extra domain A activates matrix metalloproteinase gene expression by an interleukin-1-dependent mechanism. J Biol Chem 10-22-1999;274:30756-30763.
77 Okamura Y, Watari M, Jerud ES, Young DW, Ishizaka ST, Rose J, Chow JC, Strauss JF, III: The extra domain A of fibronectin activates Toll-like receptor 4. J Biol Chem 3-30-2001;276:10229-10233.
78 Tan MH, Sun Z, Opitz SL, Schmidt TE, Peters JH, George EL: Deletion of the alternatively spliced fibronectin EIIIA domain in mice reduces atherosclerosis. Blood 7-1-2004;104:11-18.
79 van Keulen JK, de Kleijn DP, Nijhuis MM, Busser E, Velema E, Fijnheer R, van der GY, Moll FL, de Vries JP, Pasterkamp G: Levels of extra domain A containing fibronectin in human atherosclerotic plaques are associated with a stable plaque phenotype. Atherosclerosis 2007;195:e83-e91.
80 Hessian PA, Edgeworth J, Hogg N: MRP-8 and MRP-14, two abundant Ca(2+)-binding proteins of neutrophils and monocytes. J Leukoc Biol 2-1-1993;53:197-204.
81 Roth J, Vogl T, Sorg C, Sunderk÷tter C: Phagocyte-specific S100 proteins: a novel group of proinflammatory molecules. Trends in Immunology 2003;24:155-158.
82 Frosch M, Strey A, Vogl T, Wulffraat NM, Kuis W, Sunderkotter C, Harms E, Sorg C, Roth J: Myeloid-related proteins 8 and 14 are specifically secreted during interaction of phagocytes and activated endothelium and are useful markers for monitoring disease activity in pauciarticular-onset juvenile rheumatoid arthritis. Arthritis Rheum 2000;43:628-637.

83 Teigelkamp S, Bhardwaj RS, Roth J, Meinardus-Hager G, Karas M, Sorg C: Calcium-dependent complex assembly of the myeloic differentiation proteins MRP-8 and MRP-14. J Biol Chem 7-15-1991;266:13462-13467.
84 Vogl T, Ludwig S, Goebeler M, Strey A, Thorey IS, Reichelt R, Foell D, Gerke V, Manitz MP, Nacken W, Werner S, Sorg C, Roth J: MRP8 and MRP14 control microtubule reorganization during transendothelial migration of phagocytes. Blood 12-15-2004;104:4260-4268.
85 Nacken W, Roth J, Sorg C, Kerkhoff C: S100A9/S100A8: Myeloid representatives of the S100 protein family as prominent players in innate immunity. Microsc Res Tech 4-15-2003;60:569-580.
86 Ahmad A, Bayley DL, He S, Stockley RA: Myeloid Related Protein-8/14 Stimulates Interleukin-8 Production in Airway Epithelial Cells. Am J Respir Cell Mol Biol 10-1-2003;29:523-530.
87 Nacken W, Roth J, Sorg C, Kerkhoff C: S100A9/S100A8: Myeloid representatives of the S100 protein family as prominent players in innate immunity. Microsc Res Tech 4-15-2003;60:569-580.
88 Croce K, Gao H, Wang Y, Mooroka T, Sakuma M, Shi C, Sukhova GK, Packard RR, Hogg N, Libby P, Simon DI: Myeloid-related protein-8/14 is critical for the biological response to vascular injury. Circulation 8-4-2009;120:427-436.
89 Ionita MG, Vink A, Dijke IE, Laman JD, Peeters W, van der Kraak PH, Moll FL, de Vries JP, Pasterkamp G, de Kleijn DP: High levels of myeloid-related protein 14 in human atherosclerotic plaques correlate with the characteristics of rupture-prone lesions. Arterioscler Thromb Vasc Biol 2009;29:1220-1227.
90 Edgeworth J, Gorman M, Bennett R, Freemont P, Hogg N: Identification of p8,14 as a highly abundant heterodimeric calcium binding protein complex of myeloid cells. J Biol Chem 4-25-1991;266:7706-7713.
91 McCormick MM, Rahimi F, Bobryshev YV, Gaus K, Zreiqat H, Cai H, Lord RSA, Geczy CL: S100A8 and S100A9 in Human Arterial Wall: IMPLICATIONS FOR ATHEROGENESIS. J Biol Chem 12-16-2005;280:41521-41529.
92 Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MAD, Nacken W, Foell D, van der Poll T, Sorg C, Roth J: Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat Med 2007;13:1042-1049.
93 Altwegg LA, Neidhart M, Hersberger M, Muller S, Eberli FR, Corti R, Roffi M, Sutsch G, Gay S, von Eckardstein A, Wischnewsky MB, Luscher TF, Maier W: Myeloid-related protein 8/14 complex is released by monocytes and granulocytes at the site of coronary occlusion: a novel, early, and sensitive marker of acute coronary syndromes. Eur Heart J 4-2-2007;28:941-948.
94 Morrow DA, Wang Y, Croce K, Sakuma M, Sabatine MS, Gao H, Pradhan AD, Healy AM, Buros J, McCabe CH, Libby P, Cannon CP, Braunwald E, Simon DI: Myeloid-related protein 8/14 and the risk of cardiovascular death or myocardial infarction after an acute coronary syndrome in the Pravastatin or Atorvastatin Evaluation and Infection Theraphy: Thrombolysis in Myocardial Infarction (PROVE IT-TIMI 22) trial. American Heart Journal 2008;155:49-55.
95 Healy AM, Pickard MD, Pradhan AD, Wang Y, Chen Z, Croce K, Sakuma M, Shi C, Zago AC, Garasic J, Damokosh AI, Dowie TL, Poisson L, Lillie J, Libby P, Ridker PM, Simon DI: Platelet Expression Profiling and Clinical Validation of Myeloid-Related Protein-14 as a Novel Determinant of Cardiovascular Events. Circulation 5-16-2006;113:2278-2284.
96 Miller YI, Viriyakosol S, Binder CJ, Feramisco JR, Kirkland TN, Witztum JL: Minimally Modified LDL Binds to CD14, Induces Macrophage Spreading via TLR4/MD-2, and Inhibits Phagocytosis of Apoptotic Cells. Journal of Biological Chemistry 1-17-2003;278:1561-1568.
97 Miller YI, Viriyakosol S, Worrall DS, Boullier A, Butler S, Witztum JL: Toll-Like Receptor 4-Dependent and -Independent Cytokine Secretion Induced by Minimally Oxidized Low-Density Lipoprotein in Macrophages. Arterioscler Thromb Vasc Biol 6-1-2005;25:1213-1219.
98 Huo Y, Zhao L, Hyman MC, Shashkin P, Harry BL, Burcin T, Forlow SB, Stark MA, Smith DF, Clarke S, Srinivasan S, Hedrick CC, Pratico D, Witztum JL, Nadler JL, Funk CD, Ley K: Critical Role of Macrophage 12/15-Lipoxygenase for Atherosclerosis in Apolipoprotein E-Deficient Mice. Circulation 10-5-2004;110:2024-2031.
99 Bolick DT, Srinivasan S, Whetzel A, Fuller LC, Hedrick CC: 12/15 Lipoxygenase Mediates Monocyte Adhesion to Aortic Endothelium in Apolipoprotein E-Deficient Mice Through Activation of RhoA and NF-{kappa}B. Arterioscler Thromb Vasc Biol 6-1-2006;26:1260-1266.

CHAPTER III

High Levels of Myeloid-related protein 14 in Human Atherosclerotic Plaques Correlate with the Characteristics of Rupture-prone Lesions

Atherosclerosis, Thrombosis and Vascular Biology (ATVB), August 2009

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

Abstract

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 if 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 metalloprotease (MMP)-8, MMP-9 and low MMP-2 concentrations. Mrp-8 and Mrp-8/14 showed a similar trend. Within plaques, a subset of non-foam 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 histopathological features and the inflammatory status of rupture-prone atherosclerotic lesions, identifying Mrp-14 as a local marker for these plaques.

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 two 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 pro-inflammatory 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]. Upon cell activation, the two 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)-4[13] are two 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[14]. 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[15].

Taken together, these results suggest that Mrp-8, Mrp-14 and Mrp-8/14 reflect biological events in plaque progression towards 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 if 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 non-foam macrophages expressing Mrp-8 and -14 was predominant in the rupture-prone lesions; *in vitro*, the Mrp-macrophage subset did not acquired 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 two Dutch hospitals: the University Medical Center Utrecht and the St. Antonius Hospital in Nieuwegein[16]. 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)[17-20] . 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[16] and in detail in the online supplement. 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, α-actin and CD68) have been described previously[16].

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.

Immunoassays 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, Augst, Switzerland) 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 eighty plaques, from the total 186 plaques included in the present study, was 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 Mrp-14 antibody (mouse IgG1, dilution 1:200; Santa-Cruz Biotechnologies). 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.
The CD68 staining and the Mrp-8 staining were quantified using image-analyzing software (Soft Imaging Systems, Münster, Germany). Expression of Mrp-8 and Mrp-14 was detected in non-foam CD68-positive macrophages. We therefore decided to select only the non-foam 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[21], 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 non-adherent conditions in RPMI 1640 medium (BioWhittaker, Verviers, Belgium) supplemented

with 25nM Hepes, Ultraglutamin1 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 two 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, Roskilde, Denmark).The macrophages were incubated during 24 h with 10 μg/ml human oxLDL, isolated and oxidized as described previously[22, 23] or with culture medium as control. OxLDL uptake was detected with Oil-Red-O (ORO), which stains neutral lipids, as previously described[24].

Flow cytometry and Fluorescence-activated cell sorting

Fluorescence-activated cell sorting on a FACS Aria (BD Biosciences, San Jose, CA) 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 B.V., Huissen, the Netherlands). Mrp-8/14 FITC (27E10) (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-APC (R&D Systems, Minneapolis, MN); Mrp-8/14-FITC (27E10), 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., Ashland, OR) software.

Data analysis

Data are expressed as means ± SEM. Correlations between different parameters were assessed using Spearman's 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 online supplemental material. 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, however without reaching statistical significance (figure 1A and C).

A positive correlation was observed between plaque's macrophage content 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) while for Mrp-8/14 a similar but non-significant 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) (online supplemental material, 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 pro-inflammatory molecules IL-6, IL-8 in the lesion (Table 1). 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 2, a and b). Mrp-8 and Mrp-14 expression was observed in a subset of CD68 positive

macrophages (figure 2, c-f). The Mrp positive macrophages did not exhibit a foamy phenotype as CD68-foam macrophages stain negative for Mrp (figure 2, e, f). Within non-foam CD68-positive macrophage areas, the percentage of Mrp-positive macrophages is significantly higher in rupture-prone (n=55, mean 33.57 ± 3.74%) than in stable lesions (n=25, mean 9.56 ± 3.5%) (p=0.003; figure 3).

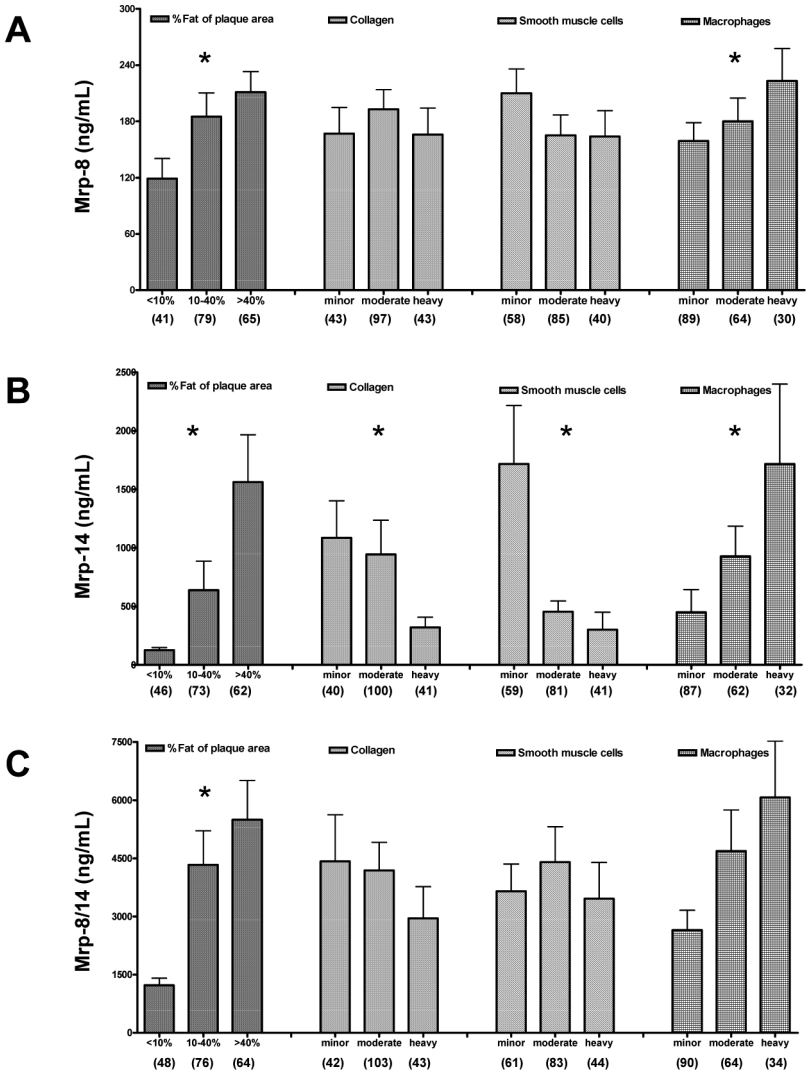


Figure 1. Mrp-8 (A), -14 (B) and -8/14 (C) in relation to the size of the lipid core as a percentage of total plaque area, the amount of collagen, smooth muscle cells and macrophages. Bars represent means ± SEM; below each bar, the number of patients per group is indicated; statistics Spearman's correlation test, significance at * $p<0.05$.

In addition to macrophages, Mrp expression was observed in a small number of neutrophilic granulocytes (figure 2, supplementary material).

MRP AND *IN VITRO* DEVELOPMENT OF MONOCYTES INTO FOAMY MACROPHAGES

Since Mrp-8 and Mrp-14 were expressed in non-foam 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[21] (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 non-adherent 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 two human primary macrophage populations, namely Mrp-8/14 positive and negative. Both populations were fed for 24 h with 10 µg/mL oxLDL and changes in the morphology were determined by light microscopy and by ORO 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).

Table 1. Correlations between Mrp plaque levels and inflammatory cytokines and proteases

	Mrp-8	Mrp-14	Mrp-8/14
Cytokines/ Chemokines			
IL-6	P = 0.048*	P = 0.001*	P = 0.114
	R = (+) 0.177	R = (+) 0.351	R = (+) 0.141
IL-8	P = 0.001*	P = 0.001*	P = 0.001*
	R = (+) 0.587	R = (+) 0.718	R = (+) 0.528
Proteases			
MMP-2	P = 0.065	P = 0.018*	P = 0.187
	R = (-) 0.222	R = (-) 0.283	R = (-) 0.155
MMP-8	P = 0.007*	P = 0.001*	P = 0.001*
	R = (+) 0.203	R = (+) 0.279	R = (+) 0.301
MMP-9	P = 0.102	P = 0.001*	P = 0.005*
	R = (+) 0.124	R = (+) 0.241	R = (+) 0.209

Spearman's correlation (R=correlation coefficient); significance at * P<0.05; IL=interleukin; MMP= matrix metalloproteinase

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).

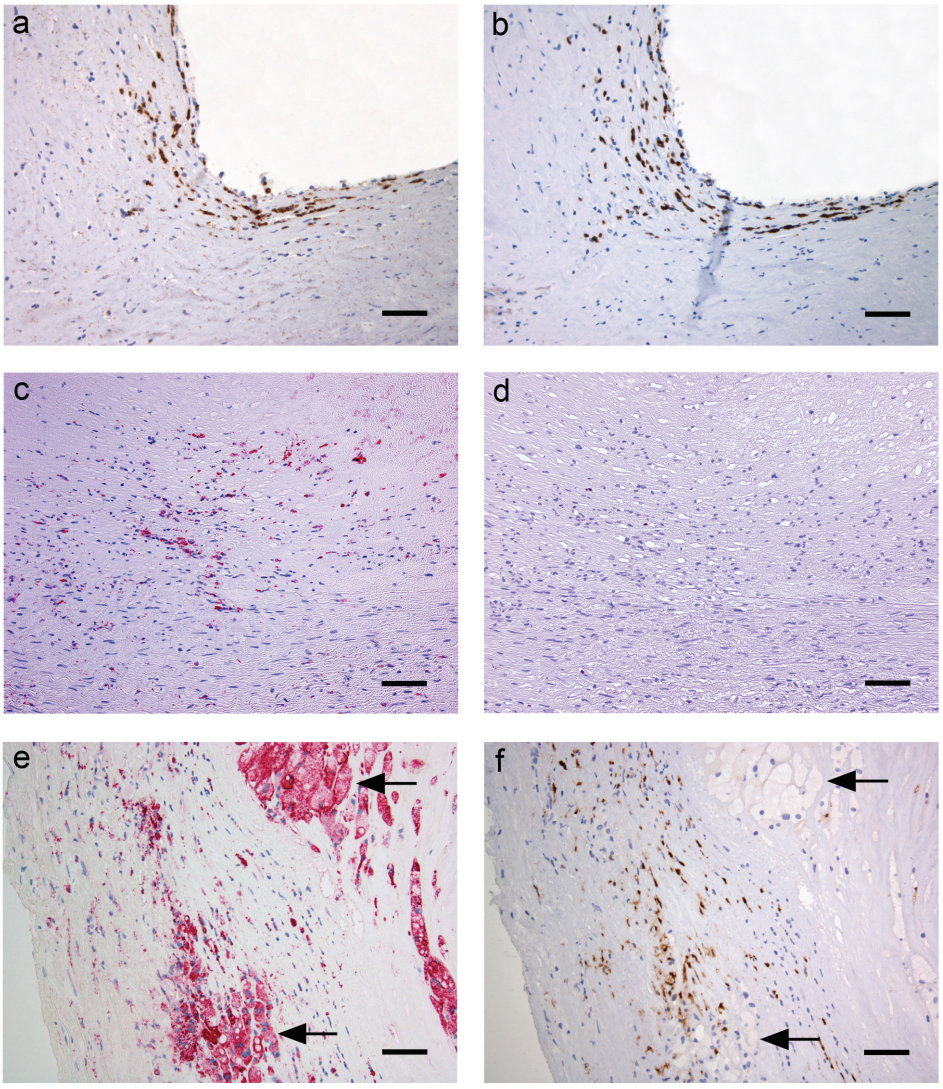


Figure 2. Expression of Mrps in carotid atherosclerotic plaque. Mrp-8 (a) and Mrp-14 (b) co-localize in consecutive sections. (c-f) Mrp-8 expression in CD68-positive macrophages. (c, d) Consecutive sections showing Mrp-8 staining (d, brown) in a small subset of CD68-positive macrophages (c, red) in a stable plaque. (e, 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.

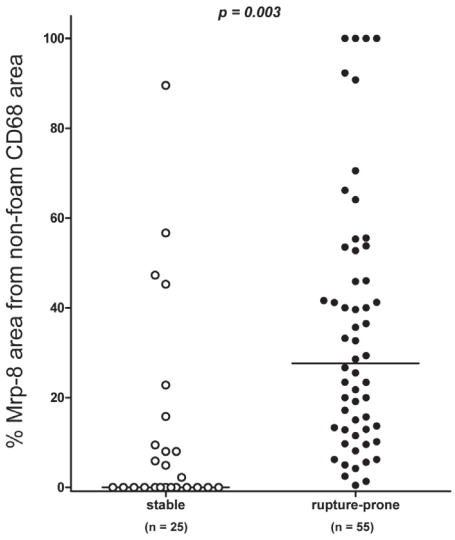


Figure 3. Quantitative assessment of the Mrp-8 area within the non-foam CD68 area in stable (n = 25) and rupture-prone (n = 55) atherosclerotic lesions. The percentage of Mrp-8 positive macrophages was significantly (p = 0.003) higher in rupture-prone plaques (mean = 34.78) compared to stable ones (mean = 12.63). Lines represent medians; statistics Mann-Whitney test.

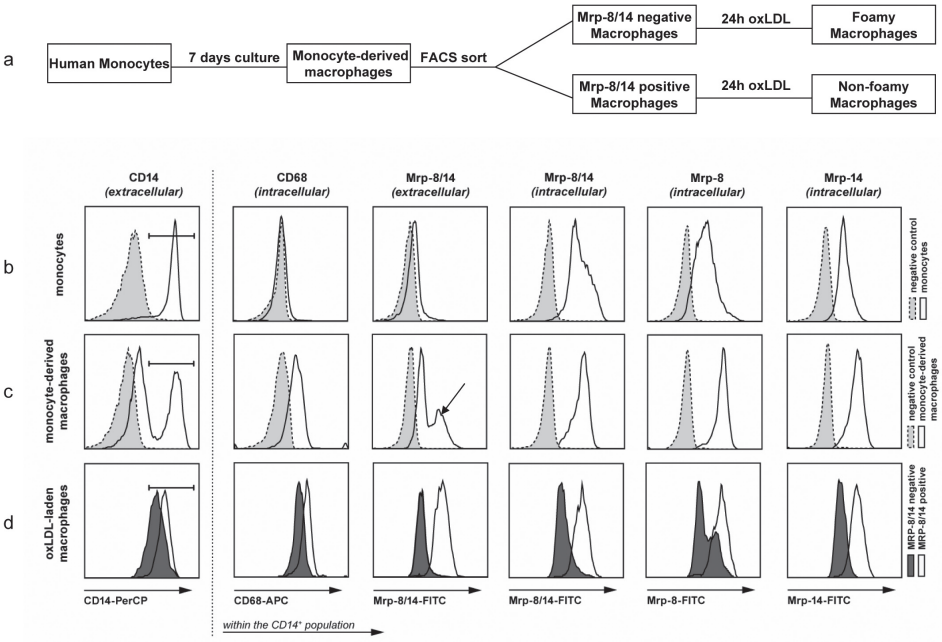


Figure 4. *In vitro* development of human monocytes into macrophages and oxLDL-loaded macrophages (a). Flow cytometry showing differential expression of extracellular CD14 and Mrp-8/14 or intracellular CD68, Mrp-8/14, Mrp-8 and Mrp-14 proteins during development of monocytes (b) into monocyte-derived macrophages (c) and oxLDL-loaded macrophages (d). Arrow points to membrane Mrp-8/14 positive macrophage population. A representative experiment out of 3 with independent individual healthy donors is shown.

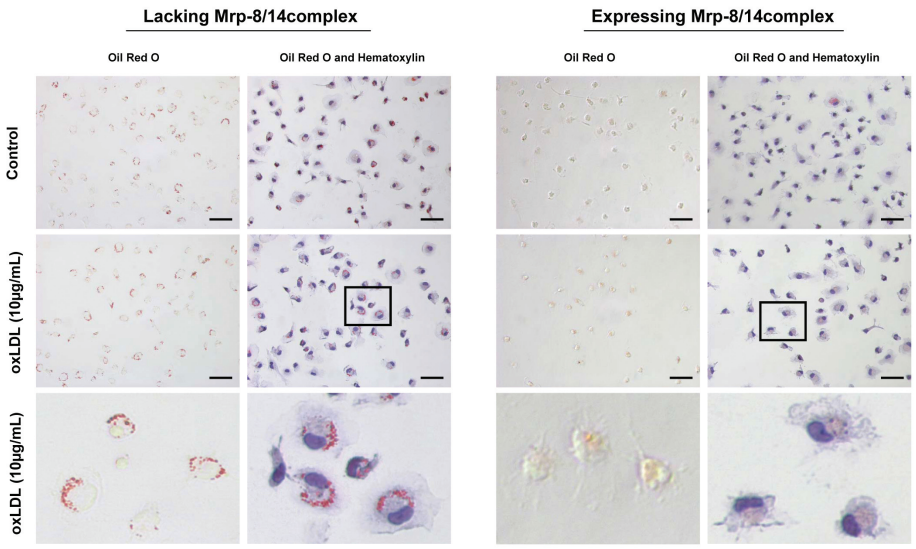


Figure 5. Macrophages expressing surface Mrp-8/14 complex did not acquire foam cell morphology upon oxLDL ingestion whereas the macrophages lacking Mrp-8/14 surface expression did acquire foam morphology. Bars 50 µm; bottom photos represent magnifications of the above black boxes.

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, MMP-9 and low levels of MMP-2. Mrp-8 showed no significant correlation with MMP-2 and MMP-9 while 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]. Pro-inflammatory 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 while IL-8 is secreted by

active macrophages, endothelial cells and T-cells[27]. MMP-2 and MMP-9 co-localize with activated smooth muscle cells and macrophages within atherosclerotic plaques[26]. MMP-8 is an extremely efficient type I collagenolytic enzyme in humans[28] and is traditionally considered a neutrophil product, a cell type not commonly observed in atheroma[29]; 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 non-foamy macrophages with a higher percentage of Mrp-8 and 14 positive area of the non-foam 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 vast 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[32], 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[33]. The Mrp-positive macrophage subset is associated with a high plaque inflammatory status as reflected by the levels of pro-inflammatory interleukins and proteases suggesting that this subset might be involved in plaque destabilization (see table V from the supplementary material). 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 (Figure 2, supplementary material). 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[34] 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 non-foam plaque macrophages, it does not exclude the importance of other possible Mrp-cellular sources within plaque (e.g. 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[35]. Altwegg et al[14] 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) when compared with systemic levels, suggesting that Mrp-8/14 is locally expressed at the site of coronary occlusion due to 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 add 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 non-foam macrophages in human plaques, are strongly associated with both histopathological 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 non-significant results obtained for Mrp-8 and -8/14 might be due to limitations of the commercial ELISA kits used for the detection of those proteins. The Mrp-8 levels might be underestimated due to 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[7], 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.

Funding Sources

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 JDL is supported by the Dutch MS Research Foundation.

Acknowledgements

The authors would like to 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.

Disclosures

The authors report that Dominique P.V. de Kleijn, Frans Moll and Gerard Pasterkamp are co-founders of Cavadis, a biomarker company. The other authors report no significant conflicts of interest. Author contributions: M.G.I. designed research, performed research, analyzed the data, and wrote the paper; A.V. designed research, and analyzed data; I.E.D. performed research, analyzed data; J.D.L. designed research, analyzed data; W.P. statistics; P.H.K. performed research; F.L.M. designed research; J.P.V. designed research; G.P. designed research; D.P.V.K. designed research, analyzed the data, and corrected paper.

Reference List

1 Falk E, Fuster V. Angina Pectoris and Disease Progression. Circulation. 1995;92:2033-2035.
2 Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons From Sudden Coronary Death : A Comprehensive Morphological Classification Scheme for Atherosclerotic Lesions. Arterioscler Thromb Vasc Biol. 2000;20:1262-1275.
3 Hessian PA, Edgeworth J, Hogg N. MRP-8 and MRP-14, two abundant Ca(2+)-binding proteins of neutrophils and monocytes. J Leukoc Biol. 1993;53:197-204.
4 Roth J, Vogl T, Sorg C, Sunderk÷tter C. Phagocyte-specific S100 proteins: a novel group of proinflammatory molecules. Trends in Immunology. 2003;24:155-158.
5 Frosch M, Strey A, Vogl T, Wulffraat NM, Kuis W, Sunderkotter C, Harms E, Sorg C, Roth J. Myeloid-related proteins 8 and 14 are specifically secreted during interaction of phagocytes and activated endothelium and are useful markers for monitoring disease activity in pauciarticular-onset juvenile rheumatoid arthritis. Arthritis Rheum. 2000;43:628-637.
6 Edgeworth J, Gorman M, Bennett R, Freemont P, Hogg N. Identification of p8,14 as a highly abundant heterodimeric calcium binding protein complex of myeloid cells. J Biol Chem. 1991;266:7706-7713.
7 McCormick MM, Rahimi F, Bobryshev YV, Gaus K, Zreiqat H, Cai H, Lord RSA, Geczy CL. S100A8 and S100A9 in Human Arterial Wall: IMPLICATIONS FOR ATHEROGENESIS. J Biol Chem. 2005;280:41521-41529.
8 Teigelkamp S, Bhardwaj RS, Roth J, Meinardus-Hager G, Karas M, Sorg C. Calcium-dependent complex assembly of the myeloic differentiation proteins MRP-8 and MRP-14. J Biol Chem. 1991;266:13462-13467.
9 Vogl T, Ludwig S, Goebeler M, Strey A, Thorey IS, Reichelt R, Foell D, Gerke V, Manitz MP, Nacken W, Werner S, Sorg C, Roth J. MRP8 and MRP14 control microtubule reorganization during transendothelial migration of phagocytes. Blood. 2004;104:4260-4268.
10 Nacken W, Roth J, Sorg C, Kerkhoff C. S100A9/S100A8: Myeloid representatives of the S100 protein family as prominent players in innate immunity. Microsc Res Tech. 2003;60:569-580.
11 Ahmad A, Bayley DL, He S, Stockley RA. Myeloid Related Protein-8/14 Stimulates Interleukin-8 Production in Airway Epithelial Cells. Am J Respir Cell Mol Biol. 2003;29:523-530.
12 Sunahori K, Yamamura M, Yamana J, Takasugi K, Kawashima M, Yamamoto H, Chazin W, Nakatani Y, Yui S, Makino H. The S100A8/A9 heterodimer amplifies proinflammatory cytokine production by macrophages via activation of nuclear factor kappa B and p38 mitogen-activated protein kinase in rheumatoid arthritis. Arthritis Research & Therapy. 2006;8:R69.
13 Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MAD, Nacken W, Foell D, van der Poll T, Sorg C, Roth J. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat Med. 2007;13:1042-1049.
14 Altwegg LA, Neidhart M, Hersberger M, Muller S, Eberli FR, Corti R, Roffi M, Sutsch G, Gay S, von Eckardstein A, Wischnewsky MB, Luscher TF, Maier W. Myeloid-related protein 8/14 complex is released by monocytes and granulocytes at the site of coronary occlusion: a novel, early, and sensitive marker of acute coronary syndromes. Eur Heart J. 2007;28:941-948.
15 Healy AM, Pickard MD, Pradhan AD, Wang Y, Chen Z, Croce K, Sakuma M, Shi C, Zago AC, Garasic J, Damokosh AI, Dowie TL, Poisson L, Lillie J, Libby P, Ridker PM, Simon DI. Platelet Expression Profiling and Clinical Validation of Myeloid-Related Protein-14 as a Novel Determinant of Cardiovascular Events. Circulation. 2006;113:2278-2284.
16 Verhoeven B, Velema E, Schoneveld A, Vries J, de Bruin P, Seldenrijk C, Kleijn D, Busser E, der Graaf Y, Mol F, Pasterkamp G. Athero-express: Differential atherosclerotic plaque expression of mRNA and protein in relation to cardiovascular events and patient characteristics. Rationale and design. European Journal of Epidemiology. 2004;19:1127-1133.
17 Beneficial effect of carotid endarterectomy in symptomatic patients with high-grade carotid stenosis. North American Symptomatic Carotid Endarterectomy Trial Collaborators. N Engl J Med. 1991;325:445-453.
18 Randomised trial of endarterectomy for recently symptomatic carotid stenosis: final results of the MRC European Carotid Surgery Trial (ECST). Lancet. 1998;351:1379-1387.

19 Barnett HJM, Taylor DW, Eliasziw M, Fox AJ, Ferguson GG, Haynes RB, Rankin RN, Clagett GP, Hachinski VC, Sackett DL, Thorpe KE, Meldrum HE, Spence JD, The North American Symptomatic Carotid Endarterectomy Trial Collaborators. Benefit of Carotid Endarterectomy in Patients with Symptomatic Moderate or Severe Stenosis. N Engl J Med. 1998;339:1415-1425.
20 Halliday A, Mansfield A, Marro J, Peto C, Peto R, Potter J, Thomas D. Prevention of disabling and fatal strokes by successful carotid endarterectomy in patients without recent neurological symptoms: randomised controlled trial. Lancet. 2004;363:1491-1502.
21 Boven LA, Van Meurs M, Van Zwam M, Wierenga-Wolf A, Hintzen RQ, Boot RG, Aerts JM, Amor S, Nieuwenhuis EE, Laman JD. Myelin-laden macrophages are anti-inflammatory, consistent with foam cells in multiple sclerosis. Brain. 2006;129:517-526.
22 Redgrave TG, Roberts DCK, West CE. Separation of plasma lipoproteins by density-gradient ultracentrifugation. Analytical Biochemistry. 1975;65:42-49.
23 Van Berkel TJ, De Rijke YB, Kruijt JK. Different fate in vivo of oxidatively modified low density lipoprotein and acetylated low density lipoprotein in rats. Recognition by various scavenger receptors on Kupffer and endothelial liver cells. J Biol Chem. 1991;266:2282-2289.
24 Chayen J., Bitensky L. Analysis of chemical components of cells and tissues; reactions for lipids. Practical histochemistry. 1991 ed. New York: Chichester; 1991. p. 45.
25 Sluijter JPG, Pulskens WPC, Schoneveld AH, Velema E, Strijder CF, Moll F, De Vries JP, Verheijen J, Hanemaaijer R, De Kleijn DPV, Pasterkamp G. Matrix Metalloproteinase 2 Is Associated With Stable and Matrix Metalloproteinases 8 and 9 With Vulnerable Carotid Atherosclerotic Lesions: A Study in Human Endarterectomy Specimen Pointing to a Role for Different Extracellular Matrix Metalloproteinase Inducer Glycosylation Forms. Stroke. 2006;37:235-239.
26 Galis ZS, Sukhova GK, Lark MW, Libby P. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. J Clin Invest. 1994;94:2493-2503.
27 Tedgui A, Mallat Z. Cytokines in Atherosclerosis: Pathogenic and Regulatory Pathways. Physiol Rev. 2006;86:515-581.
28 Hasty KA, Jeffrey JJ, Hibbs MS, Welgus HG. The collagen substrate specificity of human neutrophil collagenase. J Biol Chem. 1987;262:10048-10052.
29 Glass CK, Witztum JL. Atherosclerosis: The Road Ahead. Cell. 2001;104:503-516.
30 Herman MP, Sukhova GK, Libby P, Gerdes N, Tang N, Horton DB, Kilbride M, Breitbart RE, Chun M, Schonbeck U. Expression of Neutrophil Collagenase (Matrix Metalloproteinase-8) in Human Atheroma: A Novel Collagenolytic Pathway Suggested by Transcriptional Profiling. Circulation. 2001;104:1899-1904.
31 Zwadlo G, Schlegel R, Sorg C. A monoclonal antibody to a subset of human monocytes found only in the peripheral blood and inflammatory tissues. J Immunol. 1986;137:512-518.
32 Donato R. Intracellular and extracellular roles of S100 proteins. Microsc Res Tech. 2003;60:540-551.
33 Lau W, Devery JM, Geczy CL. A chemotactic S100 peptide enhances scavenger receptor and Mac-1 expression and cholesteryl ester accumulation in murine peritoneal macrophages in vivo. J Clin Invest. 1995;95:1957-1965.
34 Miyamoto S, Ueda M, Ikemoto M, Naruko T, Itoh A, Tamaki Si, Nohara R, Terasaki F, Sasayama S, Fujita M. Increased serum levels and expression of S100A8/A9 complex in infiltrated neutrophils in atherosclerotic plaque of unstable angina. Heart. 2008;hrt.
35 Verhoeven B, Hellings WE, Moll FL, De Vries JP, De Kleijn DPV, de Bruin P, Busser E, Schoneveld AH, Pasterkamp G. Carotid atherosclerotic plaques in patients with transient ischemic attacks and stroke have unstable characteristics compared with plaques in asymptomatic and amaurosis fugax patients. Journal of Vascular Surgery. 2005;42:1075-1081.

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 -800C, 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, α-actin and CD68. Sections were studied semiquantitatively for macrophages (CD68), smooth muscle cells (α-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 α-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 agreement[2].

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

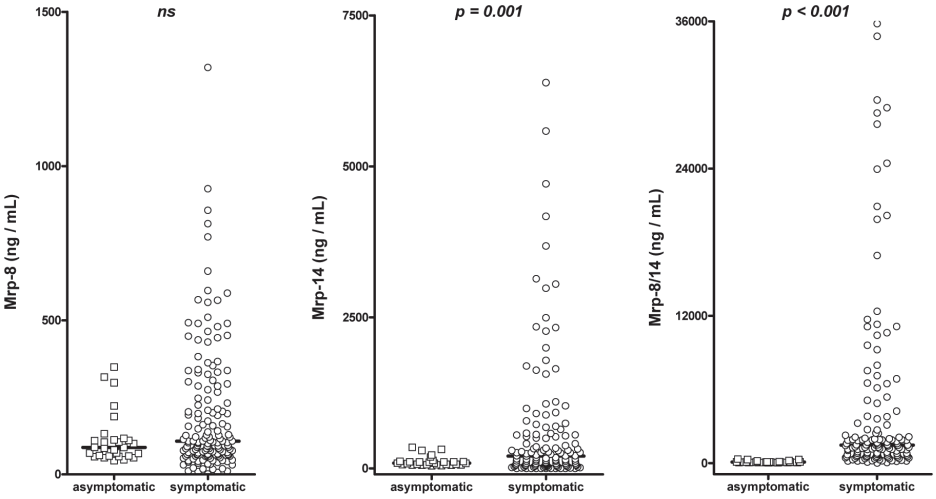


Figure 1. Distribution of Mrp-8, Mrp-14 and Mrp-8/14 levels in carotid plaques from symptomatic (n=154) and asymptomatic patients (n=32). Lines represent medians; statistics *Mann-Whitney* test, significance at $p<0.05$, *ns* = not significant.

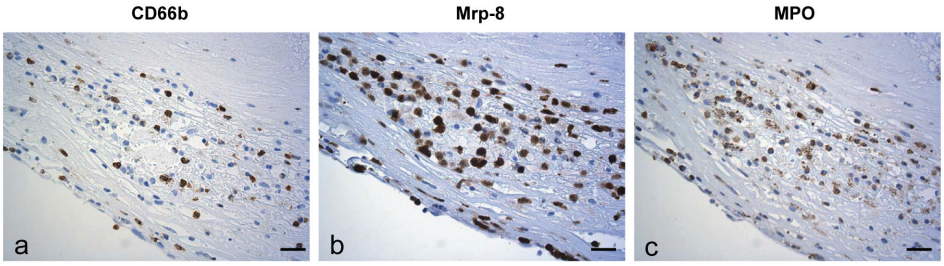


Figure 2. Consecutive sections of a rupture-prone atherosclerotic plaques showing Mrp-8 (b) and MPO (c) co-localization in CD66b-positive neutrophils (a). Bars represent 25 µm.

Table I. Clinical and histological plaque characteristics of the study population

	Number (N) N = 186	Percentage (%) 100 %
Clinical characteristics		
Age, mean	73 (48 - 93)	8.6 %
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

	Symptomatic	Asymptomatic
N=186	83 % (154)	17 % (32)
Clinical characteristics		
Age, years (mean)	71 (55-84)	74 (48-93)
Male	75 % (116)	65 % (21)
Current smoker	24 % (37)	21 % (7)
Diabetes	17 % (27)	19 % (6)
Hypercholesterolemia	56 % (86)	53 % (17)
Hypertension	64 % (98)	44 % (14)
Medication use		
Statin use	62 % (96)	47 % (15)
Aspirin use	33 % (51)	31 % (10)
ACE inhibitor	36 % (55)	19 % (6)
Oral anticoagulants	16 % (25)	9 % (3)
Corticosteroids	7 % (10)	0 % (0)
Plaque characteristics		
Large lipid core (>40%of plaque area)	33 % (50)	28 % (9)
Heavy macrophage staining	16 % (24)	16 % (5)
Heavy collagen staining	20 % (31)	19 % (6)
Heavy smooth muscle cell staining	18 % (28)	16 % (5)
Rupture-prone (overall phenotype)	59 % (92)	59 % (19)

Table III. Patient distribution per medication use (No or Yes) and Mrp plaque levels (Low versus High)

	Low	High	Low	High	Low	High
	N /Total (%)		N /Total (%)		N /Total (%)	
Statin use						
No	23/52(44)	29/52(56)	28/56(50)	28/56(50)	30/56(54)	26/56(46)
Yes	68/129(53)	61/129(47)	64/126(51)	62/126(49)	64/133(48)	69/133(52)
Aspirin use						
No	52/112(47)	60/112(53)	52/111(47)	59/111(53)	52/117(45)	65/117(55)
Yes	39/69(57)	30/69(43)	40/71(56)	31/71(44)	42/72(58)	30/72(42)
Corticosteroids use						
No	87/170(51)	83/170(49)	87/172(51)	85/172(49)	90/178(51)	88/178(49)
Yes	4/11(36)	7/11(64)	5/10(50)	5/10(50)	4/11(36)	7/11(64)

Table IV. Medication influence on Mrp plaque levels

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

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)

		Mrp-8		**Mrp-14**		**Mrp-8/14**	
		Low	High	Low	High	Low	High
Macrophages Low	IL-6	10 [4–15]	39 [8-70]	6.1 [3-9]	3 [11-76]	10 [3.5-16.7]	32 [6-59]
	IL-8	43 [23–64]	256 [106–405]	29 [16-42]	258 [109-407	53 [20-87]	241 [89-394]
	MMP-2	3.2 [2.6–3.8]	2.9 [2.4–3.5]	3.5 [2.9-4]	2.9 [2.1-3.7]	3.5 [2.8-4]	3 [2.3-3.6]
	MMP-8	5.2 [2.5–8]	5.2 [3.3-7]	5.8 [2.8-8.8]	6.7 [4.4-8.9]	4.4 [3-5.6]	8.2 [4-12]
	MMP-9	0.62 [0.4–0.8]	0.67 [0.5–0.8]	0.6 [0.4-0.8]	0.7 [0.6-0.9]	0.6 [0.4-0.8]	0.7 [0.5-0.8]
Macrophages High	IL-6	12 [6-18]	30 [7-53]	11 [4-18]	28 [9-48]	11 [5-17]	30 [8.5-52]
	IL-8	113 [48-178]	218 [156-281]	90 [13-166]	227 [159-296]	93 [34-151]	278 [197-358]
	MMP-2	3.6 [2.7-4.6]	3.8 [2.8-4.8]	4.1 [3-5]	3.7 [2.7-4.6]	3.7 [2.8-4.6]	3.8 [2.8-4.8]
	MMP-8	5 [2.5-7.6]	6.5 [4.7-8.2]	5.7 [2.5-8.8]	7.1 [5-9.3]	6 [3-9]	7 [5-9]
	MMP-9	0.6 [0.5-0.8]	0.8 [0.6-1]	0.7 [0.4-0.9]	0.8 [0.6-1]	0.6 [0.4-0.8]	0.9 [0.7-1]

Data shown as mean [95%confidence interval].

Reference List

1 Verhoeven B, Velema E, Schoneveld A, Vries J, de Bruin P, Seldenrijk C, Kleijn D, Busser E, der Graaf Y, Mol F, Pasterkamp G. Athero-express: Differential atherosclerotic plaque expression of mRNA and protein in relation to cardiovascular events and patient characteristics. Rationale and design. European Journal of Epidemiology. 2004;19:1127-1133.

2 Hellings WE, Pasterkamp G, Vollebregt A, Seldenrijk CA, De Vries JP, Velema E, De Kleijn DP, Moll FL. Intraobserver and interobserver variability and spatial differences in histologic examination of carotid endarterectomy specimens. J Vasc Surg. 2007;46:1147-1154.

CHAPTER IV

MRP-8/14 expression segregates with lipid uptake function and alternative activation (M2) features in atherosclerosis and multiple sclerosis

In preparation for publication

Mihaela G. Ionita*, I. Esmé Dijke*, Marjan van Meurs, Chaylendra Strijder, Gerard Pasterkamp, Dominique de Kleijn and Jon Laman

* These authors contributed equally to this work

Objective

High level of myeloid related protein (Mrp)-14 correlates with features of atherosclerotic plaque vulnerability, and mice deficient for Mrp-8/14 display reduced vascular injury and atherosclerosis (AS). In vitro, human Mrp-8/14 positive macrophages do not develop into foam cells upon oxLDL ingestion, suggesting that functional macrophage subsets differentially contribute to plaque progression. We addressed the hypothesis that Mrp-8/14 expression segregates with lipid uptake function, and with functional macrophage subset properties within the paradigm of classical (M1) versus alternative activation (M2) extremes. We compared and opposed AS lesions and oxLDL uptake versus multiple sclerosis (MS) lesions and uptake of myelin.

Results

The data show that Mrp-8/14 expressing macrophages in both human AS and MS lesions are non-foam cells, which populate different lesion areas than the foam macrophages. Second, using a human in vitro model of foam cell development from healthy donor monocytes, Mrp-8/14 expressing macrophages do not ingest oxLDL or myelin and fail to develop foamy morphology. Mrp-8/14 expression on macrophages is associated with enhanced expression of scavenger receptors, CD206 (mannose receptor) and CD163 (haptoglobin receptor), features consistent with anti-inflammatory action and tissue repair. Functionally, Mrp-8/14 expressing cells exposed to lipids secrete a spectrum of anti-inflammatory cytokines, in contrast to the Mrp-8/14 lacking macrophages that do ingest lipids and subsequently secrete pro-inflammatory cytokines.

Conclusion

Mrp-8/14 expressing macrophages represent a functional macrophage subset which resembles the anti-inflammatory alternatively activated macrophages side of the M1-M2 spectrum in vivo and in vitro, with distinct lipid sources for AS versus MS.

Introduction

Atherosclerosis is the underlying cause of acute ischemic attacks (cardiac and cerebral), accounting for the majority of deaths among the elderly. It is a chronic inflammatory disease of major arteries, characterized by a continuous influx of blood circulating inflammatory cells (e.g. monocytes, neutrophils, and lymphocytes). The circulating monocytes are the precursors of plaque macrophages and foam macrophages, the most abundant cells in atherosclerotic lesions[1]. Compounds driving foam cell formation include oxidized LDL, fatty acids, and local cell debris. Macrophages and foam macrophages are also present in multiple sclerosis brain lesions where foam formation is induced in macrophages by ingestion of myelin-derived lipids[2].

Blood circulating monocytes constitutively express myeloid related protein (Mrp)-8 and Mrp-14 homodimers in the cytoplasm[3]. Mrp-8 (S100A8) and Mrp-14 (S100A9) are cytoplasmic calcium-binding proteins involved in monocyte metabolism[4]. These two proteins have a high affinity for each other in the presence of calcium and form the Mrp-8/14 heterodimer which is translocated to cell membrane and/or secreted[5]. In humans, a subset of monocytes expresses the Mrp-8/14 heterodimer on the cell surface (Mrp-8/14surface positive)6]. In healthy individuals, the percentage of Mrp-8/14surface positive monocytes accounts for 0-5% of all monocytes while in type 1 diabetics the Mrp-8/14 surface expression is significantly increased[7]. This subset of monocytes has an enhanced adhesive capability, at least in type 1 diabetic patients[8], and is the preferentially migrating subpopulation of monocytes through the macrovascular endothelium[9]. In addition, this subset expresses more CD11b (complement receptor 3) than the Mrp-8/14 cell surface-negative monocytes[10].

Mrp-expressing monocytes infiltrate into atherosclerotic plaques[11] where they differentiate into macrophages. Partly plaque macrophages express Mrp-8 and -14 while those from the lesion-free intima, considered tissue resident cells, lack Mrp-expression[12]. We have previously shown that Mrp-8 and -14 are expressed by a subset of plaque macrophages which are not foam cells [13]. In addition, foam macrophages generated in vitro lack Mrp-8/14 expression while the Mrp-8/14surface positive macrophages do not phagocyte oxLDL. Interestingly, the percentage of non-foam macrophages expressing Mrp-8 and Mrp-14 is higher in rupture-prone plaques than in stable ones. In addition, the protein levels of Mrp-8, -14 and -8/14 are elevated in these lesions compared to stable plaques[11].

Functional activities of intracellular, surface-bound and secreted Mrp-8/14 are incompletely known. Intriguingly, upon release from phagocytes, complexes of Mrp-8/14 can amplify LPS-induced inflammatory responses of phagocytes. Mrp-8 interacts with the TLR4-MD2

complex, and hence Mrp-8 and -14 are endogenous activators of TLR-4[14]. Interestingly, the same group provided evidence that Mrp-8/14 acting as TLR ligands promote the development of autoreactive cytotoxic CD8 T cells[15]. Mice deficient for Mrp-14 also lack Mrp-8/14 complexes. These mice have reduced vascular damage upon experimental arterial injury, vasculitis, and atherosclerosis (combined with ApoE deficiency). In a mouse model of focal cerebral ischemia, Mrp-8/14 deficiency reduces lesion volume, brain swelling, and macrophage number[16].

In the current study, we hypothesized that Mrp-8/14 expression is associated with reduced capability of lipid uptake and foam cell formation. In addition, we investigated whether functionality of Mrp-8/14 membrane positive macrophages conforms to functional subsets as known within the M1-M2 paradigm. The overly linear M1-M2 approach has now been superseded by more subtle and useful categories based on joint functional and phenotypical data[17, 18].

We used in situ analysis to assess differential localization of Mrp-8/14 expressing macrophages in human atherosclerosis (AS) and multiple sclerosis (MS) lesions. An in vitro model of foam cell development was used to analyze atherosclerotic oxLDL and multiple sclerosis myelin uptake by macrophages lacking and expressing Mrp-8/14surface as well as cytokine release and scavenger receptors. This showed that macrophages lacking Mrp-8/14surface phenotypically as well as functionally resemble the M1 (classically activated) cells while those macrophages expressing Mrp-8/14surface resemble the M2 (alternatively activated) cells. These findings add to our understanding of functional macrophage subsets in lesion development and vulnerability, and could add to current treatment strategies[19].

Methods

In situ analysis of human atherosclerotic plaques and multiple sclerosis lesions

Ninety carotid plaques were carefully dissected from the carotid arteries and immediately transferred to the laboratory for further processing as described previously[20]. Plaque 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 Mrp-14 antibody (mouse IgG1, dilution 1:200; Santa-Cruz Biotechnologies). 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.

MS brain tissues were obtained from The Netherlands Brain Bank (NBB), Netherlands Institute for Neuroscience, Amsterdam. All material was collected from donors from whom a written informed consent for brain autopsy and the use of the material and clinical information for research purposes had been obtained by the NBB. MS lesions from three different human brains were snap frozen in liquid nitrogen and processed as described here: consecutive sections were stained with mouse anti-human Mrp-8 (mouse IgG2b, dilution 1: 80; Santa-Cruz Biotechnologies, Santa-Cruz, CA) and with mouse anti-human Mrp-14 antibody (mouse IgG1, dilution 1:80; Santa-Cruz Biotechnologies) monoclonal antibodies. Rabbit poly HRP- anti-mouse (Dako, Denmark) was used as secondary antibody. The signal was visualized using 3-amino-9-ethylcarbazole (AEC). Sections were counterstained with hematoxylin.

In vitro generation of human oxLDL-laden macrophages

Human monocyte-derived macrophages (MDM) were generated as previously described [21, monocytes were isolated from anonymous healthy blood donor buffy coats (at least n = 3 per experiment) 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 non-adherent conditions in RPMI 1640 medium (BioWhittaker, Verviers, Belgium) supplemented with 25nM Hepes, Ultraglutamin1 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 two 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, Roskilde, Denmark).The macrophages were incubated during 24 h with 10 µg/ml human oxLDL, isolated and oxidized as described previously[22, 23] ; during 24 – 48 h with 50 µg/ml non-labeled or DiI labeled human myelin, isolated and labeled as described previously, or with culture medium as control. Uptake of OxLDL and non-labeled myelin was detected with Oil-Red-O (ORO), which stains neutral lipids, as previously described[24]. Uptake of DiI-labeled was detected by flow cytometry, described below.

Flow cytometry and fluorescence-activated cell sorting

Fluorescence-activated cell sorting on a FACS Aria (BD Biosciences, San Jose, CA) was performed to separate 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 B.V., Huissen, the Netherlands). Mrp-8/14 FITC (27E10) (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-APC (R&D Systems); Mrp-8/14-FITC (clone 27E10), Mrp-8-FITC (clone CF145) and Mrp-14-FITC (clone MRP1H9) (all from Santa Cruz); CD36-RPE; HLA-DR-APC_Cy7; CD80-PE_Cy5; CD163; and CD206 (all from BD Biosciences). The samples were measured on an LSR II (BD Biosciences) and analyzed using FACS Diva version 6.1.1 (BD Biosciences) and Flow Jo Version 7.6.1 (Tree Star Inc., Ashland, OR) software.

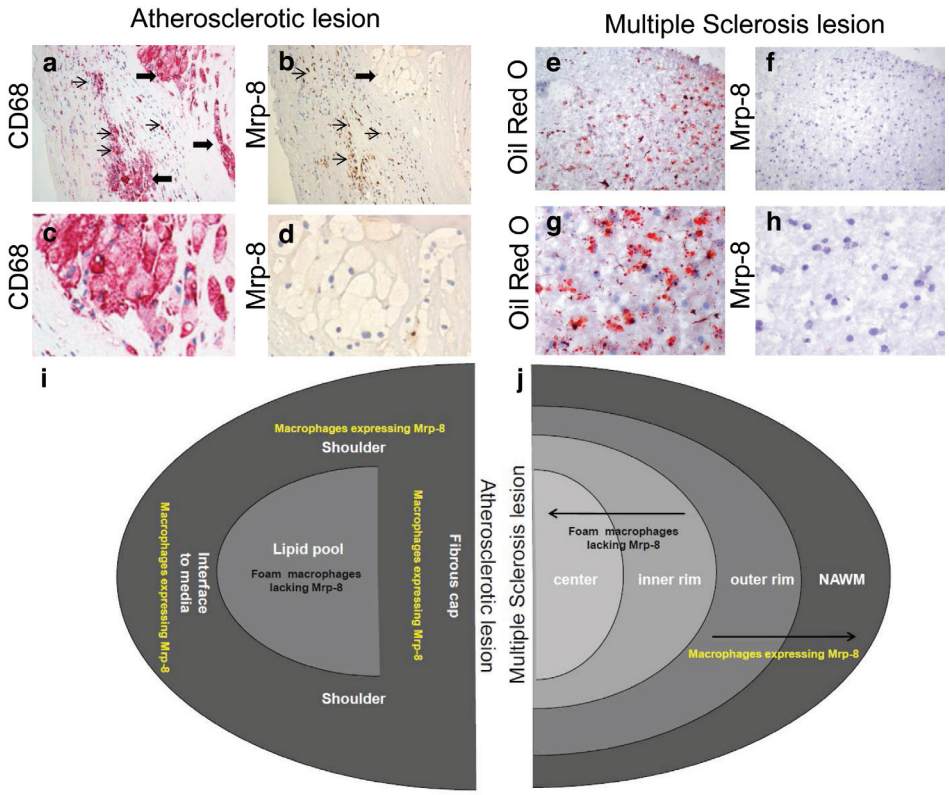


Figure 1. Foam cell morphology and Mrp-8/14 expression are mutually exclusive in AS plaques and MS lesions. Consecutive sections of human carotid AS plaque (a-d) and MS lesion (e-h) showing foam macrophages lacking Mrp-8 expression; (a) Cd68 expression in plaque macrophages, foam (thick arrows) and non-foam (thin arrows); (b) Mrp-8 expression (in brown) in a group of non-foam plaque macrophages (thin arrows); foam macrophages (thick arrows) lack Mrp-8, shown at higher magnification in c and d; (e) Oil red O positive foam macrophages indicative of neutral lipid content; (f) consecutive section showing the same foam macrophages lacking Mrp-8 expression; (g and h) magnifications of e and f, respectively; (i) schematic representation of different plaque regions with localization of Mrp-8 expressing macrophages; (j) schematic representation of different regions of an MS lesion25 showing localization of Mrp-8 expressing macrophages; NAWM = normal appearing white matter.

Cytokine measurements

Conditioned media (from three different donors) of plated (12-well flat-bottom culture plates; Nunc, Roskilde, Denmark) Mrp-8/14surface negative and positive sorted MDMs was collected 24 h after cells were fed with oxLDL or myelin. The conditioned media (0.5 ml/condition) were loaded onto a human cytokine antibody array V (RayBio) designed to detect 80 different human cytokines; the assay was performed according to the manufacturer's protocol. MDM culture media was used as negative control. The intensity of the signals was detected with a Molecular Imager ChemiDocTM XRS+ system (Bio-Rad). The MDMs culture media was negative. Positive control (present on each membrane, by manufacturer) was used to normalize for the variation between the membranes. Cytokine levels were quantified using Image Lab software (Bio-Rad) and are reported as pixel intensity/mm2.

Data analysis

Data were analyzed with SPSS 17.0. The statistical significance of the difference between two groups was determined using Mann-Whitney test; probability values <0.05 were considered significant. The flow cytometry data of the markers CD68, CD36, CD80, CD163, CD206, CD80, CD11b, CD16 and MPO derived from 3 different experiments and shown in figure 3, has been corrected for the variance between experiments. For each marker, the X-median fluorescence of unstained cells in one experiment was considered as standard. The X-median fluorescence values of the other 2 experiments were divided by the standard value resulting in a correction factor for each marker. The X-median fluorescence of Mrp-8/14surface positive and negative cells of each marker was then corrected according to the correction value.

Results

Foam macrophages within human AS plaques and MS lesions lack Mrp-expression

Both AS plaques and MS lesions are characterized by presence of macrophage foam cells. This allows a compare and contrast approach for Mrp-8/14 expression and function, since the foam cell lipid source in AS is thought to be mainly oxLDL and fatty acids, while in MS the lipid source is mainly myelin, and perhaps cell debris of leukocytes and inflamed brain tissue. In situ analysis of human carotid plaque specimens revealed that Mrp-8 and Mrp-14 co-localize in cells within plaques as expected and shown before[10]. Conversely, the CD68-positive macrophages with a foam-cell morphology lack Mrp-expression. In contrast, the CD68-positive non-foam macrophages express both Mrp-8 (figure 1a-d) and Mrp-14 (data not shown). The Mrp-8 expressing macrophages were observed in all plaque regions, with prevalence in the fibrous and shoulder areas (figure 1i).

Similarly, foam macrophages within human MS lesions are Mrp-8 and Mrp-14 negative (figure 1 e-h) while the Mrp-positive macrophages are non-foam cells (not shown). In these lesions, the Mrp-8 expressing macrophages were seen within the normal appearing white matter (NAWM) and pre-active areas (outer rim in figure 1j) and not in areas of demyelination which are abundantly populated by foam macrophages (inner rim and center in figure 1j).

Surface expression of Mrp-8/14 on Monocyte Derived Macrophages associates with a characteristic receptor repertoire

Since Mrp-8/14 expression and foam cell formation are mutually exclusive in AS and MS lesions, we reasoned that the surface receptor repertoire would differ. To experimentally model that under controlled conditions, buffy coat monocytes from healthy blood bank donors were employed. Monocytes were identified based on their high expression of surface CD14.

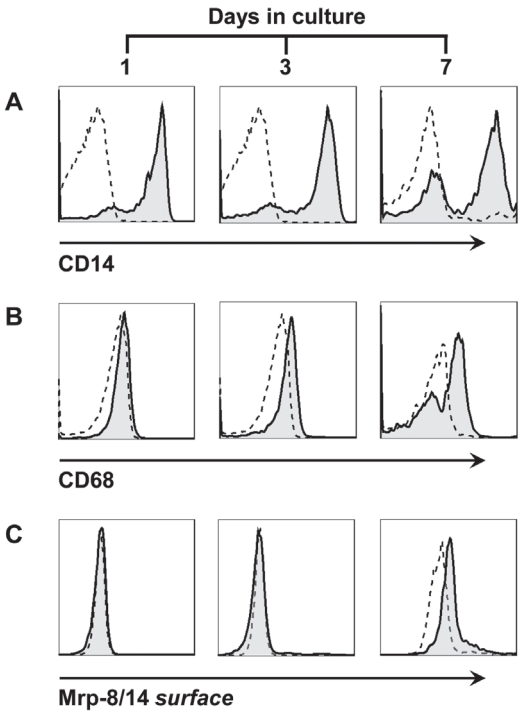


Figure 2. Monocytes from healthy human donors were cultured for 7 days under non-adherent conditions and without further cytokine stimulation; one representative example of three experiments is shown. (A) Surface CD14, (B) intracellular CD68 and (C) surface Mrp-8/14 expression on monocytes (culture days 1 and 3) and monocyte-derived macrophages (day 7) is given. Dashed histogram: unlabeled control cells; colored histogram: the population after staining.

In addition, these cells had a low expression of intracellular CD68. After 7 days of culture under non-adherent conditions and without further cytokine stimulation, the surface expression of CD14 was decreased (figure 2A) and the intracellular expression of CD68 was increased (figure 2B) which is conform to the predicted phenotype of MDM. At day 7, on average 25% (ranging from 20 to 40%) of the cultured MDM were Mrp-8/14surface positive while the rest were negative (figure 2C). Intracellularly, on average 45% of the MDM were Mrp-8/14 positive, 70% were Mrp-8 positive and 60% were Mrp-14 positive (data not shown). To gain more insight into the phenotype of the Mrp-8/14surface positive and negative macrophages, we analyzed the expression of a selection of markers differentially expressed on functional macrophage subsets: CD11b, CD16, MPO (myeloperoxidase), CD80, MHC-II and the scavenger receptors CD36, CD68, CD163 and CD206 (also known as the mannose receptor). All MDM showed surface CD11b and intracellular MPO expression as expected. The expression of these two markers was markedly higher on the Mrp-8/14surface positive cells than on the negative ones: on average, 1.7 fold higher CD11bsurface and 2.2 fold higher intracellular MPO expression (figure 3). Mrp-8/14surface positive MDMs had 1.8 fold higher CD80 cell membrane expression compared to the Mrp-8/14surface negative population (figure 3).

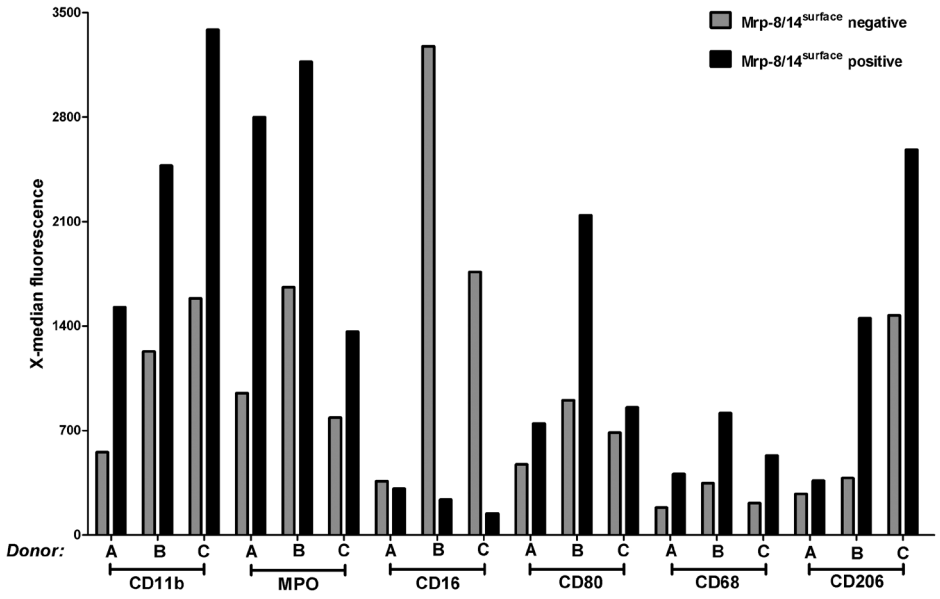


Figure 3. Expression of cell surface CD11b, CD16, CD80, CD68 and CD206 and intracellular MPO on unsorted monocyte-derived macrophages at day 7 of in vitro culture without addition of growth factors or lipid exposure. Three different donors are shown.

Interestingly, the frequency of MDM expressing surface CD16 was higher in the Mrp-8/14surface negative population than in the positive one: on average 59% (ranging from 46% to 67%) of the Mrp-8/14surface negative MDM were CD16 positive in contrast to 43% (ranging from 37% to 51%) of the Mrp-8/14surface positive MDMs ($p = 0.03$). Moreover, within the Mrp-8/14surface negative MDM expressing CD16 (59%), 78% showed a high CD16 expression while the rest had an intermediate expression. In contrast, from the Mrp-8/14surface positive MDM expressing CD16 (43%), only 20% showed a high CD16 expression, and the vast majority of the cells showed an intermediate expression. We have previously shown that human MDM expressing surface bound Mrp-8/14 do not take up oxLDL in vitro, and therefore do not acquire a foamy phenotype26.

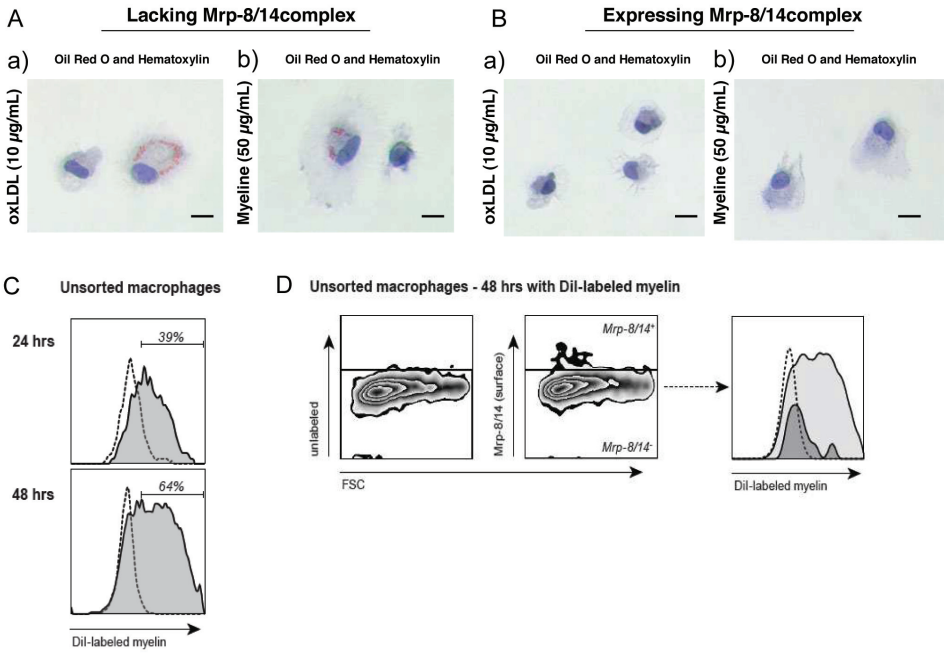


Figure 4. Sorted (A and B) and unsorted (C and D) Mrp-8/14surface positive and negative monocyte-derived macrophages (MDMs) were fed with human oxLDL and labeled or unlabeled human myelin for 24 and 48hours. One representative example of three experiments is shown. (A) Sorted MDMs lacking surface Mrp-8/14 did uptake oxLDL (a) and myelin (b) after 24 h. (B) Sorted MDMs expressing surface Mrp-8/14 did not uptake oxLDL (a) and myelin (b) after 24 h. (C) Flow-cytometry histograms showing that the frequency of macrophages that ingested DiI-labeled myelin increased with time; the percentage represents the frequency of macrophages that has taken up DiI-labeled myelin (background is subtracted). (D): In contrast to Mrp-8/14surface negative cells, the vast majority of the Mrp-8/14surface positive cells did not take up DiI-labeled myelin. Dashed histogram: control cells; colored histogram: cells fed with 10µg/ml DiI-labeled myelin →light grey: Mrp-8/14surface negative cells; dark grey: Mrp-8/14surface positive cells; in red, Oil Red O staining visualizes accumulation of neutral lipids in the cytoplasm; in blue, hematoxylin staining of the cell nucleus.

Here, we investigated whether the expression of the lipid scavenger receptors CD36 and CD68, as well as receptors CD206 and CD163, and CD11b, MHC-II plus CD80 for antigen presentation/costimulation, differed between the Mrp-8/14surface positive and negative MDM, before feeding them with oxLDL or myelin. The expression of CD36 was similar on both MDM populations (data not shown). The expression of CD68surface was 2.4 fold higher on the Mrp-8/14surface positive cells than on the negative ones (figure 3). In addition, the Mrp-8/14surface positive MDM had 1.9 fold higher CD206 membrane expression (figure 3) and a slightly higher CD163 (not shown) membrane expression compared to the Mrp-8/14surface negative population. Both populations showed similar MHC-II membrane expression (data not shown).

	Lacking Mrp-8/14surface			Expressing Mrp-8/14surface		
	Donor 1	*Donor 2*	*Donor 3*	*Donor 1*	*Donor 2*	*Donor 3*
CYTOKINE *						
GRO / CXCL-1,-2,-3	17771.4	13944.0	6888.5	7173.1	1618.1	9752.5
IL-1α	811.9	2574.7	0.0	0.0	487.5	0.0
IL-6	7182.3	9015.5	3158.2	3861.9	1639.4	4989.3
IL-8 / CXCL-8	133625.2	47367.3	114180.1	59154.4	5078.0	200290.6
MIP-1δ	3037.0	2281.0	2069.5	2239.8	2014.3	0.0
SDF-1 / CXCL-12	3301.0	4058.9	1503.3	918.2	1373.0	1681.4
VEGF (165&121)	3481.6	0.0	5099.6	2931.7	0.0	3350.1
EOTAXIN / CCL-1	2498.4	3005.4	0.0	556.3	961.8	1668.0
MCP-4 / CCL-13	475.0	1563.9	1834.6	278.7	140.8	0.0
NAP-2N / CXCL-7	40596.7	21902.0	32796.7	10726.9	19860.5	39045.2
PARC / CCL-18	3432.8	1175.6	2701.6	1361.1	2217.6	1498.4
IL-5	2721.8	0.0	539.4	1737.4	241.0	3176.1
IL-10	3405.5	1433.7	2551.2	1088.2	5042.6	10650.5
IL-12p40	2248.7	0.0	726.7	1679.9	538.2	1518.3
IFN-γ	3628.3	0.0	2561.1	3838.9	1925.1	5332.4
MCP-2 / CCL-8	612.1	0.0	1046.3	1590.7	1318.9	4181.1
MCP-3 / CCL-7	0.0	464.3	59.2	581.1	1141.5	1634.6
RANTES / CCL-5	7396.5	5953.2	9501.3	14092.3	7740.2	8497.9
TARC	2999.2	1505.7	4713.6	3316.3	3466.0	4065.5
TGF-β1 (active)	0.0	0.0	1377.6	423.2	1695.5	0.0
OSTEOPONTIN	0.0	3790.1	1827.5	3601.3	3577.7	3271.0
PIGF	5482.6	3138.8	7919.5	6229.5	5823.8	8487.1
TIMP-2	3106.5	264.9	1684.9	154.8	3779.3	3075.7

Figure 5. Cytokines secreted by MDMs lacking and expressing Mrp-8/14surface, after feeding oxLDL (10 µg/ml) for 24 h. Three individual donors are shown.*Cytokine levels in pixel intensity/mm2; white = no difference; green = low expression (ratio of mean lacking / mean expressing < 0.6); red = high expression (ratio of mean lacking/mean expressing > 1.4).

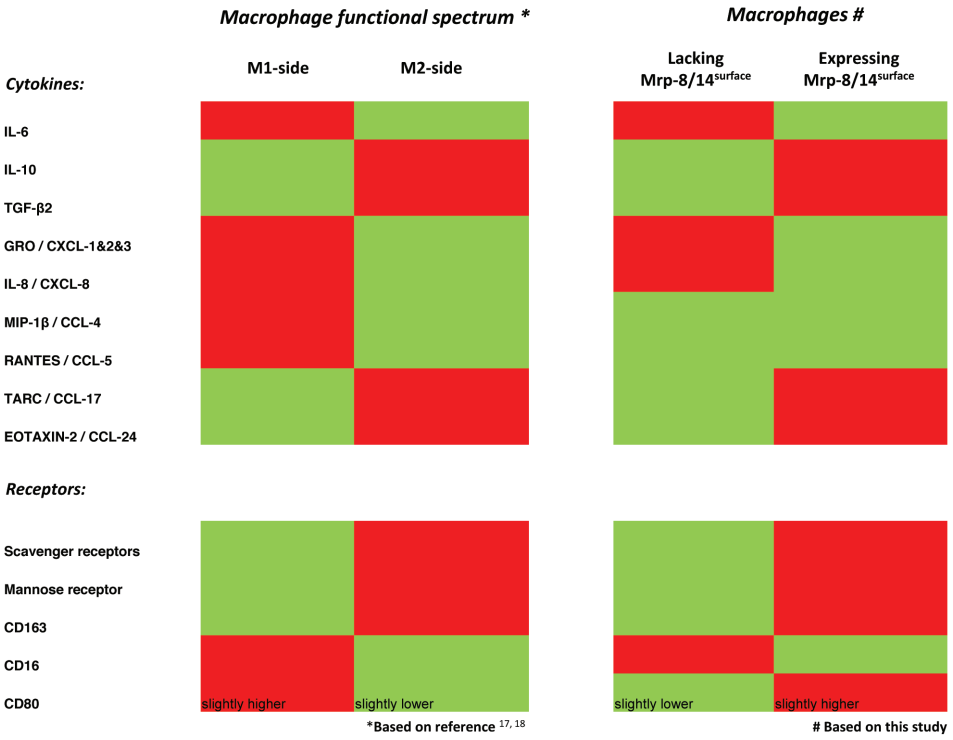


Figure 6. Similarities between M1-M2 functional extremes of the human macrophage spectrum and macrophages lacking or expressing Mrp-8/14surface.

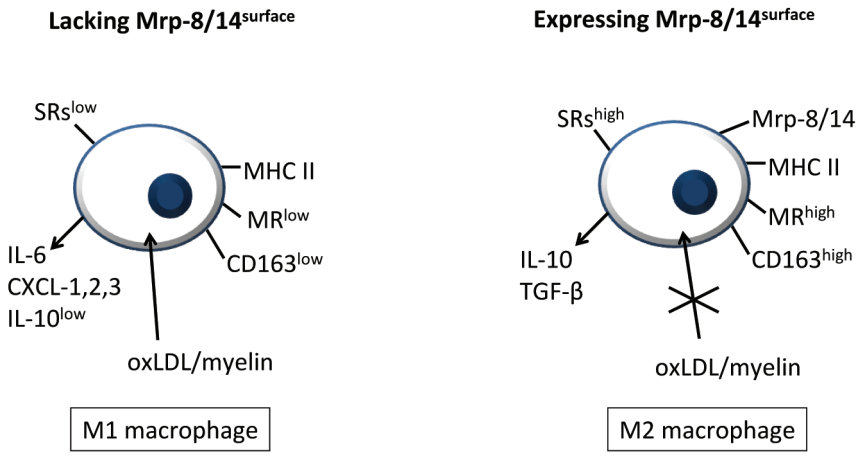


Figure 7. Proposed phenotype and function of the Mrp-8/14surface negative and positive macrophages in atherosclerosis and multiple sclerosis.

Mrp-8/14surface positive MDM do not take up/ingest oxLDL and myelin

In AS and MS lesions, foam cells do not express Mrp-8/14. To analyze whether this reflects a true lack of uptake capability, the ingestion of lipids by the Mrp-8/14surface positive and negative MDM, we sorted the positive and negative population and cultured these cells for 24 and 48 h under adherent conditions with human oxLDL (10µg/ml) and human myelin (50µg/ml). Mrp-8/14surface negative MDM ingested oxLDL in vitro (figure 4Aa). In contrast, Mrp-8/14surface positive MDMs did not take up oxLDL (figure 4Ba).
Culturing unsorted MDMs with DiI-labeled myelin (10µg/ml) for 24 and 48 h revealed that the percentage of MDM that ingested myelin increased with time (figure 4C). Moreover, the DiI-labeled myelin was predominantly ingested by Mrp-8/14surface negative cells; the vast majority of Mrp-8/14surface positive cells did not take up myelin (figure 4D).

The same effect was seen when sorted Mrp-8/14surface positive and negative MDMs were fed with non-labeled myelin (50 µg/ml) for 24 h: MDMs lacking surface Mrp-8/14 did ingest myelin (figure 4Ab) while those expressing Mrp-8/14 did not (figure 4Bb). Interestingly, unlike the lipids oxLDL and myelin, the uptake of the sugar dextran was not hampered in the Mrp-8/14surface positive cell population (data not shown).
Mrp-8/14surface positive MDM secrete anti-inflammatory cytokines, upon ingestion of oxLDL and myelin
Next, we asked if MDMs lacking and expressing Mrp-8/14surface secrete different cytokine profiles consistent with known macrophage subset functions, after feeding them two distinct lipid preparations.
Sorted MDM lacking and expressing Mrp-8/14surface were plated and fed for 24 h with human oxLDL (10 µg/ml) and human myelin (50 µg/ml), after which the cell-conditioned media were collected and used for cytokine semi-quantification.

Discussion

Foam cell formation is a characteristic of both AS and MS. Atherosclerotic plaques are abundantly populated by foam macrophages, mainly derived from infiltrating blood monocytes which ingest and accumulate lipids (e.g. oxLDL, fatty acids) present in the vessel intima. In MS lesions, infiltrating monocytes and local microglia accumulate myelin debris rich in oligodendrocyte membrane lipids and transform into foam macrophages [27]. It is of note that the size and structure of the lipid sources are different in AS versus MS: while oxLDL are relatively small particles, myelin debris actually consists of large particles maintaining the lamellar structure of myelin sheath, originally consisting of live multiple membrane windings by oligodendrocytes. In vitro, foam macrophages appear to have

mainly an anti-inflammatory phenotype: TNF production is diminished in TNF stimulated lipid-laden macrophages[28]; LDL uptake by macrophages induces IL-10 production[29]; myelin-laden macrophages produce, among others, anti-inflammatory cytokines IL-10, TGF-beta and CCL-18[30].

Diversity and plasticity of macrophages are since long acknowledged. Macrophages can express a continuous spectrum of activation/deactivation programs in response to the different microenvironments they encounter during their life-span[19]. It is now well understood that the M1-M2 paradigm based on in vitro differentiation is overly linear and more nuance is required for the definition of functional subsets. Definition solely by surface phenotyping is generally not sufficient, while functional characterization is hampered by differences in experimental conditions, such as the concentration and degree of LDL oxidation. In atherosclerosis, macrophages are heterogeneous in terms of tissue factor[31], CCL-18[32], MPO[33] and Mrp-8 and Mrp-14[26, 34].

In atherosclerotic plaques as well as in active multiple sclerosis lesions, the infiltrated macrophages express Mrp-8 and Mrp-14[35, 36]. Previously, we have shown that Mrp-8 and Mrp-14 are expressed by a subset of non-foam macrophages within atherosclerotic plaques, while plaque foam macrophages lack Mrp-expression[26]. Furthermore, in vitro MDM expressing membrane surface Mrp-8/14 do not take up oxLDL and do not acquire a foamy phenotype; in contrast, MDM lacking membrane surface Mrp-8/14 do. In the current study, we confirmed and extended these observations and provide further evidence that Mrp-8/14surface expressing macrophages do not phagocytes human modified lipids (i.e. oxLDL and myelin) in vitro. We demonstrated this with two different methodologies: first by sorting the Mrp-8/14surface expressing macrophages from the population lacking Mrp-8/14surface and subsequently feeding them with oxLDL or myelin (figure 4A and B). The second method entailed plating the mixed population of macrophages (expressing and lacking Mrp-8/14surface) with myelin and subsequent flow cytometry confirmation of myelin uptake by the macrophages lacking Mrp-8/14surface and not by those expressing this marker (figure 4D).

The expectation was that the Mrp-8/14surface expressing macrophages would have a diminished expression of scavenger receptors, explaining that these macrophages do not take up oxLDL. In contrast, we show that the expression of CD36, a common class B scavenger receptor for oxLDL on macrophages[37] is similar on macrophages lacking or expressing Mrp-8/14surface. Another receptor implicated in oxLDL scavenging and uptake is surface CD68[38, 39], a class D receptor[37]. Mrp-8/14surface expressing macrophages showed a higher CD68 surface expression than the ones lacking Mrp-8/14surface. Thus, unexpectedly, the macrophages expressing Mrp-8/14surface showed similar or higher expression of lipid scavenger receptors compared to the ones lacking it, although the former do not take up / ingest lipids. The overall phagocytic capacity of the Mrp-8/14surface expressing macrophages was not hampered, since these cells ingested the sugar dextran

to comparable extent (data not shown). Therefore, the macrophages expressing surface Mrp-8/14 have an altered phagocyte capacity for lipids. Two other receptors, the scavenger receptor CD163 (hemoglobin scavenger receptor) and the mannose receptor CD206 were higher expressed on the surface of macrophages with membrane Mrp-8/14 than on those lacking the complex. Whether Mrp-8/14 is involved in the mechanisms leading to an enhanced expression of the aforementioned receptors on macrophages' surface or whether it interacts with these receptors, is currently under investigation using silencing RNA approaches.

It was previously shown that Mrp-8/14surface positive monocytes have higher CD11b expression than the negative population[40]. Similarly, in this study the expression of CD11b was higher on macrophages expressing Mrp-8/14surface than on those without (figure 3). CD11b mediates leukocyte adhesion and migration and is involved in immune processes such as cellular activation, chemotaxis, and cell-mediated cytotoxicity[41]. Therefore, the macrophages carrying membrane Mrp-8/14 and high CD11b potentially have an enhanced migration capability.

Macrophages expressing Mrp-8/14surface also had high intracellular MPO expression. MPO is a pro-oxidant enzyme which has been found in macrophages within plaques, where it might play a role in pathogenesis of atherosclerosis by secreting the reactive oxygen species hypochlorous acid (HOCl)[42]; HOCl activates the pro-forms of the matrix metalloproteinases and inactivates the inhibitors of the metalloproteinases, thereby stimulating extracellular matrix degradation in two ways[33]. Moreover, this differential MPO expression is in line with the heterogeneity of macrophages in human atherosclerotic lesions where MPO-positive and MPO-negative subsets coexist[33]. Also in human blood, two distinct monocyte populations exist with high MPO versus low MPO monocytes, in circulating human blood[33]. Consistent with these observations, we generated monocyte-derived macrophages with low and high MPO. Interestingly, the macrophages expressing surface Mrp-8/14 were MPO high suggesting that these cells may be involved in tissue degradation. This can prove relevant for atherosclerosis, considering that the proportion of macrophages expressing Mrp-8 and Mrp-14 is higher in rupture-prone plaques than in stable ones[26].

The binding of soluble Mrp-8/14 to TLR-4 on phagocytes induces the activation of a positive feedback loop resulting in production of cytokines, chemokines and reactive oxygen species (ROS)[43]. Here we show that Mrp-8/14surface expressing macrophages, in the presence of oxLDL and myelin, secrete anti-inflammatory cytokines (e.g. IL-10 and TGF-β) compared to macrophages lacking Mrp-8/14surface expression which secrete pro-inflammatory cytokines (e.g. IL-6).

Coupling this evidence with the fact that the Mrp-8/14surface expressing macrophages are CD206 and CD163 high, we propose that the Mrp-8/14surface expressing cells are a subset of alternatively activated macrophages (also known as the M2- macrophages).

In comparison with the interferon-γ-mediated classical activation of macrophages in vitro, the alternative pathway of macrophage activation is prototypically induced by the T helper 2 (Th2) cytokines IL-4 and IL-13 in vitro[44]. Typically, M1- macrophages express opsonic receptors such as CD16 and produce high levels of the pro-inflammatory cytokines IL-6, IL-8, RANTES, GRO (CXCL1, 2 and 3) and CCL-4. In contrast M2-macrophages express abundant levels of non-opsonic receptors (e.g. mannose receptor) and scavenger receptors and they make high levels of the anti-inflammatory cytokines IL-10 and TGF-β[45, 46]. Macrophages lacking or expressing surface Mrp-8/14 follow the M1 – M2 dichotomy in terms of expressed receptors and secreted cytokines: cells lacking Mrp-8/14surface show similarities with the M1-macrophages while those expressing Mrp-8/14surface with the M2-macrophages (summarized in figure 6).

In human carotid atherosclerotic plaques, both classically and alternatively activated macrophage subsets have been described, but these two macrophage types display a different tissue distribution. The alternatively activated macrophages are found distant from the lipid core and are not foam cells but are CD68-non foam plaque macrophages [47]. Similarly we showed that in human carotid plaques, Mrp-8 and Mrp-14 are expressed by CD68-non foam plaque macrophages26. It is not known whether the alternatively activated macrophages identified in human carotid plaques by Bouhlel and colleagues[47] express Mrp-8 and Mrp-14. In conclusion, we provide functional and pathologic evidence underlining macrophage subset heterogeneity in AS and MS, since we identified two distinct macrophage populations in these lesions: expressing and lacking surface Mrp-8/14. Based on the in vivo and in vitro observations presented here, we propose the following model (see figure 7): in AS plaques and MS lesions, macrophage subsets expressing and lacking surface Mrp-8/14 coexist. The population lacking Mrp-8/14surface effectively ingests modified lipids leading to foam cell formation and secretion of pro-inflammatory cytokines in these cells; the cells expressing Mrp-8/14surface do not phagocytes lipids leading to foam cell formation, have an enhanced tissue destruction capability facilitates their migration in the tissue, and secrete anti-inflammatory cytokines. The Mrp-8/14surface expressing macrophages may be functionally involved in the resolution of the inflammatory reaction generated by the macrophages lacking surface Mrp-8/14.

Acknowledgments

This work was supported by an EC FP6 grant to the Immunath consortium (037400). The authors acknowledge discussions within the EC COST Action BM0603 Inflammation in Brain Disease Neurinfnet, and networking support from COST. The authors thank Dr. Pieter Leenen for valuable discussions and critical reading of the manuscript.

Reference List

1 Woollard KJ, Geissmann F. Monocytes in atherosclerosis: subsets and functions. Nat Rev Cardiol 2010 February;7(2):77-86.

2 Boven LA, Van Meurs M, Van Zwam M, Wierenga-Wolf A, Hintzen RQ, Boot RG, Aerts JM, Amor S, Nieuwenhuis EE, Laman JD. Myelin-laden macrophages are anti-inflammatory, consistent with foam cells in multiple sclerosis. Brain 2006 February 1;129(2):517-26.

3 Hessian PA, Edgeworth J, Hogg N. MRP-8 and MRP-14, two abundant Ca(2+)-binding proteins of neutrophils and monocytes. J Leukoc Biol 1993 February;53(2):197-204.

4 Donato R. Intracellular and extracellular roles of S100 proteins. Microsc Res Tech 2003 April 15;60(6):540-51.

5 Nacken W, Roth J, Sorg C, Kerkhoff C. S100A9/S100A8: Myeloid representatives of the S100 protein family as prominent players in innate immunity. Microsc Res Tech 2003 April 15;60(6):569-80.

6 Zwadlo G, Schlegel R, Sorg C. A monoclonal antibody to a subset of human monocytes found only in the peripheral blood and inflammatory tissues. J Immunol 1986 July 15;137(2):512-8.

7 Bouma G, Coppens JM, Lam-Tse WK, Luini W, Sintnicolaas K, Levering WH, Sozzani S, Drexhage HA, Versnel MA. An increased MRP8/14 expression and adhesion, but a decreased migration towards proinflammatory chemokines of type 1 diabetes monocytes. Clin Exp Immunol 2005 September;141(3):509-17.

8 Bouma G, Coppens JM, Lam-Tse WK, Luini W, Sintnicolaas K, Levering WH, Sozzani S, Drexhage HA, Versnel MA. An increased MRP8/14 expression and adhesion, but a decreased migration towards proinflammatory chemokines of type 1 diabetes monocytes. Clin Exp Immunol 2005 September;141(3):509-17.

9 Nacken W, Roth J, Sorg C, Kerkhoff C. S100A9/S100A8: Myeloid representatives of the S100 protein family as prominent players in innate immunity. Microsc Res Tech 2003 April 15;60(6):569-80.

10 Eue I, Pietz B, Storck J, Klempt M, Sorg C. Transendothelial migration of 27E10+ human monocytes. Int Immunol 2000 November 1;12(11):1593-604.

11 Eue I, Langer C, Eckardstein A, Sorg C. Myeloid related protein (MRP) 14 expressing monocytes infiltrate atherosclerotic lesions of ApoE null mice. Atherosclerosis 2000 August;151(2):593-7.

12 McCormick MM, Rahimi F, Bobryshev YV, Gaus K, Zreiqat H, Cai H, Lord RSA, Geczy CL. S100A8 and S100A9 in Human Arterial Wall: IMPLICATIONS FOR ATHEROGENESIS. J Biol Chem 2005 December 16;280(50):41521-9.

13 Ionita MG, Vink A, Dijke IE, Laman JD, Peeters W, van der Kraak PH, Moll FL, De Vries JP, Pasterkamp G, De Kleijn DPV. High Levels of Myeloid-Related Protein 14 in Human Atherosclerotic Plaques Correlate With the Characteristics of Rupture-Prone Lesions. Arterioscler Thromb Vasc Biol 2009 August 1;29(8):1220-7.

14 Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MAD, Nacken W, Foell D, van der Poll T, Sorg C, Roth J. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat Med 2007 September;13(9):1042-9.

15 Loser K, Vogl T, Voskort M, Lueken A, Kupas V, Nacken W, Klenner L, Kuhn A, Foell D, Sorokin L, Luger TA, Roth J, Beissert S. The Toll-like receptor 4 ligands Mrp8 and Mrp14 are crucial in the development of autoreactive CD8+ T cells. Nat Med 2010 June;16(6):713-7.

16 Ziegler G, Prinz V, Albrecht MW, Harhausen D, Khojasteh U, Nacken W, Endres M, Dirnagl U, Nietfeld W, Trendelenburg G. Mrp-8 and -14 mediate CNS injury in focal cerebral ischemia. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease 2009 December;1792(12):1198-204.

17 Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. Immunity 2010 May 28;32(5):593-604.

18 Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nat Rev Immunol 2008 December;8(12):958-69.

19 Mantovani A, Garlanda C, Locati M. Macrophage diversity and polarization in atherosclerosis: a question of balance. Arterioscler Thromb Vasc Biol 2009 October;29(10):1419-23.

20 Verhoeven B, Velema E, Schoneveld A, Vries J, de Bruin P, Seldenrijk C, Kleijn D, Busser E, der Graaf Y, Mol F, Pasterkamp G. Athero-express: Differential atherosclerotic plaque expression of mRNA and protein in relation to cardiovascular events and patient characteristics. Rationale and design. European Journal of Epidemiology 2004 December 15;19(12):1127-33.

21 Boven LA, Van Meurs M, Van Zwam M, Wierenga-Wolf A, Hintzen RQ, Boot RG, Aerts JM, Amor S, Nieuwenhuis EE, Laman JD. Myelin-laden macrophages are anti-inflammatory, consistent with foam cells in multiple sclerosis. Brain 2006 February 1;129(2):517-26.
22 Redgrave TG, Roberts DCK, West CE. Separation of plasma lipoproteins by density-gradient ultracentrifugation. Analytical Biochemistry 1975 May 12;65(1-2):42-9.
23 Van Berkel TJ, De Rijke YB, Kruijt JK. Different fate in vivo of oxidatively modified low density lipoprotein and acetylated low density lipoprotein in rats. Recognition by various scavenger receptors on Kupffer and endothelial liver cells. J Biol Chem 1991 February 5;266(4):2282-9.
24 Chayen J., Bitensky L. Analysis of chemical components of cells and tissues; reactions for lipids. Practical histochemistry. 1991 ed. New York: Chichester; 1991. p. 45.
25 Boven LA, Van Meurs M, Van Zwam M, Wierenga-Wolf A, Hintzen RQ, Boot RG, Aerts JM, Amor S, Nieuwenhuis EE, Laman JD. Myelin-laden macrophages are anti-inflammatory, consistent with foam cells in multiple sclerosis. Brain 2006 February 1;129(2):517-26.
26 Ionita MG, Vink A, Dijke IE, Laman JD, Peeters W, van der Kraak PH, Moll FL, de Vries JP, Pasterkamp G, de Kleijn DPV. High Levels of Myeloid-Related Protein 14 in Human Atherosclerotic Plaques Correlate With the Characteristics of Rupture-Prone Lesions. Arterioscler Thromb Vasc Biol 2009 August 1;29(8):1220-7.
27 Li H, Cuzner ML, Newcombe J. Microglia-derived macrophages in early multiple sclerosis plaques. Neuropathol Appl Neurobiol 1996 June;22(3):207-15.
28 Ares MP, Stollenwerk M, Olsson A, Kallin B, Jovinge S, Nilsson J. Decreased inducibility of TNF expression in lipid-loaded macrophages. BMC Immunol 2002 October 6;3:13.
29 Varadhachary AS, Monestier M, Salgame P. Reciprocal induction of IL-10 and IL-12 from macrophages by low-density lipoprotein and its oxidized forms. Cell Immunol 2001 October 10;213(1):45-51.
30 Boven LA, Van Meurs M, Van Zwam M, Wierenga-Wolf A, Hintzen RQ, Boot RG, Aerts JM, Amor S, Nieuwenhuis EE, Laman JD. Myelin-laden macrophages are anti-inflammatory, consistent with foam cells in multiple sclerosis. Brain 2006 February 1;129(2):517-26.
31 Wilcox JN, Smith KM, Schwartz SM, Gordon D. Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. Proc Natl Acad Sci U S A 1989 April;86(8):2839-43.
32 Dollery CM, Owen CA, Sukhova GK, Krettek A, Shapiro SD, Libby P. Neutrophil elastase in human atherosclerotic plaques: production by macrophages. Circulation 2003 June 10;107(22):2829-36.
33 Sugiyama S, Okada Y, Sukhova GK, Virmani R, Heinecke JW, Libby P. Macrophage myeloperoxidase regulation by granulocyte macrophage colony-stimulating factor in human atherosclerosis and implications in acute coronary syndromes. Am J Pathol 2001 March;158(3):879-91.
34 McCormick MM, Rahimi F, Bobryshev YV, Gaus K, Zreiqat H, Cai H, Lord RSA, Geczy CL. S100A8 and S100A9 in Human Arterial Wall: IMPLICATIONS FOR ATHEROGENESIS. J Biol Chem 2005 December 16;280(50):41521-9.
35 McCormick MM, Rahimi F, Bobryshev YV, Gaus K, Zreiqat H, Cai H, Lord RSA, Geczy CL. S100A8 and S100A9 in Human Arterial Wall: IMPLICATIONS FOR ATHEROGENESIS. J Biol Chem 2005 December 16;280(50):41521-9.
36 Bruck W, Porada P, Poser S, Rieckmann P, Hanefeld F, Kretzschmar HA, Lassmann H. Monocyte/macrophage differentiation in early multiple sclerosis lesions. Ann Neurol 1995 November;38(5):788-96.
37 Moore KJ, Freeman MW. Scavenger receptors in atherosclerosis: beyond lipid uptake. Arterioscler Thromb Vasc Biol 2006 August;26(8):1702-11.
38 van der Kooij MA, von der Mark EM, Kruijt JK, van Velzen A, van Berkel TJC, Morand OH. Human MonocyteûDerived Macrophages Express an 184120-kD Ox-LDL Binding Protein With Strong Identity to CD68. Arterioscler Thromb Vasc Biol 1997 November 1;17(11):3107-16.
39 Ramprasad MP, Terpstra V, Kondratenko N, Quehenberger O, Steinberg D. Cell surface expression of mouse macrosialin and human CD68 and their role as macrophage receptors for oxidized low density lipoprotein. Proc Natl Acad Sci U S A 1996 December 10;93(25):14833-8.
40 Eue I, Pietz B, Storck J, Klempt M, Sorg C. Transendothelial migration of 27E10+ human monocytes. Int Immunol 2000 November 1;12(11):1593-604.
41 Solovjov DA, Pluskota E, Plow EF. Distinct roles for the alpha and beta subunits in the functions of integrin alphaMbeta2. J Biol Chem 2005 January 14;280(2):1336-45.

42 Hazell LJ, Arnold L, Flowers D, Waeg G, Malle E, Stocker R. Presence of hypochlorite-modified proteins in human atherosclerotic lesions. J Clin Invest 1996 March 15;97(6):1535-44.
43 Ehrchen JM, Sunderkotter C, Foell D, Vogl T, Roth J. The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. J Leukoc Biol 2009 September;86(3):557-66.
44 Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. Immunity 2010 May 28;32(5):593-604.
45 Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol 2004 December;25(12):677-86.
46 Stoger JL, Goossens P, de Winther MP. Macrophage heterogeneity: relevance and functional implications in atherosclerosis. Curr Vasc Pharmacol 2010 March;8(2):233-48.
47 Bouhlel MA, Derudas B, Rigamonti E, Dievart R, Brozek J, Haulon S, Zawadzki C, Jude B, Torpier G, Marx N, Staels B, Chinetti-Gbaguidi G. PPARgamma activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. Cell Metab 2007 August;6(2):137-43.

CHAPTER V

High neutrophil numbers in human carotid atherosclerotic plaques are associated with characteristics of rupture-prone lesions

Atherosclerosis, Thrombosis and Vascular Biology (ATVB). 2010 September 30

Mihaela G. Ionita, Pleunie Van Den Borne, Louise M. Catanzariti, Frans L. Moll, Jean-Paul P.M. De Vries, Ph.D., Gerard Pasterkamp, Aryan Vink And Dominique P.V. De Kleijn

Objective

Neutrophils are inflammatory cells with tissue destruction capabilities that have been found at the site of an atherosclerotic plaque rupture or erosion. Poor evidence exists for neutrophil infiltration in human carotid atherosclerotic plaques and its association with plaque morphology has not yet been described. We scored the number of plaque neutrophils and related it to plaque morphology and inflammatory status.

Methods and Results

A set of 355 human carotid plaques were stained for the neutrophil marker CD66b. High neutrophil numbers were found in plaques with a large lipid core, high macrophage numbers and low collagen amount and smooth muscle cell numbers. High neutrophil numbers were associated with high interleukin-8 ($p<0.001$), matrix metalloprotease (MMP)-8 ($p = 0.005$) and MMP-9 ($p<0.001$) plaque levels. High micro vessel density within plaques was correlated with high neutrophil numbers ($p=0.014$). In addition, low numbers of neutrophils were associated with female gender and use of β-blockers.

Conclusion

These results show for the first time that neutrophil-numbers are strongly associated with the histopathological features of rupture-prone atherosclerotic lesions and suggest a role for neutrophils in plaque destabilization.

INTRODUCTION

Chronic inflammation plays a key role in the pathogenesis and progression of atherosclerosis and later in the destabilization and rupture of an atherosclerotic plaque, leading to adverse cardiovascular events. The involvement of inflammatory cells such as macrophages and T cells in atherogenesis is well documented, while the neutrophil granulocytes, also present in atherosclerotic lesions are detected in much lower numbers.

Neutrophils have been observed at the site of plaque erosion or rupture in atherectomy specimens from patients with unstable angina and in autopsy samples from patients with acute myocardial infarction (AMI)[185, 186]. Histological analysis of plaques from cerebral arteries has shown that the expression of neutrophil elastase is increased in late-stage plaques[187]. Epidemiological studies have shown that neutrophil counts in peripheral blood positively correlate with coronary atherosclerotic risk[188] and the risk of AMI[189].

Mouse studies reveal accumulation of neutrophils in the luminal plaque region and adventitia of aortic plaques of mice that lack apolipoprotein E (ApoE)[190] as well as in lesions of LDL-receptor deficient mice[191]. Neutrophils exert most of their functions via preformed granule proteins, most of these proteins are found in atherosclerotic lesions (e.g. alarmins, human neutrophil peptides, elastase, cathepsin G, proteinase-3)[192]. Neutrophils, similar to monocytes, are phagocytic cells involved in innate immunity[193]. Next to pathogen recognition and destruction, neutrophils contribute to tissue damage by secreting enzymes such as myeloperoxidase (MPO), elastase, esterase and matrix metalloproteinase-9 (MMP-9)[194]. MPO and MMP-9 expressed in mouse and human atherosclerotic lesions[195], have been shown to have prognostic value in atherothrombosis [196, 197,198].

Although the presence of neutrophils in atherosclerotic specimens (carotid, coronary, femoral) has been previously reported, no study so far strictly and specifically investigated neutrophil localization within plaques, their association with plaque characteristics and clinical data. We hypothesized that the number of neutrophils per plaque correlates with the features of high risk, rupture-prone atherosclerotic lesions.

In this observational study, we determined the total number of neutrophils per plaque in a large human cohort, assessed their localization within plaques and the correlation with plaque characteristics. We also studied the association of neutrophils with gender and β-blocker therapy and report an association between high neutrophil plaque numbers and the features of the rupture-prone plaque. Next to this, low neutrophil numbers were associated with female gender and history of β-blocker medication. These results might imply a role for neutrophils in rupture-prone plaque development and bring evidence for *in vivo* β-blocker therapy induced neutrophil-dependent plaque stabilization.

Methods

Athero-Express Biobank

Athero-Express is an ongoing longitudinal cohort study, initiated in 2002 by two Dutch hospitals: the University Medical Center Utrecht and the St. Antonius Hospital in Nieuwegein [199] .

Plaque processing

All 355 carotid plaques used in this study, were carefully dissected from the carotid arteries and immediately transferred to the laboratory for further processing as described previously[199]. In short, in the laboratory the atherosclerotic fragments were dissected 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 adjacent segments were used for protein isolation.

Human carotid endarterectomy specimens

Patients included for CEA were asymptomatic (no clinical symptoms related to the carotid luminal stenosis > 75%, n = 46) and symptomatic (n = 309); with minor clinical presentations (i.e., transient ischemic attack, amaurosis fugax and retinal infarction; n = 234) or major presentations (i.e., stroke; n = 75).

(Immuno)histochemistry

Plaque segments were fixed in formalin and embedded in paraffin. Consecutive sections were stained with Hematoxylin & Eosin (H&E), Elastin von Gieson, Picrosirius red and α-actin, CD68 and CD34 immunostains[199, 200]. To determine the plaque phenotype, sections were scored as described previously[199].

To visualize neutrophils, consecutive sections were boiled in citrate buffer and stained with mouse anti-human CD66b (dilution 1:100; AbD Serotec, Oxford, UK) and myeloperoxidase (MPO) (dilution 1:10000; Dako, Glostrup, Denmark) monoclonal antibodies. 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 antibodies was used as negative control. The signals were visualized using diaminobenzidine. Sections were counterstained with hematoxylin.

In addition to the immunostains, we used a third staining to detect neutrophils. Naphthol AS-D Chloroacetate Esterase (Leder) reaction is a common procedure to identify the neutrophils in tissues. Sections adjacent to the ones used for immunohistochemistry were boiled in citrate buffer and subsequently developed in naphthol AS-D chloroacetate for 45 minutes. Sections were counterstained with hematoxylin.

Presence of neutrophils was analyzed using image-analyzing software (Soft Imaging Systems, Munster, Germany). Tissue areas with CD66b-positive cells were selected for further analysis. The areas with CD66b-positive cells within loose blood in between the tissue due to the surgical procedure were excluded. The numbers of CD66b-positive cells were counted and expressed as the number of neutrophils per plaque. Using a magnification of ×100, CD34-positive microvessels were counted in three areas of the plaque with the highest microvessel density (MVD) as described previously[201]. Subsequently, the average MVD per square millimeter of these areas was calculated for each plaque.
Intra plaque haemorrhage (IPH) was scored using H&E and fibrin (Mallory's phosphotungstic acid-hematoxilin) and anti smooth muscle actin immunostains[202].

Protein isolation

Segments adjacent to the ones used for histology were used for protein isolation as described previously[199]. In short, plaque segments were frozen in liquid nitrogen and stored at -800˚C until further use; protein extraction was performed according to a standard protocol using Tris. Levels of interleukin (IL)-8 were measured by a multiplex suspension array system according to the manufacturer's protocol (Bender Med Systems, Vienna, Austria). Matrix metalloproteinase MMP-8 and MMP-9 activities were measured using the Biotrak activity assays RPN 2635 and RPN 2634 (Amersham Biosciences, Buckinghamshire, UK), respectively. Measurements of IL-8, MMP-8 and -9 are standard for all Athero-Express patients and part of these data (8.5%) have been used in another article[203].

Data analysis

Statistics were performed with SPSS 15.0

Correlations between plaque characteristics, cytokines and MMPs and patient clinical data were assessed using Spearman's correlation test and the difference between two groups was determined using Mann-Whitney test. For these analyses, the number of neutrophils per plaque was used as a continuous variable.
For the associations mentioned in table 1, the number of neutrophils per plaque (as continuous variable) was divided into two equal groups using the median (31.0) as a cut-off point. Multivariate analysis was used to adjust for confounders (e.g. β-blockers and gender): the neutrophil data were binned into two groups (low and high) using the median as a cut-off point; a binary logistic model with a probability for stepwise entry (0.01) and removal (0.05) was performed. Probability values <0.05 were considered significant.

Results

Neutrophil identification and localization within plaque

Clinical characteristic of the study population in relation with neutrophil numbers are shown in table 1 and table 2. Three staining methods were used to identify the neutrophils in sections of atherosclerotic plaques (CD66b, Leder and MPO). The analysis showed that CD66b positivity was detected in neutrophils and not in other cell types within plaque; Leder staining was mainly found in neutrophils and resembled the CD66b distribution; MPO expression was found in neutrophils and in a subset of plaque macrophages (supplement material, figure I).

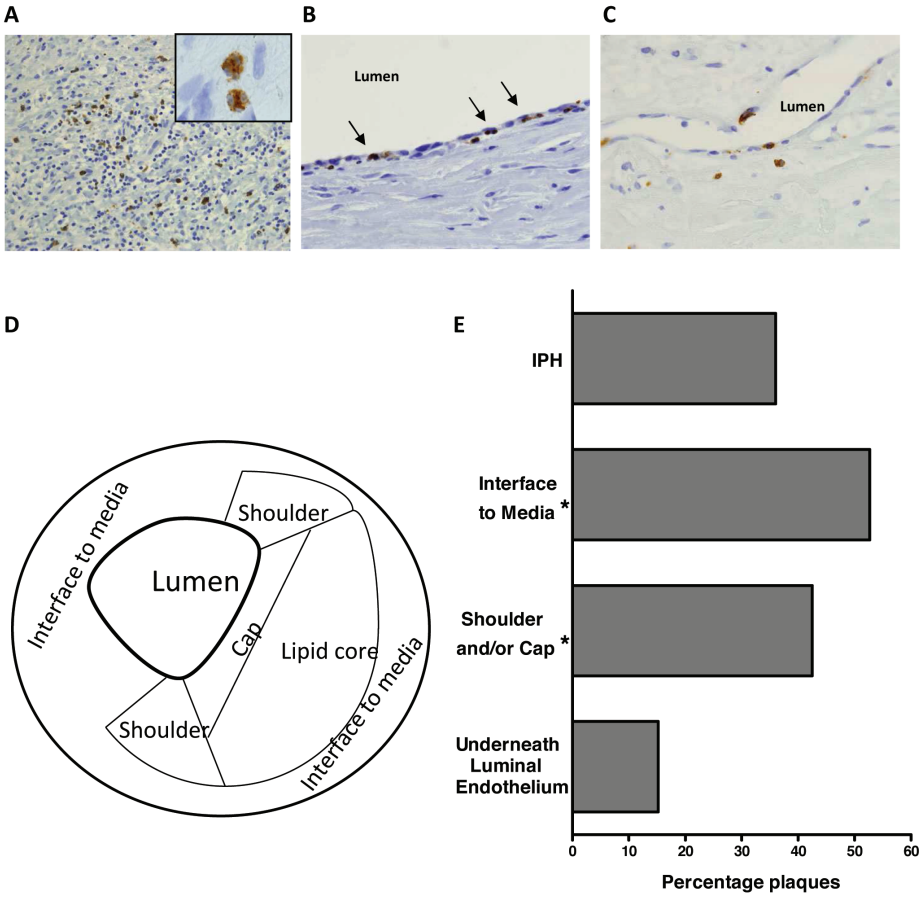


Figure 1. Neutrophil localization in human carotid plaques (A-E). (A) CD66b - positive neutrophils in the shoulder; (B) CD66b – positive neutrophils underneath the luminal endothelium (black arrows); (C) CD66b - positive neutrophils around a plaque micro-vessel; (D) schematic representation of different plaque areas; (E) distribution of CD66b-positive neutrophils within plaque areas. IPH indicates intra plaque hemorrhage; * mostly observed around microvessels.

Further analysis of the CD66b-positivity of plaque neutrophils showed a heterogeneous distribution for neutrophils within plaque tissue. Neutrophils were found in different regions of the plaque: in the fibrous cap or in the shoulder, in the interface to media (also called the base of a plaque) or in areas with intra plaque bleeding (hemorrhage) (figure 1). In addition, neutrophils were observed lying underneath the luminal endothelium (figure 1B) or in the vicinity of micro vessels in the plaque (figure 1C).

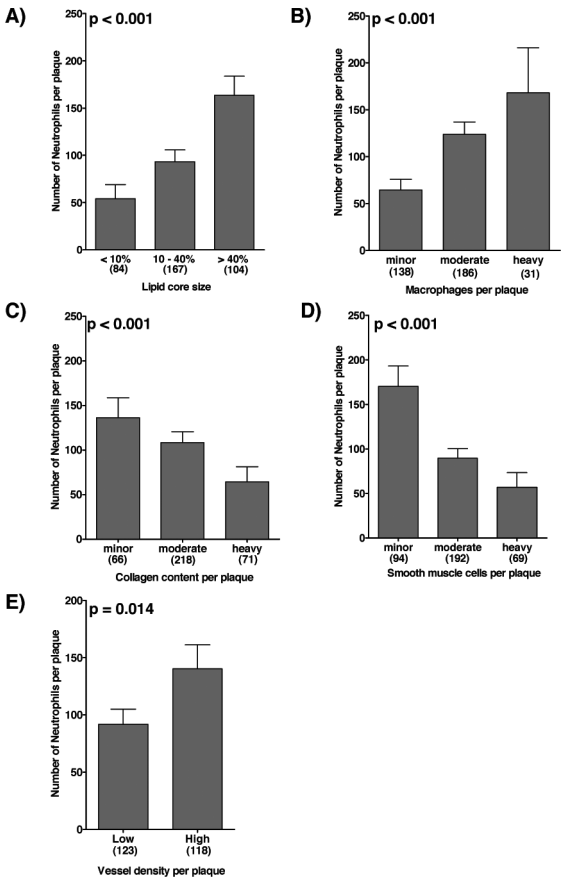


Figure 2. Neutrophils and plaque characteristics (A-E). Number of Neutrophils per plaque in relation to the size of the lipid core (A), macrophages (B), collagen (C), smooth muscle cells (D) and micro-vessels (E). Bars represent means ± SEM; below each bar, the number of patients per group is indicated; statistics Spearman's correlation test.

Neutrophils and plaque histology

The number of neutrophils per plaque was assessed and compared with different plaque histological characteristics: neutrophil numbers were positively correlated with the size of the lipid core ($p < 0.001$), the amount of macrophages ($p < 0.001$) and micro vessels ($p = 0.014$); and negatively correlated with the amount of collagen ($p < 0.001$) and smooth muscle cells ($p < 0.001$; figure 2).

Neutrophils and IL-8, MMP-8 and MMP-9

Next, we investigated the association of plaque neutrophils with IL-8, a neutrophil chemo attractant protein and MMP-8 and MMP-9, two proteases expressed by neutrophils. A positive correlation was observed between the number of neutrophils and the levels of IL-8 (Spearman's correlation coefficient $r = 0.407$, $P = <0.001$), active MMP-8 (Spearman's correlation coefficient $r = 0.239$, $P = 0.005$) and active MMP-9 (Spearman's correlation coefficient $r = 0.305$, $P = <0.001$).

Table 1. Patient characteristics related to the number of neutrophils in the common carotid endarterectomy specimens:

	Neutrophils		
	Low	High	P-value
Medians, [IQR]	8 [2 - 17]	110 [58 - 240]	
Number of patients (n)	178	177	
Age, mean (sd)	72 (10)	73 (9)	0.83
Male, n	102	134	0.05
Hypertension, n	115	124	0.94
Diabetes, n	41	37	0.56
Current Smoker, n	46	37	0.22
Hypercholesterolemia, n	113	117	0.94
History: Angina pectoris, n	60	51	0.17
History: Myocardial infarction, n	27	31	0.68
History: Vascular intervention, n	29	24	0.65
History: Carotid intervention, n	16	9	0.25
Symptomatic carotid stenosis, n	149	159	0.076
Time between TIA/Stroke until CEA, median [IQR]	57 [24 – 110]	58 [21 – 106]	0.88
Medication			
Statins	151	147	0.23
Aspirin	47	36	0.07
ACE inhibitors	55	56	0.90
Beta-blockers	100	83	0.12

TIA indicates transient ischemic attack; CEA indicates carotid endarterectomy; [IQR] indicates interquartile range.

Neutrophils and clinical patient characteristics

Having established that neutrophils are associated with characteristics of rupture-prone plaques, we investigated whether clinical parameters differed between patients with high and low neutrophils (equal groups, using the median 31.0 as a cut-off point) within plaques (table 1).

Table 2 shows the number of neutrophils in plaque for each patient characteristic. The number of plaque-neutrophils was significantly different between males and females. Lower neutrophil numbers were observed in plaques from females compared with plaques from males (figure 3A; $p < 0.001$). In addition, an association between the number of neutrophils per plaque and the use of β-blockers was found (table 2). The number of neutrophils was lower in plaques from patients treated with β-blockers prior to their CEA compared to plaques from untreated patients ($p = 0.039$, figure 3B).

In addition, patients on β-blocker treatment longer than 1 year had lower plaque neutrophil numbers compared to patients treated for less than 1 year ($p = 0.049$; supplement material, figure IVB). A trend towards lower neutrophil numbers in plaques from patients treated with selective β1-blockers (e.g. Metoprolol, Bisoprolol, Atenolol) than in plaques from patients treated with non-selective drugs (e.g. Sotalol) was observed (supplement material, figure IVA). Baseline patient characteristics in relation to beta blocker therapy are presented in the supplement material (table 1).

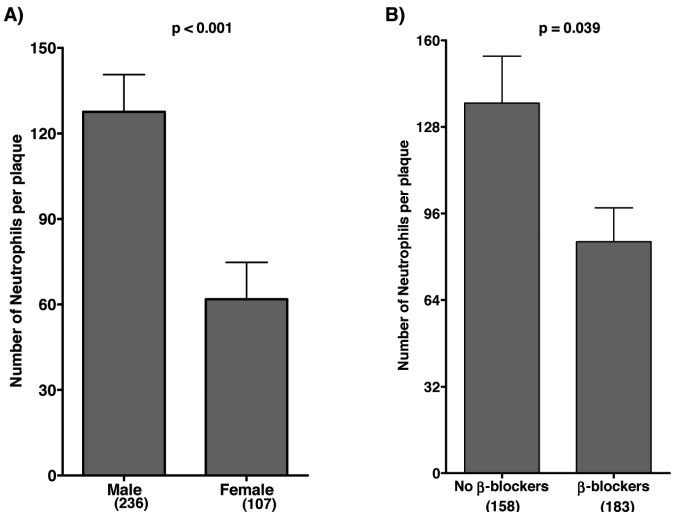


Figure 3. Neutrophils in relation to gender and β-blocker therapy. (A) The number of Neutrophils per plaque and gender (male and female); (B) the number of neutrophils per plaque and beta-blocker therapy. Bars represent means ± SEM; below each bar, the number of patients per group is indicated; statistics Mann-Whitney test.

Table 2. Neutrophil numbers in relation to clinical characteristics:

Characteristic Groups (n)	Number of Neutrophils Median [interquartile range]	P - value
Age		
≤ 70 (n = 153)	33.0 [7 -150]	
> 70 (n = 190)	32.5 [8 - 105]	p = 0.525
Gender		
Female (n = 107)	16.0 [5 – 54]	
Male (n = 236)	43.5 [11 – 143]	p < 0.001*
Smoker		
No (n = 246)	42.0 [8 - 122]	
Yes (n = 83)	28.0 [7 – 82]	p = 0.357
Hypertension (HT)		
No (n = 87)	30.0 [6 – 110]	
Yes (n = 239)	38.0 [9 – 117]	p = 0.816
Hypercholesterolemia		
No (n = 95)	36.0 [12 – 108]	
Yes (n = 230)	32.0 [7 – 110]	p = 0.714
Diabetes Mellitus (DM)		
No (n = 248)	36.5 [8 – 112]	
Yes (n = 78)	26.0 [5 – 110]	p = 0.199
Carotid stenosis		
Asymptomatic (n = 46)	21.0 [6 – 65]	
Symptomatic (n = 309)	37.0 [8 – 118]	p = 0.085
History: Myocardial infarction (MI)		
No (n = 276)	32.0 [8 – 106]	
Yes (n = 58)	41.0 [7 – 167]	p = 0.621
History: Angina Pectoris (AP)		
No (n = 203)	42.0 [8 – 121]	
Yes (n = 111)	28.0 [7 – 97]	p = 0.104
Medication used:		
Statins		
No (n = 57)	50.0 [16 – 120]	
Yes (n = 298)	29.0 [7 – 106]	p = 0.107
Aspirin		
No (n = 262)	38.5 [8 – 119]	
Yes (n = 83)	25.0 [8 – 70]	p = 0.134
ACE inhibitors		
No (n = 231)	31.5 [8 – 112]	
Yes (n = 111)	28.0 [7 – 89]	p = 0.691
Beta-blockers (β-blockers)		
No (n = 158)	43.0 [8 – 136]	
Yes (n =183)	25.0 [7 – 85]	p = 0.039*

ACE = angiotensin conversion enzyme; *Mann-Whitney* test, *significance at p < 0.05

Multivariate analysis showed that the association between neutrophil numbers and the characteristics of rupture-prone plaque is independent of β-blocker therapy and gender (table 3). No difference in neutrophil plaque numbers between patients presenting with clinical manifestations of the carotid stenosis (symptomatic) and those without symptoms (asymptomatic) was observed (p = 0.085; table 2 and supplement material, figure II).

Table 3. Association between neutrophil numbers and plaque characteristics, adjusted for potential confounders

	Neutrophil Numbers (Low versus High)
	OR [95%CI]
Characteristics	
Lipid core size	1.9 [1.29 – 2.79] *p = 0.001**
Macrophage influx	1.7 [1.23 – 2.53] *p= 0.002**
Smooth muscle cell influx	0.7 [0.48 – 0.99] *p = 0.047**
Collagen amount	1.0 [0.65 – 1.57] *p = 0.002**
Gender	1.5 [0.92 – 2.62] *p = 0.099**
Beta-blockers use	0.7 [0.46 – 1.19] *p = 0.221**

Discussion

Atherosclerosis is a chronic inflammatory disease. Different inflammatory cell types infiltrate through vessel's damaged endothelium into the intima, where they initiate a chronic inflammatory process. The role of macrophages and T-lymphocytes in the process of atherogenesis is already well established. A wide range of functions relevant to atherosclerosis are now attributed to mast cells and dendritic cells[204]. Neutrophils, although present in much lower numbers within an atherosclerotic lesion, are now regaining interest in respect to atherosclerosis development and progression.

We used different markers to identify the neutrophils in human CEA specimens: CD66b, MPO and esterase. The analysis of consecutive sections stained for anti-CD66b, MPO and esterase showed that the CD66b-positive cells expressed MPO and esterase, demonstrating that the CD66b-positive cells in plaques are neutrophils (as previously reported[205]).

The localization of neutrophils within the plaque proved to be heterogeneous; neutrophils were found infiltrated in the cap, in the shoulder and in areas towards the media (also known as the base of the plaque, figure 1). In these areas, neutrophils were mainly found around micro vessels (figure 1C, E); in the cap, neutrophils were also observed underneath the luminal endothelium (figure 1B, E). These may represent two distinct routes by which neutrophils infiltrate into the plaque. Rotzius et al[206] recently showed in ApoE deficient

mice (by use of flow cytometry on the whole aorta and confocal microscopy on whole mounted plaque) how neutrophils infiltrate and abundantly accumulate in the shoulder regions of the plaque where they outnumber the amount of infiltrating monocytes. In addition, they observed that neutrophils were the main leukocyte subset which interacted with the lesion endothelium. Naruko et al[207] in coronary arteries and Leclercq et al[208] in carotid arteries, reported the localization of neutrophils in the vicinity of intraplaque vessels. In our study, an association between high numbers of neutrophils and high vessel density per plaque was observed (figure 2E), providing further support for neutrophil infiltration through small vessels. The presence of (neo)vessels, expressing adhesion molecules (ICAM, VCAM) within an atherosclerotic lesion may facilitate the influx of inflammatory cells, thus possibly contributing to plaque destabilization and rupture[209]. Leclercq et al[210] observed in the shoulder regions of carotid plaques, areas positive for P-selectin (a neutrophil adhesion molecule required for their diapedesis) surrounded by neutrophils. Thus, the infiltration of neutrophils from intra plaque micro vessels, into atherosclerotic lesions could be an active phenomenon. However, since our study is purely observational, we cannot exclude the possibility that the observed neutrophils around plaque micro vessels and underneath the luminal endothelium might be an effect of CEA; it is known that the surgical procedure can induce diapedes of neutrophils. No correlation between the time interval in which the carotid artery was opened and plaque was removed (on average 34 ± 11min) and the number of neutrophils per plaque was found (Spearman's correlation coefficient, $R= -0.059$, $p = 0.350$).

In 36 percent of the plaques, neutrophils were observed in areas with intra plaque hemorrhage (figure 1E), this being a third possible route by which neutrophils come into the plaque. A histological analysis of human carotid endarterectomy (CEA) specimens suggested that intra plaque hemorrhage could convey neutrophils into the lesion[211]. Moreover, intra plaque hemorrhage along with angiogenesis, contribute to plaque destabilization and possible rupture[212].

High neutrophil numbers were found in plaques with features of rupture-prone lesions (bigger lipid core, heavy macrophage influx, and minor collagen and smooth muscle cells). The aforementioned histological characteristics of rupture-prone plaques originate from cross-sectional observations in coronary lesions; however, these characteristics also apply for carotid plaques[213].

Different studies reported the presence of neutrophils at sites of rupture of human atherosclerotic lesions (coronary, carotid and cerebral) and mouse studies suggest a role for neutrophils in plaque formation[214]. Previous human neutrophil studies were either based on autopsy specimens (coronary[215], cerebral[216]) or atherectomy specimens[217]. In the coronary specimens obtained postmortem, neutrophils were detected in atherosclerotic lesions of patients who died of acute myocardial infarction and not in the lesions of patients who died from noncardiovascular disease[218]. A study in CEA specimens compared the culprit

zone with the adjacent plaque segments and reported the presence of neutrophils in the culprit lesion and an association between neutrophil infiltration and intraplaque hemorrhage[219].

A positive association between the number of neutrophils and plaque levels of IL-8, MMP-8 and MMP-9 was observed. IL-8 is a well known neutrophil chemo-attractant molecule and high IL-8 levels are found in rupture-prone plaques[220]. It is secreted by active macrophages, endothelial cells and T-cells[221] and may contribute to neutrophil migration into the plaque. IL-8 is also known as a neutrophil stimulator, inducing the release of MMP-9 from neutrophil's tertiary granules[222]. In addition, MMP-8 (also known as neutrophil collagenase) is highly expressed by neutrophils. This might explain the strong correlation between the high number of neutrophils and the high levels of IL-8, MMP-8 and MMP-9 in plaques and might point to neutrophils as a source of MMP-8 and MMP-9 in human plaques. IL-8 levels were significantly associated with MMP-8 and MMP-9 levels (data not shown), suggesting that IL-8 might stimulate MMP-8 and MMP-9 expression in those plaques and is in accordance with the finding that MMP-8 and MMP-9 levels are elevated in rupture-prone plaques[223]. This study, however, can only measure at one point in time which limits firm conclusions on regulatory mechanism. Also, the source of these proteases in human plaques is heterogeneous; plaque's vascular endothelial cells, smooth muscle cells and macrophages can produce and secrete MMP-9 and MMP-8[224]. Therefore, neutrophils could only be counted as one of the cellular sources of matrix mettalloproteases in human plaques, next to macrophages and smooth muscle cells.

We and others previously demonstrated that carotid plaques stabilize over time following stroke [225, 226]; the levels of cytokines and the number of infiltrated macrophages decrease immediately after a stroke/TIA until CEA. Therefore, we assessed the relation between neutrophils and time between stroke/TIA and CEA; no significant association between the two was found (Spearman's correlation, $r = -0.029$, $p = 0.645$, data not shown).

It would be interesting to know whether the numbers of plaque infiltrated neutrophils correlate with the numbers of blood circulating neutrophils. In this study, in a subgroup of 25 patients, no correlation between the number of neutrophils in plaque and the number of circulating granulocytes was observed (Spearman's correlation, $r = 0.249$, $p = 0.230$; supplement material, figure III). This might be due to the limited number of patients for which both plaque and blood were available.

An association between gender and neutrophil numbers in plaques was observed (figure 3A and Table 2). The plaques from females showed lower neutrophil counts compared to plaques from males. This is in accordance with earlier results from our group which shown, in a large cohort of patients undergoing carotid endarterectomy, that females have a more stable, less inflammatory plaque phenotype compared with males[227]. Gender differences in cardiovascular disease are since long a source of investigation; pre-menopausal women are protected against atherosclerosis and its complications, however post-menopausal their

risk of cardio-vascular complications increases by a factor of 2 or 3[228]. Women have smaller arteries and therefore might have smaller plaques. In the current study, the degree of carotid stenosis (partially representing the plaque size) did not differ between women and men (supplement material, table II).

An association between the number of neutrophils per plaque and the use of β-blockers was observed. Plaques from patients with records of β-blockers use had significantly lower neutrophil numbers compared to plaques from patients without a history of β-blockers use (figure 3B and Table 2). The use of β-blockers showed no association with the different plaque characteristics (size of the lipid core, amount of collagen, macrophages and smooth muscle cells) or with the levels of IL-8, MMP-8 and MMP-9 (data not shown). Use of beta-blockers for longer than 1 year might induce a significant reduction in the number of infiltrating neutrophils, since an inverse association between the time of beta-blockers treatment and the number of plaque neutrophils was observed (supplement material, figure IV). It has been described that β-blockers have accessory effects on neutrophils; several *in vitro* studies demonstrated a β-blocker-induced inhibition of neutrophil chemotaxis and release of cytoplasmic products[229, 230]. Our observation is in line with these previous findings suggesting that beta-blockers could reduce neutrophil influx and might therefore reduce progression to rupture-prone plaque lesion. New prospective clinical studies are, however, necessary to confirm this hypothesis.

In summary, we show that within human carotid atherosclerotic plaques high number of neutrophils is associated with morphological characteristics and the inflammatory status of rupture-prone lesions. This points to neutrophils, known for their tissue destruction capabilities, as potential contributors to the destabilization and subsequent rupture of an atherosclerotic plaque.

Acknowledgements

The authors would like to acknowledge Petra van der Kraak - Homoet for excellent technical assistance.

Reference List

1 Naruko T, Ueda M, Haze K, van der Wal AC, van der Loos CM, Itoh A, Komatsu R, Ikura Y, Ogami M, Shimada Y, Ehara S, Yoshiyama M, Takeuchi K, Yoshikawa J, Becker AE. Neutrophil Infiltration of Culprit Lesions in Acute Coronary Syndromes. Circulation. 2002;106 :2894-2900.

2 Tavora F, Ripple M, Li L, Burke A. Monocytes and neutrophils expressing myeloperoxidase occur in fibrous caps and thrombi in unstable coronary plaques. BMC Cardiovascular Disorders. 2009;9 :27.

3 Larionov S, Dedeck O, Birkenmeier G, Thal DR. Expression of alpha2-macroglobulin, neutrophil elastase, and interleukin-1alpha differs in early-stage and late-stage atherosclerotic lesions in the arteries of the circle of Willis. Acta Neuropathol .2007; 113:33-43.

4 Kostis JB, Turkevich D, Sharp J. Association between leukocyte count and the presence and extent of coronary atherosclerosis as determined by coronary arteriography. The American Journal of Cardiology. 1984 ;53:997-999.

5 Friedman GD, Klatsky AL, Siegelaub AB. The leukocyte count as a predictor of myocardial infarction. N Engl J Med .1974;290:1275-1280.

6 Zernecke A, Bot I, Djalali-Talab Y, Shagdarsuren E, Bidzhekov K, Meiler S, Krohn R, Schober A, Sperandio M, Soehnlein O, Bornemann J, Tacke F, Biessen EA, Weber C. Protective Role of CXC Receptor 4/ CXC Ligand 12 Unveils the Importance of Neutrophils in Atherosclerosis. Circ Res.2008;102:209-217.

7 van LM, Gijbels MJ, Duijvestijn A, Smook M, van de Gaar MJ, Heeringa P, de Winther MP, Tervaert JW. Accumulation of myeloperoxidase-positive neutrophils in atherosclerotic lesions in LDLR-/- mice. Arterioscler Thromb Vasc Biol. 2008 ;28:84-89.

8 Soehnlein O, Weber C. Myeloid cells in atherosclerosis: initiators and decision shapers. Semin Immunopathol. 2009;31:35-47.

9 Dale DC, Boxer L, Liles WC. The phagocytes: neutrophils and monocytes. Blood. 2008 ;112:935-945.

10 Pugin J, Widmer MC, Kossodo S, Liang CM, Preas HL, II, Suffredini AF. Human Neutrophils Secrete Gelatinase B In Vitro and In Vivo in Response to Endotoxin and Proinflammatory Mediators. Am J Respir Cell Mol Biol .1999;20:458-464.

11 Weber C, Zernecke A, Libby P. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. Nat Rev Immunol .2008 ;8:802-815.

12 Exner M, Minar E, Mlekusch W, Sabeti S, Amighi J, Lalouschek W, Maurer G, Bieglmayer C, Kieweg H, Wagner O, Schillinger M. Myeloperoxidase Predicts Progression of Carotid Stenosis in States of Low High-Density Lipoprotein Cholesterol. Journal of the American College of Cardiology. 2006 ;47:2212-2218.

13 Buffon A, Biasucci LM, Liuzzo G, D'Onofrio G, Crea F, Maseri A. Widespread Coronary Inflammation in Unstable Angina. N Engl J Med .2002;347:5-12.

14 Blankenberg S, Rupprecht HJ, Poirier O, Bickel C, Smieja M, Hafner G, Meyer J, Cambien F, Tiret L, for the AtheroGene Investigators. Plasma Concentrations and Genetic Variation of Matrix Metalloproteinase 9 and Prognosis of Patients With Cardiovascular Disease. Circulation. 2003;107:1579-1585.

15 Verhoeven BA, Velema E, Schoneveld AH, de Vries JP, de BP, Seldenrijk CA, de Kleijn DP, Busser E, van der GY, Moll F, Pasterkamp G. Athero-express: differential atherosclerotic plaque expression of mRNA and protein in relation to cardiovascular events and patient characteristics. Rationale and design. Eur J Epidemiol. 2004;19:1127-1133.

16 Post S, Peeters W, Busser E, Lamers D, Sluijter JPG, Goumans MJ, de Weger RA, Moll FL, Doevendans PA, Pasterkamp G, Vink A. Balance between Angiopoietin-1 and Angiopoietin-2 Is in Favor of Angiopoietin-2 in Atherosclerotic Plaques with High Microvessel Density. Journal of Vascular Surgery. 2008;45:244-250.

17 Hellings WE, Peeters W, Moll FL, Piers SRD, van Setten J, Van der Spek PJ, De Vries JP, Seldenrijk KA, De Bruin PC, Vink A, Velema E, De Kleijn DPV, Pasterkamp G. Composition of Carotid Atherosclerotic Plaque Is Associated With Cardiovascular Outcome. A Prognostic Study. Circulation. 2010; 121: 1941-1950

18 Ionita MG, Vink A, Dijke IE, Laman JD, Peeters W, van der Kraak PH, Moll FL, De Vries JP, Pasterkamp G, De Kleijn DPV. High Levels of Myeloid-Related Protein 14 in Human Atherosclerotic Plaques Correlate With the Characteristics of Rupture-Prone Lesions. Arterioscler Thromb Vasc Biol. 2009;29:1220-1227.

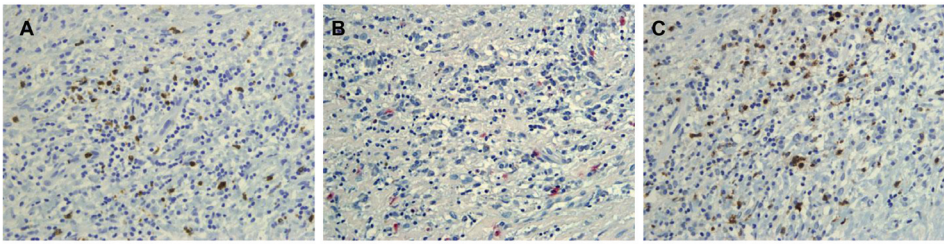
19 Rotzius P, Thams S, Soehnlein O, Kenne E, Tseng CN, Bjorkstrom NK, Malmberg KJ, Lindbom L, Eriksson EE. Distinct Infiltration of Neutrophils in Lesion Shoulders in ApoE-/- Mice. Am J Pathol. 2010;epub

20 Leclercq A, Houard X, Philippe M, Ollivier V, Sebbag U, Meilhac O, Michel JB. Involvement of intraplaque hemorrhage in atherothrombosis evolution via neutrophil protease enrichment. J Leukoc Biol. 2007;82:1420-1429.
21 de Boer OJ, van der Wal AC, Teeling P, Becker AE. Leucocyte recruitment in rupture prone regions of lipid-rich plaques: a prominent role for neovascularization? Cardiovasc Res. 1999;41:443-449.
22 Virmani R, Kolodgie FD, Burke AP, Finn AV, Gold HK, Tulenko TN, Wrenn SP, Narula J. Atherosclerotic Plaque Progression and Vulnerability to Rupture: Angiogenesis as a Source of Intraplaque Hemorrhage. Arterioscler Thromb Vasc Biol. 2005; 25:2054-2061.
23 Redgrave JNE, Lovett JK, Gallagher PJ, Rothwell PM. Histological Assessment of 526 Symptomatic Carotid Plaques in Relation to the Nature and Timing of Ischemic Symptoms: The Oxford Plaque Study. Circulation. 2006;113:2320-2328.
24 Verhoeven B, Hellings WE, Moll FL, de Vries JP, de Kleijn DP, de BP, Busser E, Schoneveld AH, Pasterkamp G. Carotid atherosclerotic plaques in patients with transient ischemic attacks and stroke have unstable characteristics compared with plaques in asymptomatic and amaurosis fugax patients. J Vasc Surg. 2005 ;42:1075-1081.
25 Tedgui A, Mallat Z. Cytokines in Atherosclerosis: Pathogenic and Regulatory Pathways. Physiol Rev. 2006;86:515-581.
26 Chakrabarti S, Patel KD. Regulation of matrix metalloproteinase-9 release from IL-8-stimulated human neutrophils. J Leukoc Biol. 2005;78:279-288.
27 Galis ZS, Sukhova GK, Lark MW, Libby P. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. J Clin Invest.1994; 94:2493-2503.
28 Herman MP, Sukhova GK, Libby P, Gerdes N, Tang N, Horton DB, Kilbride M, Breitbart RE, Chun M, Schonbeck U. Expression of Neutrophil Collagenase (Matrix Metalloproteinase-8) in Human Atheroma: A Novel Collagenolytic Pathway Suggested by Transcriptional Profiling. Circulation. 2001;104:1899-1904.
29 Peeters W, Hellings WE, de Kleijn DPV, de Vries JPPM, Moll FL, Vink A, Pasterkamp G. Carotid Atherosclerotic Plaques Stabilize After Stroke: Insights Into the Natural Process of Atherosclerotic Plaque Stabilization. Arterioscler Thromb Vasc Biol. 2009 ; 29:128-133.
30 Redgrave JNE, Lovett JK, Gallagher PJ, Rothwell PM. Histological Assessment of 526 Symptomatic Carotid Plaques in Relation to the Nature and Timing of Ischemic Symptoms: The Oxford Plaque Study. Circulation. 2006;113:2320-2328.
31 Hellings WE, Pasterkamp G, Verhoeven BAN, De Kleijn DPV, De Vries JP, Seldenrijk KA, van den Broek T, Moll FL. Gender-associated differences in plaque phenotype of patients undergoing carotid endarterectomy. Journal of Vascular Surgery. 2007 ;45:289-296.
32 Perez-Lopez FR, Larrad-Mur L, Kallen A, Chedraui P, Taylor HS. Review: Gender Differences in Cardiovascular Disease: Hormonal and Biochemical Influences. Reproductive Sciences. 2010; 17:511-531.
33 Dunzendorfer S, Wiedermann CJ. Modulation of Neutrophil Migration and Superoxide Anion Release by Metoprolol. Journal of Molecular and Cellular Cardiology. 2000 ;32:915-924.
34 Djanani A, Kaneider NC, Meierhofer C, Sturn D, Dunzendorfer S, Allmeier H, Wiedermann CJ. Inhibition of neutrophil migration and oxygen free radical release by metipranolol and timolol. Pharmacology. 2003;68:198-203.

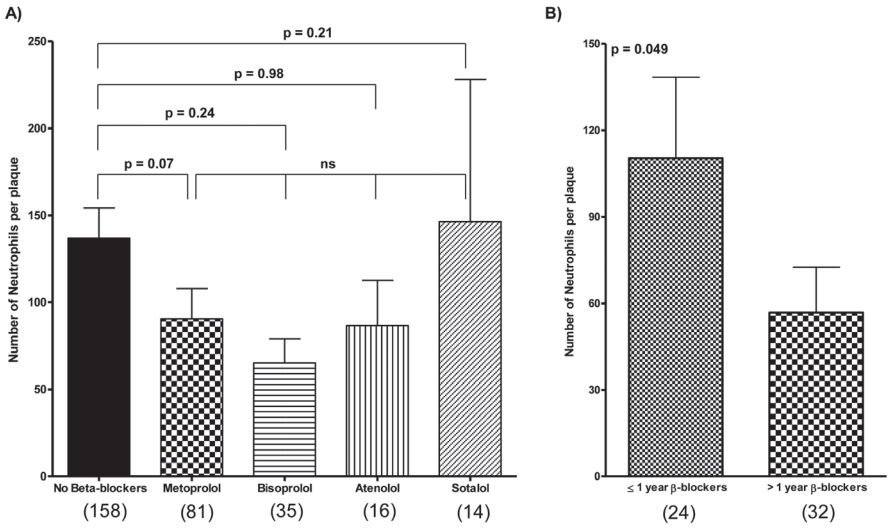
Supplemental Material

Supplemental table. Baseline characteristics in relation to β-blockers treatment

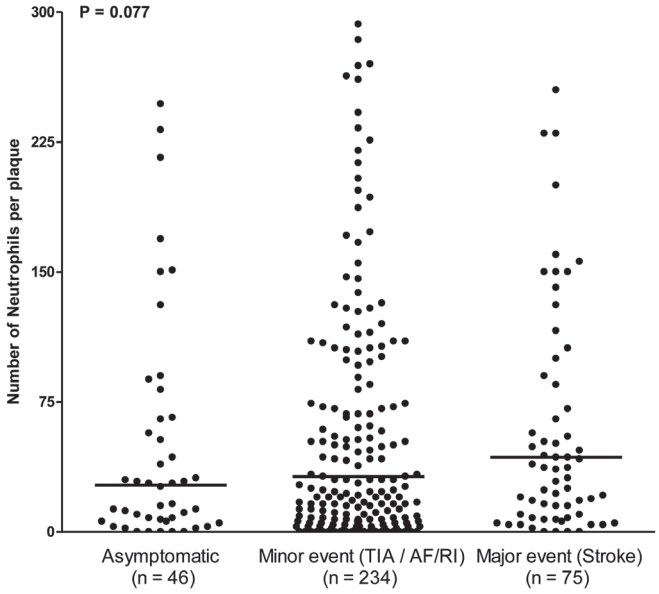
	β-blocker naïve (n = 183)	β-blocker treatment (n = 158)	P
Characteristics			
Age, y	70.3 ± 0.8	72.5 ± 0.7	0.04
Gender, n (%)			
Male	121 (69.9)	101 (65.5)	0.17
Female	52 (30.1)	53 (34.5)	
Smoker, n (%)	39 (22.5)	44 (28.5)	0.58
Asymptomatic, n (%)	19 (10.9)	21 (13.6)	
Symptomatic, n (%)	147 (84.9)	125 (81.1)	
History			
Hypertension, n (%)	113 (65.3)	116 (75.3)	0.84
DM, n (%)	37 (21.4)	37 (24)	1.0
Hypercholesterolemia, n (%)	116 (67)	103 (66.8)	0.88
Angina pectoris, n (%)	41 (23.6)	65 (42.2)	0.02
Myocardial infarction, n (%)	14 (8.09)	41 (26.6)	0.001
CABG, n (%)	16 (9.2)	26 (16.8)	0.12
Vascular intervention, n (%)	22 (12.7)	24 (15.5)	0.65
Carotid intervention, n (%)	43 (24.8)	34 (22)	0.81
Serum Cholesterol, mmol/L	4.2 ± 1.2	4.2 ± 1.1	0.80
HDL, mmol/L	1.1 ± 0.4	1 ± 0.4	0.99
LDL, mmol/L	2.5 ± 0.9v	2.4 ± 0.9	0.93
Triglycerides, mmol/l	1.3 ± 0.6	1.4 ± 0.7	0.70
Medication			
Statins, n (%)	142 (82)	130 (84.4)	0.35
Aspirin, n (%)	33 (19)	43 (28)	0.12
ACE inhibitors, n (%)	41 (24)	60 (39)	0.38
Plaque histology			
Small lipid core			
(0 - 40% of plaque area), n (%)	117 (67.3)	115 (74.6)	0.89
Minor macrophage staining	61 (35.2)	67 (43.5)	0.59
Minor collagen staining	31 (17.9)	26 (16.8)	0.50
Minor smooth muscle cell staining	45 (31.2)	44 (28.5)	0.91



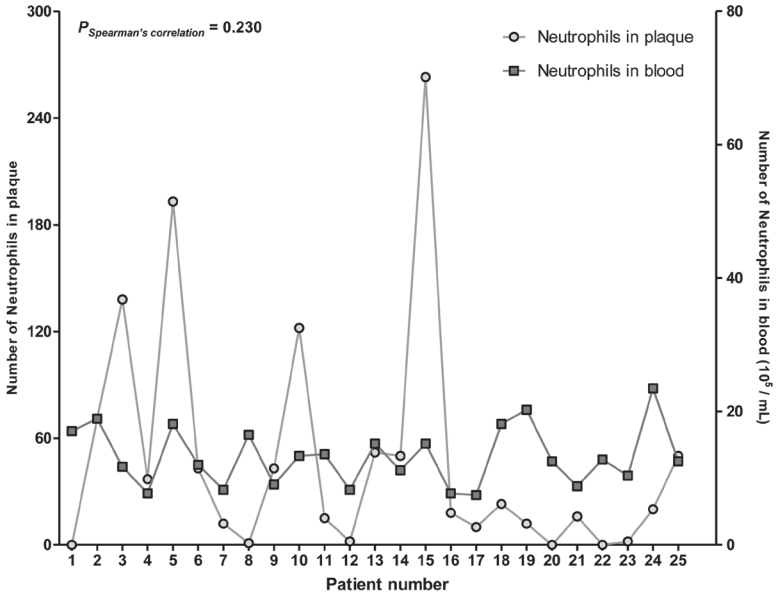
Supplemental figure I. Sections of rupture-prone plaque (A-C) showing CD66b (A), esterase (B) and MPO (C) expression in neutrophils.



Supplemental figure II. Neutrophils and β-blocker therapy. (A) Number of Neutrophils per plaque in relation to different beta-blockers and (B) to the time of treatment (less or more than 1 year). Bars represent means ± SEM; below each bar, the number of patients per group is indicated; statistics Mann-Whitney test, significance at $p < 0.05$.



Supplemental figure III. Number of neutrophils in plaque in relation to clinical presentation: asymptomatic (46) versus symptomatic (309), reported as minor or major events. TIA = transient ischemic attack; AF = amaurosis fugax; RI = retinal infarct. Kruskal-Wallis test, significance at $p < 0.05$.



Supplemental figure IV. Number of neutrophils in plaque in relation to the number of blood circulating granulocytes.

CHAPTER VI

Temporal changes in myeloid-related protein 8/14 plaque and plasma levels after cerebral ischemia

In preparation for publication

Mihaela G. Ionita, Wouter Peeters, Frans L. Moll, Jean-Paul P.M. De Vries, Gerard Pasterkamp And Dominique P.V. De Kleijn

Objective

Mrp-8/14 plaque levels are a marker of plaque vulnerability. It unknown whether Mrp-8/14 (plaque and plasma) levels associate with symptomatic atherosclerosis and how the Mrp-8/14 levels change in time after a cerebrovascular ischemic event. Here, we investigated the association between Mrp-8/14 plaque and plasma levels and the type and time after cerebrovascular ischemic event.

Methods and Results

In 230 consecutive patients (183 symptomatic and 47 asymptomatic) undergoing carotid endarterectomy, Mrp-8/14 levels (plaque and plasma) and the percentage of Mrp-8 positive macrophages were determined. For each symptomatic patient, the time between the latest cerebrovascular ischemic event and carotid endarterectomy was recorded. Plaque Mrp-8/14 levels showed an inverse association with the time interval between the latest ischemic cerebrovascular event and surgery ($p = 0.049$); plasma Mrp-8/14 levels and Mrp-8 and Mrp-14 (plaque and plasma) levels showed no correlation. The percentage of Mrp-expressing non-foam macrophages showed a similar trend, albeit not significant.

Conclusion

Mrp-8/14 plaque levels are high shortly after an ischemic cerebrovascular event, but decrease at later time points, supporting the concept that an atherosclerotic plaque stabilize after an event.

INTRODUCTION

Most of ischemic strokes and transient ischemic attacks (TIAs) occur due to a reduction in the blood supply to the brain, after significant stenosis of a carotid artery. The degree of carotid stenosis is associated with the incidence of stroke in symptomatic patients[231]. A large percentage of stroke patients have a history of TIA[232] while effective treatment of patients with TIAs diminishes the incidence of stroke in those patients[233].

Previous histological studies have shown that for most strokes and TIAs, the pathological substrate is an atherosclerotic plaque built within the vessel wall along the carotid artery; in addition, these plaques have a rupture-prone phenotype, with large lipid pools, intense macrophage infiltration and reduce collagen and smooth muscle cells numbers[234]. The rupture or erosion of an atherosclerotic plaque and subsequent thrombus formation may result in a downstream ischemic symptom. Sequential ruptures and/or erosions might happen during plaque natural evolution which may contribute to further plaque development. Histopathological studies have shown that immediately after a major event (e.g. stroke), a plaque reveals morphological and biological features of instability/vulnerability ; in time following the initial event, the plaque undergoes cellular and molecular changes leading to its stabilization[235, 236].

We previously reported that high levels of myeloid related protein (Mrp) -8, -14 and -8/14 associate with the rupture-prone plaques, which make these proteins candidate markers and potential contributors to plaque destabilization[237]. Mrps are proteins expressed in the cytoplasm of monocytes, macrophages and neutrophils[238, 239]. Mrp-8 actively binds to Mrp-14 and form the Mrp-8/14 complex that is translocated to cell membrane and/or secreted[240]. Only a subset of non-foam macrophages expresses Mrp in human plaques and this subset is more numerous in the rupture-prone lesions[237].

In patients with coronary heart disease, the serum Mrp-8/14 levels associate with the ischemic event and either remain constant or change in time after the event: Katashima et al[241] showed that in unstable angina patients, with vulnerable coronary plaques, the Mrp-8/14 levels remained unchanged for one week while in acute myocardial infarction (AMI) patients, the levels reached a peak (50% increase) at 3-5 days after the onset of AMI and showed still a high level at 6-8 days after (all compared to day 1, considered the baseline value).

Whether high plasma Mrp-8/14 levels also associate with cerebrovascular ischemic events and how the plasma Mrp-8/14 levels fluctuate in time after such an event, it is so far unknown. Considering all this, we hypothesized that the Mrp- levels (plaque and plasma) are higher in patients with symptomatic atherosclerosis than in those patients without complains and that in symptomatic patients Mrp-levels are high shortly after an acute cerebrovascular ischemic event (stroke, TIA) and decrease in time following the event, consistent with the already established plaque stabilization. For this, in a cohort of patients with carotid atherosclerotic disease undergoing carotid endarterectomy (CEA),

we investigated the association between Mrp-8/14 levels (plaque and plasma) with patients' clinical presentation (asymptomatic or symptomatic) and with the time between event onset and CEA. We found that high Mrp-8/14 (plaque and plasma) levels associate with symptomatic atherosclerotic disease while only plaque levels change in time after the ischemic cardiovascular event until CEA. The fact that plaque Mrp-8/14 levels are high shortly after a cerebrovascular event and decrease at later time points supports the concept of plaque stabilization after an event.

Methods

Study population and design

Athero-Express is an ongoing longitudinal cohort study, initiated in 2002 by two 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 CEA and relate these to recurrence of cardiovascular events during follow up, as described previously[242].

In this study a random set of 230 patients presenting with cerebral ischemia (i.e. transient ischemic attack or stroke) or free of clinical symptoms but with a carotid luminal stenosis greater than 75%, undergoing carotid endarterectomy were included. At baseline clinical parameters including cardiovascular risk factors and medication use were documented and recorded. Exclusion criteria for follow-up were: unwillingness or physical incapability to participate (e.g. severe dementia).

Clinical presentations of atherosclerotic carotid disease, prior to CEA and the time scale

Patients included for CEA were asymptomatic (no clinical symptoms related to the carotid luminal stenosis > 75%, n = 47) and symptomatic (n = 183); with minor clinical presentations (i.e., transient ischemic attack, amaurosis fugax and retinal infarction; n = 127) or major presentations (i.e., stroke; n = 56). The time between the onset of symptoms and CEA (in days) was recorded as previously described13. We reported an association between the time of symptoms onset until CEA and cellular and molecular changes in plaques. In short, we showed that symptomatic plaques are associated with a rupture-prone phenotype and remodel into more stable plaques over time following stroke; therefore these temporal plaque phenotypic changes should be taken into account when analyzing plaque Mrp-8/14 as a biomarker.

Symptomatic patients were categorized into 4 different groups according to the time elapsed from the latest cerebrovascular ischemic event until the carotid endarterectomy: 0-30 days, 31 - 90 days, 91-180 days and >180 days, as described earlier[243].

Plaque processing

All 230 carotid plaques used in this study, were carefully dissected from the carotid arteries and immediately transferred to the laboratory for further processing as described previously[199]. In short, in the laboratory the atherosclerotic fragments were dissected 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, alpha-actin and CD68) have been described previously. The adjacent segments were used for protein isolation.

Immunohistochemistry for Mrp-8

Plaque (n = 170) 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. 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.

The CD68 staining and the Mrp-8 staining were quantified using image-analyzing software (Soft Imaging Systems, Münster, Germany). Expression of Mrp-8 was detected in non-foam CD68-positive macrophages. We therefore decided to select only the non-foam 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).

Immunoassays of Mrp-8, -14 and -8/14

Levels of Mrp-8/14 heterodimers were measured with a commercial ELISA (Bühlmann Laboratories AG, Schönenbuch, Switzerland). For each patient, 50 µg of plaque Tris-protein and 5µl heparin plasma were used. The detection limits for plaque and plasma Mrp-8/14 were 10µg/g and 4ng/ml, respectively. The average inter-assay variability was 2.5%. The ELISA kit is specific for the Mrp-8/14 heterodimers and the cross-reactivity with Mrp-8 and -14 homodimers is minimal (according to the manufacturer).

Data analysis

Statistics were performed with SPSS 17.0

Correlations between the Mrp levels and patient clinical data were assessed using Spearman's correlation test and the difference between two groups was determined using Mann-Whitney test. Probability values <0.05 were considered significant.

Results

Baseline characteristics

The baseline characteristics of the study population are mentioned in table 1. From the cohort of 230 patients, 183 were symptomatic with the majority of patients presenting with TIA (n = 79, 43%) or stroke (n = 46, 25%).

Mrp levels and the type of cerebrovascular event prior and leading to CEA Plasma Mrp-8/14 levels were higher in patients who suffered a major event compared to asymptomatic patients (p = 0.001, figure 1A) and minor events (p = 0.002, figure 1A). The same trend, although not significant, was observed in patients with minor events compared to asymptomatic ones (figure 1).

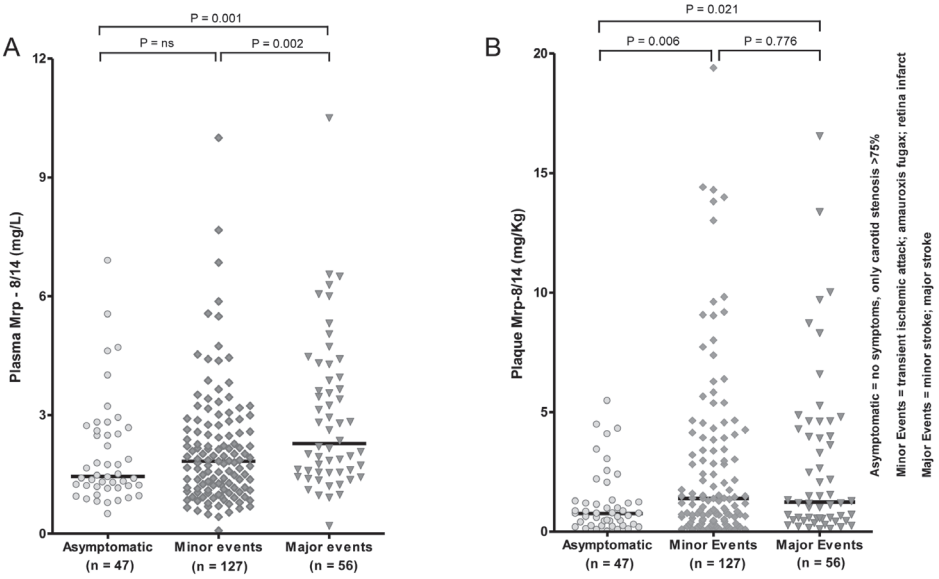


Figure 1. Mrp-8/14 plasma (A) and plaque (B) levels in relation to baseline manifestations of atherosclerotic carotid disease (asymptomatic, minor or major events) prior to carotid endarterectomy. Lines represent medians; on the X-axis, below each group, the number of patients is mentioned. Statistics Mann-Whitney; significance at $p < 0.05$.

Plaque Mrp-8/14 levels were higher in patients with clinical manifestations of carotid disease compared to asymptomatic ones (figure 1B); minor events compared to asymptomatic (p = 0.006) and major events compared to asymptomatic (p = 0.021). No significant difference was observed between Mrp-8/14 plaque levels in patients with minor compared to major events (p = 0.776, figure 1B).

Mrp -8/14 plaque and plasma levels and the time between latest symptom onset and CEA

Time between latest symptom (i.e. stroke, TIA) onset and CEA showed a weak inverse correlation with plaque Mrp-8/14 levels (Spearman's correlation coefficient, r = -0.16, p = 0.045). Figure 2 shows the relation between Mrp-8/14 plaque levels and the time elapsed from latest symptom onset until CEA. The levels of Mrp-8/14 in plaques collected within 90-180 days after an event were lower compared to the levels measured in plaques collected within the first 30 days (median [IQR]; 0.69 [0.33-2.44] vs 2.68 [1.07-5.09], respectively; p = 0.001) or within 30-90 days (median [IQR]; 0.69 [0.33-2.44] vs 1.4 [0.64-4.76], respectively; p = 0.018). In plaques obtained from patients operated later than 180days after an event, the levels of Mrp-8/14 were higher than in plaques from 90-180 days (median [IQR]; 1.52 [0.92-4.61] vs 0.69 [0.33-2.44], respectively; p = 0.008).

Table 1. Baseline characteristics in relation to the time between symptom onset and carotid endarterectomy

	Symptomatic					Asymptomatic
	0-30	31-90	91-180	>180	P-value	
N	28	55	55	25		47
Clinical presentation						
TIA, % (n)	54% (15)	46% (25)	49% (27)	48% (12)	-	
Stroke, % (n)	18% (5)	29% (16)	33% (18)	28% (7)	-	
Age, years mean (sd)	74 (8.8)	72 (8.3)	73 (9.6)	74 (8.4)	0.780	72 (8.2)
Male gender, % (n)	86% (24)	58% (32)	67% (37)	84% (21)	0.024	57% (27)
Hypertension, % (n)	64% (18)	75% (41)	66% (36)	68% (17)	0.707	77% (36)
Diabetes, % (n)	18% (5)	26% (14)	16% (9)	4% (1)	0.139	17% (8)
Current Smoker, % (n)	25% (7)	29% (16)	26% (14)	12% (3)	0.419	28% (13)
Hypercholesterolemia, % (n)	54% (15)	53% (29)	51% (28)	56% (14)	0.980	64% (30)
History:vascular intervention	14% (4)	15% (8)	6% (3)	12% (3)	0.322	36% (17)
Statin use, % (n)	64% (18)	69% (38)	64% (35)	64% (16)	0.887	72% (34)
Aspirin use, % (n)	29% (8)	31% (17)	42% (23)	60% (15)	0.136	49% (23)
Oral anticoagulants, % (n)	14% (4)	15% (8)	15% (8)	16% (4)	0.918	11% (5)

TIA = transient ischemic attack

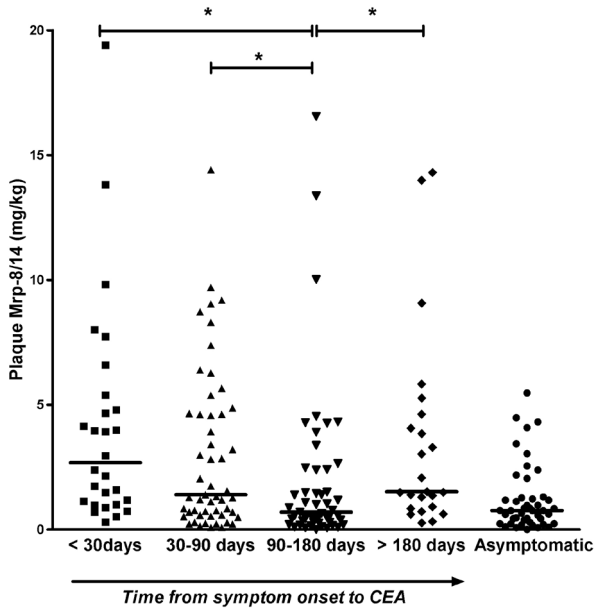


Figure 2. Time (in days) between latest symptom (stroke or transient ischemic attack) onset until carotid endarterectomy (CEA) and asymptomatic patients group in relation to Mrp-8/14 plaque levels. Lines represent medians; statistics *Mann-Whitney* test, significance at * $p < 0.05$.

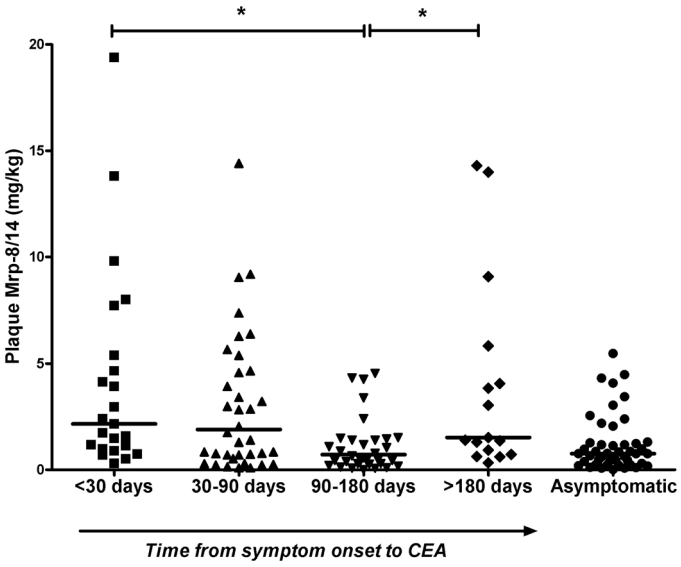


Figure 3. Time (in days) between TIA onset until CEA in relation to Mrp-8/14 plaque levels. Lines represent medians; statistics Mann-Whitney test; significance at * $p < 0.05$.

A similar trend was observed in subgroup analysis; time between TIA onset and CEA showed a weak inverse correlation with Mrp-8/14 plaque levels (Spearman's correlation coefficient, r = -0.22; p = 0.049) while patients who suffered from a stroke showed a similar trend, albeit not significant (Spearman's correlation coefficient, r = -0.09, p = 0.532). Consistent with the aforementioned trends, the levels of Mrp-8/14 in plaques collected within 90-180 days after a TIA were lower compared to the levels measured in plaques collected within the first 30 days (median [IQR]; 0.65 [0.30-1.40] vs 2.96 [1.16-5.02], respectively; p = 0.003) or after 180 days (median [IQR]; 0.65 [0.30-1.40] vs 2.19 [0.82-4.95], respectively; p = 0.041; figure 3).
Plasma Mrp-8/14 showed no correlation with the time between stroke or TIA and CEA (Spearman's correlation coefficient, r = -0.018, p = 0.824; data not shown).

Mrp-8 and Mrp-14 plaque and plasma levels and the time between latest symptom onset and CEA

The time between latest symptom (stroke, TIA) onset and CEA showed no correlation with Mrp-8 levels (plaque: Spearman's correlation coefficient, r = -0.11, p = 0.236; plasma: Spearman's correlation coefficient, r = 0.03; p = 0.761), Mrp-14 levels (plaque: Spearman's correlation coefficient, r = 0.13, p = 0.174; plasma: Spearman's correlation coefficient, r =0.03; p = 0.747; figure 4).

Mrp-expressing macrophages and the time between latest symptom onset and CEA

We previously shown that Mrp-8 and Mrp-14 (co-localized in plaques) are expressed by subset of plaque non-foam macrophages which associates with rupture-prone lesions[237]. Here, we addressed the association between the subset of Mrp-8 expressing plaque macrophages (shown as percentage) and the time between symptom onset and CEA. In a subgroup of 170 plaques, a trend towards more Mrp-positive macrophages soon after the event (within 30days) and less afterwards (between 30 and 180 days) was observed, albeit not significant (Kruskal-Wallis test, p = 0.78; figure 5).

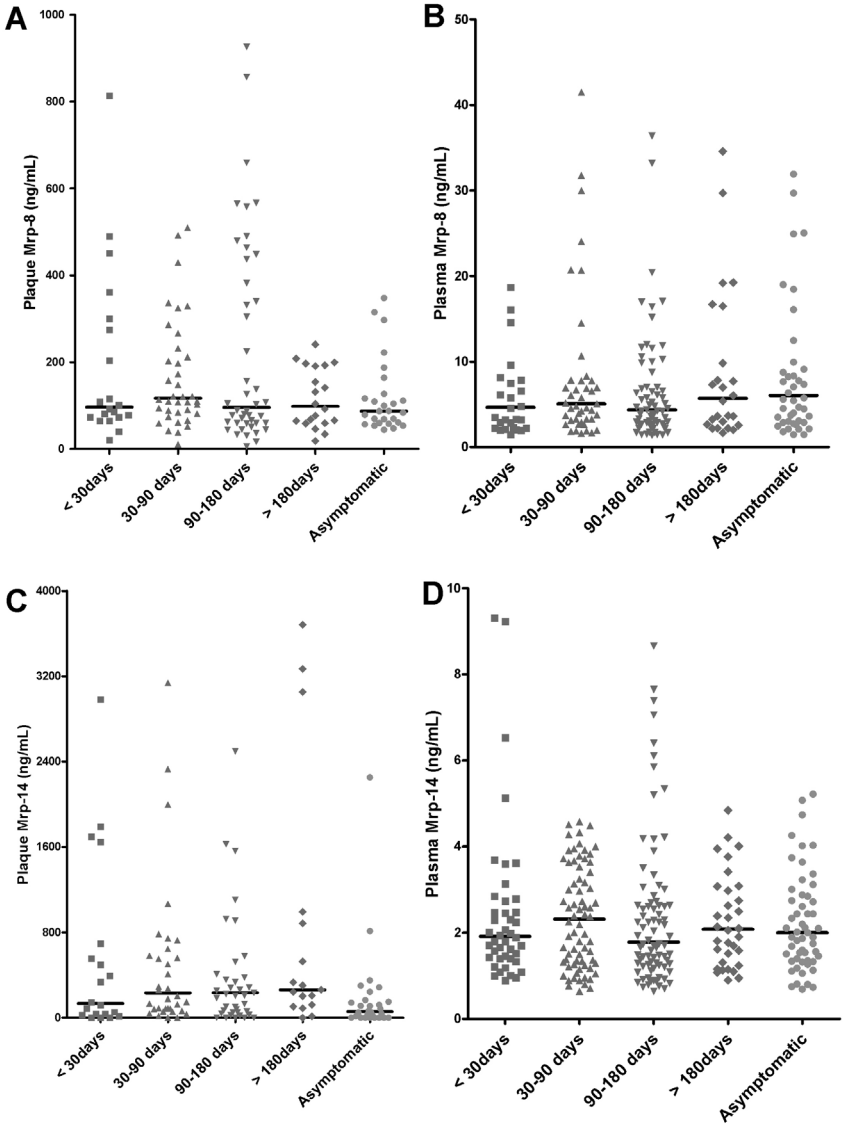


Figure 4. Time (in days) from latest symptom (stroke or transient ischemic attack) onset until CEA and asymptomatic patients group in relation to Mrp-8 levels (A plaque; B plasma) and Mrp-14 levels (C plaque; D plasma). Lines represent medians.

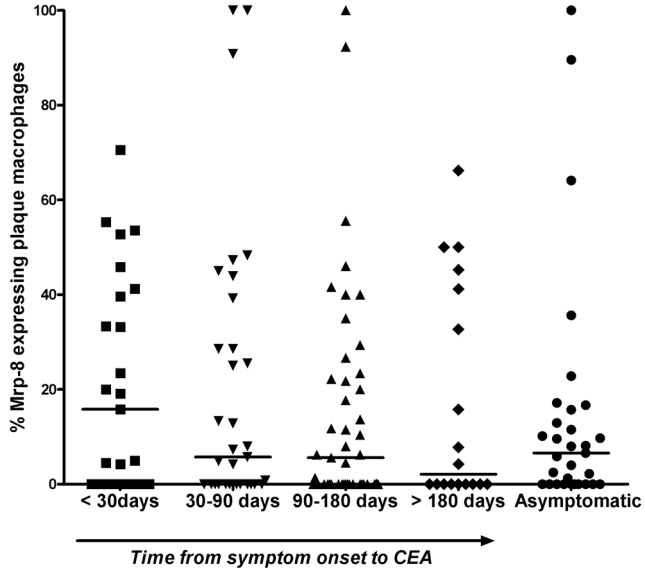


Figure 5. Percentage Mrp-8 positive plaque non-foam macrophages in relation to time (in days) between latest symptom onset until CEA. Lines represent medians.

Discussion

Atherosclerotic plaques are commonly built within the vessel wall of large arteries and the natural course of an atherosclerotic lesion it is, so far unknown. Carotid plaques are often discovered during medical routine screenings of the carotid arteries in high-risk profile patients or when a patient presents to the physician with complains of cerebral ischemia (i.e. TIA, stroke). In the common practice, atherosclerotic plaques which provoke carotid stenosis larger than 70%, asymptomatic or symptomatic, are surgically removed. The severity of the cerebral ischemia determines the rapidity of the intervention. Nowadays, stroke patients are scheduled for carotid endarterectomy much sooner than TIA or asymptomatic patients. In the *Athero-Express* study (ongoing since 2002), patients who underwent a CEA are included. We and others have shown, in patients undergoing CEA, that soon after a cerebro-vascular event, plaques stabilize at molecular and histological levels: following stroke, plaques demonstrated significant decrease of macrophage content after 180days; at protein level, interleukin (IL)-6, IL-8 levels and caspase 3-7 activity strongly decreased within 90 days[235, 236].

In rupture-prone carotid plaques, the levels of Mrp-8, Mrp-14 and Mrp-8/14 are higher than in stable plaques[237], therefore Mrp-8/14 could be considered a marker of plaque destabilization and rupture.

In the current study, we showed that the both plaque and plasma Mrp-8/14 levels are higher in patients who suffered a recent stroke or TIA than in asymptomatic patients. Adding this observation to the previous evidence that symptomatic patients often have plaques with an unstable/rupture-prone phenotype while the asymptomatic ones have more stable lesions[244], we suggest that Mrp-8/14 could be involved in the plaque destabilization preceding or leading to rupture.

Consistent with this idea, we hypothesized that Mrp-8/14 levels (plaque and plasma) are high soon after the acute cerebrovascular ischemic event and decrease afterwards during plaque stabilization post event. For this, we investigated to which extent the Mrp-8/14 levels (plaque and plasma) fluctuate in time after a stroke or TIA until CEA. Interestingly, we showed here that Mrp-8/14 plaque levels are high within the first 30 days after an event and decrease afterwards, being significantly lower at 180 days; from 180 days onwards, plaque Mrp-8/14 levels appear to increase again, being significantly higher than in the 90-180 days interval (figure 2). This effect was mainly observed in patients who suffered a TIA (only a trend for stroke patients). This is relevant, in the context of clinical recurrent pattern of cardiovascular events (increased incidence of stroke and recurrent TIAs) from 90 days onwards following a TIA[245]. It also underlines the idea of atherosclerosis as a process with repetitive cellular and molecular changes resulting in sequential ruptures and/or erosions with thrombus formation; further, it strengthens the association of Mrp-plaque levels with the high-risk, rupture prone plaque demonstrated elsewhere[237].

Although high Mrp-8/14 plasma levels associate with symptomatic atherosclerosis (stroke and TIA), after the cerebrovascular event the levels did not changed in time (0 to 180 days) until CEA. In coronary disease patients, after an AMI, the serum Mrp-8/14 levels reached a peak (50% increase) at 3-5 days after the onset of AMI and showed still a high level at 6-8 days after (all compared to day 1, considered the baseline value)[246]. No study to our knowledge documented the time interval in which Mrp-8/14 levels are detectable in plasma or serum of patients with cardiovascular disease. Here, we bring proof that Mrp-8/14 levels are circulating unchanged in plasma of patients with cerebrovascular ischemia for at least 180 days.

Mrp-8 and Mrp-14 are expressed by subset of non-foam macrophages within human carotid plaques. The proportion of plaques showing high macrophage intensity (moderate/heavy staining) is high within 30 days after an acute ischemic event, decreases over time until 180 days but increases after more than 180 days (in TIA patients)[235]. In the current study, we showed a similar trend for the subset of plaque macrophages expressing Mrps; the incidence of plaques containing high percentage of Mrp-8 expressing macrophages was higher in plaques excised within the first 30 days after an event and showed a tendency of decreasing with time. This observation might imply that the macrophages expressing Mrp are involved in the processes leading to plaque disruption. The fate of these cells during the changes that occur in a plaque after rupture remains unknown. The levels of cytokines and matrix degrading proteases also fluctuate in time after an event: following stroke, IL-6

and IL-8 levels strongly decrease within 90 to 180 days. IL-6 and other cytokine levels (IL-2,-4,-5,-8,-10,-12p70, IFN-gamma and TNF-alpha) decrease initially but increase at later time points (>180 days); the same trend was seen for the matrix degrading proteases[235].

In summary, we conclude that plaque Mrp -8/14 levels and not plasma levels decrease over time after a cerebral event until 180 days after the event. After 180 days levels increase suggesting that after stabilization features of plaque vulnerability reappear.

Reference List

1 Rothwell PM, Gutnikov SA, Warlow CP. Reanalysis of the Final Results of the European Carotid Surgery Trial. Stroke 2003 February 1;34(2):514-23.

2 Brainin M, McShane LM, Steiner M, Dachenhausen A, Seiser A. Silent Brain Infarcts and Transient Ischemic Attacks : A Three-Year Study of First-Ever Ischemic Stroke Patients: The Klosterneuburg Stroke Data Bank. Stroke 1995 August 1;26(8):1348-52.

3 Johnston SC, Gress DR, Browner WS, Sidney S. Short-term Prognosis After Emergency Department Diagnosis of TIA. JAMA 2000 December 13;284(22):2901-6.

4 Verhoeven B, Hellings WE, Moll FL, de Vries JP, de Kleijn DP, de BP, Busser E, Schoneveld AH, Pasterkamp G. Carotid atherosclerotic plaques in patients with transient ischemic attacks and stroke have unstable characteristics compared with plaques in asymptomatic and amaurosis fugax patients. J Vasc Surg 2005 December;42(6):1075-81.

5 Peeters W, Hellings WE, de Kleijn DP, de Vries JP, Moll FL, Vink A, Pasterkamp G. Carotid atherosclerotic plaques stabilize after stroke: insights into the natural process of atherosclerotic plaque stabilization. Arterioscler Thromb Vasc Biol 2009 January;29(1):128-33.

6 Redgrave JN, Lovett JK, Gallagher PJ, Rothwell PM. Histological assessment of 526 symptomatic carotid plaques in relation to the nature and timing of ischemic symptoms: the Oxford plaque study. Circulation 2006 May 16;113(19):2320-8.

7 Ionita MG, Vink A, Dijke IE, Laman JD, Peeters W, van der Kraak PH, Moll FL, de Vries JP, Pasterkamp G, de Kleijn DPV. High Levels of Myeloid-Related Protein 14 in Human Atherosclerotic Plaques Correlate With the Characteristics of Rupture-Prone Lesions. Arterioscler Thromb Vasc Biol 2009 August 1;29(8):1220-7.

8 Foell D, Wittkowski H, Vogl T, Roth J. S100 proteins expressed in phagocytes: a novel group of damage-associated molecular pattern molecules. J Leukoc Biol 2007 January;81(1):28-37.

9 Hessian PA, Edgeworth J, Hogg N. MRP-8 and MRP-14, two abundant Ca(2+)-binding proteins of neutrophils and monocytes. J Leukoc Biol 1993 February 1;53(2):197-204.

10 Roth J, Burwinkel F, van den Bos C, Goebeler M, Vollmer E, Sorg C. MRP8 and MRP14, S-100-like proteins associated with myeloid differentiation, are translocated to plasma membrane and intermediate filaments in a calcium-dependent manner. Blood 1993 September 15;82(6):1875-83.

11 Katashima T, Naruko T, Terasaki F, Fujita M, Otsuka K, Murakami S, Sato A, Hiroe M, Ikura Y, Ueda M, Ikemoto M, Kitaura Y. Enhanced expression of the S100A8/A9 complex in acute myocardial infarction patients. Circ J 2010 March 25;74(4):741-8.

12 Verhoeven BA, Velema E, Schoneveld AH, de Vries JP, de BP, Seldenrijk CA, de Kleijn DP, Busser E, van der GY, Moll F, Pasterkamp G. Athero-express: differential atherosclerotic plaque expression of mRNA and protein in relation to cardiovascular events and patient characteristics. Rationale and design. Eur J Epidemiol 2004;19(12):1127-33.

13 Peeters W, Hellings WE, de Kleijn DPV, de Vries JPPM, Moll FL, Vink A, Pasterkamp G. Carotid Atherosclerotic Plaques Stabilize After Stroke: Insights Into the Natural Process of Atherosclerotic Plaque Stabilization. Arterioscler Thromb Vasc Biol 2009 January 1;29(1):128-33.

14 Verhoeven BA, Velema E, Schoneveld AH, de Vries JP, de BP, Seldenrijk CA, de Kleijn DP, Busser E, van der GY, Moll F, Pasterkamp G. Athero-express: differential atherosclerotic plaque expression of mRNA and protein in relation to cardiovascular events and patient characteristics. Rationale and design. Eur J Epidemiol 2004;19(12):1127-33.

15 Verhoeven B, Hellings WE, Moll FL, De Vries JP, De Kleijn DPV, de Bruin P, Busser E, Schoneveld AH, Pasterkamp G. Carotid atherosclerotic plaques in patients with transient ischemic attacks and stroke have unstable characteristics compared with plaques in asymptomatic and amaurosis fugax patients. Journal of Vascular Surgery 2005 December;42(6):1075-81.

16 Johnston SC, Gress DR, Browner WS, Sidney S. Short-term Prognosis After Emergency Department Diagnosis of TIA. JAMA 2000 December 13;284(22):2901-6.

CHAPTER VII

High myeloid - related protein 8/14 levels are related to an increased risk of recurrent cardiovascular events after carotid endarterectomy

Stroke. 2010 September

Mihaela G. Ionita, Louise M. Catanzariti, Michiel L. Bots, Jean-Paul P.M. De Vries, Frans L. Moll, Siu Kwan Sze, Gerard Pasterkamp And Dominique P.V. De Kleijn

Objective

Myeloid-related protein (Mrp) 8/14 complex is the functional relevant form of Mrp-8 and Mrp-14. Mrp-8/14 complex is actively formed in the cytoplasm of circulating neutrophils and monocytes and then secreted. Plasma Mrp-8/14 complex is emerging as a new biomarker that may discriminate between patients with an acute coronary syndrome and those with stable coronary heart disease. Little is known about the predictive value of Mrp - 8/14 plaque and plasma levels for cardiovascular events after atherectomy.

Methods and Results

Plasma and plaque Mrp-8/14 levels were determined by ELISA in 230 consecutive patients (mean age 73), who underwent carotid endarterectomy. Patients were followed for three years for recurrent cardiovascular events (vascular death, nonfatal vascular event and peripheral intervention). During follow-up, 62 patients experienced an event. Baseline Mrp-8/14 levels were higher in patients who experienced an event than in event-free patients (plasma 0.78±0.63 versus 0.57±0.67 mg/L, p = 0.030 and plaque 0.54±1.23 versus 0.08±1.51mg/kg, p = 0.027). In a Cox model, a 1 unit increase in log Mrp-8/14 was associated with an increased risk of recurrent events (plasma, HR 1.51, 95% CI [1.02 – 2.23], p = 0.040 and plaque, HR 1.23, 95% CI [1.04 – 1.46], p = 0.018). After multivariate adjustment for risk factors (both plasma and plaque Mrp-8/14) and plaque characteristics (only plaque Mrp-8/14), the HR remained the same for both plasma (HR 1.50, 95%CI [1.01 – 2.30], p = 0.046) and plaque (HR 1.20, 95%CI [1.01 – 1.44], p = 0.042).

Conclusion

High Mrp-8/14 plasma and plaque levels are related to an increased risk of adverse cardiovascular events following a carotid endarterectomy, independent of traditional cardiovascular risk factors.

Introduction

Disruption and thrombosis of an atherosclerotic plaque may lead to severe clinical presentations such as acute ischemic attacks in the heart and brain, with morbidity and mortality for the patient. Inflammation is pivotal for the development and destabilization of an atherosclerotic plaque; therefore a large number of inflammatory proteins have been investigated in prospective studies and they have been found to relate to cardiovascular events[260].

Myeloid - related protein (Mrp) 8 and 14 are inflammation-associated proteins, expressed by inflammatory cells like neutrophils, monocytes[239] and macrophages[261]. Upon cell activation, Mrp-8 and Mrp-14 form the Mrp-8/14 complex, which is transferred to the cell membrane and/or secreted[262]. The Mrp-8/14 complex is considered the functional relevant form of Mrp-8 and Mrp-14, being a cytokine and chemokine-like protein[263]. Mrp-8/14 is expressed in both mouse[264] and human atherosclerotic plaques[237]. Mice lacking the Mrp-8/14 have reduced atherosclerotic lesion area and macrophage accumulation within the lesions[264], supporting a role for Mrp in plaque formation and development. In human carotid endarterectomy (CEA) specimens, we previously described an association between high Mrp-8, -14 and -8/14 levels and the features of the rupture-prone lesions[237], suggesting a role for high levels of Mrp - proteins in plaque destabilization and disruption.

In human plasma, Mrp-8/14 levels were shown to be useful markers for monitoring disease activity in inflammatory diseases, like rheumatoid arthritis and Crohn disease[265]. In addition, plasma as well as local (thrombi) Mrp-8/14 levels are elevated in patients with an acute coronary syndrome (ACS) compared to patients with stable coronary artery disease (CAD) or patients with normal coronary arteries[30]. Moreover, levels of systemic Mrp-8/14 appear to increase prior to markers of myocardial necrosis (myoglobin, creatine kinase – MB and troponin) and high levels are associated with increased risk of recurrent cardiovascular events[31]. Plasma Mrp-8/14 levels were higher in patients with ST-segment-elevation MI compared to patients with stable CAD[32] and the risk of the first cardiovascular event increased with each quartile of plasma Mrp-8/14.

Taken together, these studies demonstrate that high Mrp-8/14 plasma levels are related to an increased risk of cardiovascular events in healthy (primary event) and coronary diseased patients (secondary event). Mrp-8/14 is expressed in human carotid plaques and high levels are associated with rupture-prone lesions; this might point to Mp-8/14 as a marker for plaque vulnerability. No study so far has evaluated the association of high Mrp-8/14 plaque levels and the occurrence of recurrent cardiovascular events after atherectomy.

To investigate this, we measured Mrp-8/14 levels locally (carotid plaque) and systemically (plasma) in patients undergoing CEA and studied the association with the occurrence of recurrent cardiovascular events during three years of follow-up.

Methods

Study population and design

Athero-Express is an ongoing longitudinal cohort study, initiated in 2002 by two 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, in patients undergoing CEA, the expression of atherosclerotic tissue derived biological markers in relation to plaque phenotype and the recurrence of cardiovascular events during follow up, as described previously[242].

In this study a set of 230 consecutive patients undergoing CEA between April 1, 2002 and March 1, 2006 were included. At baseline clinical parameters including cardiovascular risk factors and medication use were documented and recorded. Exclusion criteria for follow-up were: unwillingness or physical incapability to participate (e.g. severe dementia).

Clinical presentations of atherosclerotic carotid disease, prior to CEA.

Patients included for CEA were asymptomatic (no clinical symptoms related to the carotid luminal stenosis > 75%, n = 47) and symptomatic (n = 183); with minor clinical presentations (i.e., transient ischemic attack, amaurosis fugax and retinal infarction; n = 127) or major presentations (i.e., stroke; n = 56). The time between the onset of symptoms and CEA (in days) was recorded as previously described[235]. We reported an association between the time of symptoms onset until CEA and cellular and molecular changes in plaques[235]. In short, we showed that symptomatic plaques are associated with a rupture-prone phenotype and remodel into more stable plaques over time following stroke; therefore these temporal plaque phenotypic changes should be taken into account when analyzing plaque Mrp-8/14 as a biomarker.

Follow-up

Patients underwent clinical follow-up 1 year after CEA and completed postal questionnaires 1, 2 and 3 years after the surgery. Adjudication of the outcome events was done by a committee, consisting of three surgeons, who were blinded to laboratory results. All endpoints were independently assessed by two members of the committee.

Clinical Outcome

The primary outcome was defined as a composite of events including: any death of vascular origin (fatal stroke, fatal myocardial infarction, sudden death, and other vascular death), non-fatal stroke, non-fatal myocardial infarction, and any arterial vascular intervention

that had not already been planned at the time of inclusion (i.e. carotid surgery or angioplasty, coronary artery bypass, percutaneous coronary artery intervention, peripheral vascular surgery or angioplasty). In addition, we defined three subgroups regarding the clinical outcome in different vascular territories: coronary, stroke and peripheral; and a composite group of major outcomes. Coronary outcomes include myocardial infarction (fatal and non-fatal), coronary artery bypass, coronary artery intervention and sudden death. Stroke outcomes include non-fatal and fatal stroke. Peripheral outcomes include leg amputation and peripheral arterial intervention that had not been planned at the time of inclusion. Major outcomes include myocardial infarction (fatal and non-fatal), stroke (fatal and non-fatal), coronary artery bypass, coronary artery intervention and sudden death.

Carotid endarterectomy specimens and blood collection

All carotid plaques were dissected from the carotid arteries during surgery and immediately transferred to the laboratory for further processing as described previously[242]. 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; adjacent segments were used for protein isolation. Plaques were categorized as no/minor staining or moderate/heavy staining for the following stains: Hematoxylin and Eosin (HE), Pico Sirius Red (PSR, for total collagen), anti-CD68 immunostain (for macrophages) and anti alpha-1 actin immunostain (SMA-1, for vascular smooth muscle cells). Using the HE and PSR stains, the size of the lipid core was estimated as percentage of total plaque area and divided in to three categories: <10%, 10-40% and >40%. Overall plaque phenotype is based on the size of the lipid core, the amount of collagen and the extent of macrophage and smooth muscle cell (SMC) infiltration. Plaques with a lipid core size greater than 40% of the plaque area, with high macrophage infiltration, low collagen levels, and low SMC infiltration were identified as rupture-prone plaques. Intra plaque hemorrhage was scored using HE, fibrin (Mallory's phosphotungstic acid-hematoxilin) and anti smooth muscle actin; immunostains are reported in this study as absent (no) or present (yes). Blood is withdrawn prior to surgical incision for CEA and plasma is stored at -800 C until further use.

Immunoassays for Mrp-8/14

Levels of Mrp-8/14 heterodimers were measured with a commercial ELISA (Bühlmann Laboratories AG, Schönenbuch, Switzerland). For each patient, 50 µg of plaque Tris-protein and 5µl heparin plasma were used. The detection limits for plaque and plasma Mrp-8/14 were 10µg/g and 4ng/ml, respectively. The average inter-assay variability was 2.5%. The ELISA kit is specific for the Mrp-8/14 heterodimers and the cross-reactivity with Mrp-8 and -14 homodimers is minimal (according to the manufacturer).

Data analysis

Medians with inter quartile ranges (IQRs), means with standard deviations (SDs) or proportions for baseline clinical characteristics were computed for patients with and without secondary cardiovascular events during three years of follow-up. The distributions of plasma and plaque Mrp-8/14, low density lipoprotein (LDL), triglycerides (TG) and high sensitivity C reactive protein (CRP) were skewed therefore they were log transformed. The differences between two variables were tested by the t-test (continuous versus continuous/categorical) or by chi-square (categorical versus categorical). The correlation between two variables was assessed by Spearman's or Pearson's correlation test. Cox proportional hazard models were used to assess the independent relation with recurrent CV events for a 1 unit increase of log transformed Mrp-8/14 (plasma or plaque) data. Results are presented as hazard ratios (HRs) with their 95% confidence intervals (CIs). First, crude hazard ratios were calculated (Cox-regression, Enter method). Next, results were adjusted for age (continuous), male gender (yes/no), smoking status (yes/no), hypertension (yes/no), hypercholesterolemia (yes/no), diabetes mellitus (yes/no), history of coronary interventions, peripheral interventions, stroke or MI (yes/no), the use of statins prior to operation (yes/no). Results for plasma Mrp-8/14 were further adjusted for serum CRP (continuous). Results for plaque Mrp-8/14 were further adjusted for plaque characteristics (i.e., large lipid core (yes/no), high macrophage infiltration (yes/no), low collagen (yes/no), low SMC infiltration (yes/no), intra plaque hemorrhage (yes/no)) and for time between symptom onset and CEA (continuous). A backward method leaving variables with a p-value > 0.1 step-wise out was used. Statistically significant associations with clinical outcome were defined as a 95% confidence interval (CI) not including 1 or a p-value < 0.05. For statistical analyses SPSS 15.0 was used (SPSS Inc., Chicago, Illinois).

Results

Baseline characteristics

The current study is based on a cohort of 230 patients suffering from carotid atherosclerotic disease. The cohort was 68% male, with an average age of 73 years, a mean BMI of 26.3 (± 3.9), 70% were hypertensive, 54% had hypercholesterolemia and 19% had diabetes mellitus. Baseline characteristics are provided in Table 1. 20% of the patients reported a previous manifestation of atherosclerotic disease (i.e. previous stroke, myocardial infarction, coronary or peripheral intervention). At the time of inclusion for CEA, 80% of the patients were symptomatic with minor clinical presentations (i.e., transient ischemic attack, amaurosis fugax and retinal infarction; n = 127) or major presentations (i.e., stroke; n = 56); 47 patients were asymptomatic. Prior to CEA, 67% were on statin therapy, while a

Table 1. Characteristics of the study population, by cardiovascular event status during follow-up

Variable	All patients (n = 230)	Non-Event (n = 168)	Event (n = 62)	p-value
Age, mean/sd, years	73/8.6	72.1/8.3	74.7/9.3	0.039*
Males, % (n)	68% (157)	65% (109)	77% (48)	0.047*
Current smoker, % (n)	26% (60)	25% (42)	29% (18)	0.412
BMI, mean/sd	26.3/3.9	26.4/4.2	26.3/3.3	0.902
Hypertension, % (n)	70% (160)	69% (115)	73% (45)	0.546
Hypercholesterolemia, % (n)	54% (125)	53% (88)	60% (37)	0.532
Diabetes, % (n)	19% (43)	19% (32)	18% (11)	0.822
History: Coronary intervention, % (n)	22% (51)	17% (29)	35% (22)	0.003*
History: Peripheral intervention, % (n)	20% (46)	18% (31)	24% (15)	0.217
History: Stroke, % (n)	30% (70)	31% (52)	29% (18)	0.476
History: MI, % (n)	21% (49)	18% (31)	29% (18)	0.190
Plaque Mrp-8/14†, mean/sd	0.21/1.47	0.08/1.51	0.54/1.23	0.027*
Plasma Mrp-8/14†, mean/sd	0.63/0.66	0.57/0.67	0.78/0.63	0.030*
TG†, mean/sd	0.44/0.49	0.45/0.50	0.44/0.49	0.927
LDL†, mean/sd	0.98/0.34	1.00/0.32	0.93/0.38	0.247
HDL, mean/sd	1.16/0.37	1.19/0.39	1.05/0.32	0.005*
CRP†, mean/sd	1.20/1.22	1.16/1.19	1.34/1.31	0.354
Symptomatic carotid stenosis, % (n)	80% (183)	80% (135)	77% (48)	0.353
Time between event onset and CEA, days, median (IQR)	90 (43.5-146)	92 (48-146.5)	69 (19-136.5)	0.095
Statines, % (n)	67 % (154)	64% (107)	76% (47)	0.201
Aspirin, % (n)	43% (98)	41% (69)	47% (29)	0.631
Oral Anticoagulants, % (n)	15% (34)	12% (20)	23% (14)	0.111
Plaque characteristics				
Large lipid core, % (n) (>40% of plaque area)	27% (63)	26% (44)	31% (19)	0.509
High macrophage infiltration, % (n)	46% (105)	44% (74)	50% (31)	0.455
Low collagen amount, % (n)	17% (40)	16% (26)	23% (14)	0.237
Low SMCs infiltration, % (n)	24% (56)	24% (40)	26% (16)	0.730
Intraplaque hemorrhage, % (n)	77% (178)	77% (130)	77% (48)	0.628

BMI indicates body mass index; MI indicates myocardial infarction; TG indicates triglycerides; LDL indicates low density lipoprotein in mmol/l ; HDL indicates high density lipoprotein in mmol/l ; CRP indicates C reactive protein in mg/L; IQR indicates interquartile range; SMCs indicates smooth muscle cells; * p< 0.05; † Log transformed.

smaller percentage was using aspirin (43%) or oral anticoagulants (15%; Table 1).
During three years of follow-up (mean follow-up 2.46 years, range 0.01 to 3.00), 62 patients reached a primary outcome; 14 patients had a non- or a fatal stroke; 11 patients had non- or fatal MI; 26 patients had a peripheral intervention and 21 patients died of cardiovascular disease. Patients who developed a recurrent event during follow-up were significantly older, were more frequently men, had lower HDL levels and had more coronary interventions (percutaneous coronary intervention or surgery) in the past (table 1). Mrp-8/14 (plasma and plaque) levels were considerably higher in patients with recurrent events than in those without (table 1). Serum CRP levels were correlated with plasma Mrp-8/14 levels (Pearson's correlation coefficient, $r = 0.50$, $p < 0.001$); no correlation with plaque Mrp-8/14 levels was observed ($r = 0.088$; $p = 0.189$). Time between symptom onset and CEA showed a weak inverse correlation with plaque Mrp-8/14 levels (Spearman's correlation coefficient, $r = -0.16$, $p = 0.045$); no correlation with plasma Mrp-8/14 was observed ($r = -0.018$; $p = 0.824$). No correlation between plasma and plaque Mrp-8/14 levels was observed (Spearman's correlation coefficient $r = 0.121$, $p = 0.068$). High plaque Mrp-8/14 levels were correlated with the size of the lipid core ($r = 0.235$; $p < 0.001$), collagen amount ($r = - 0.209$; $p = 0.001$), SMC infiltration ($r = -0.235$; $p < 0.001$) and presence of intra plaque hemorrhage ($r = 0.242$; $p = 0.001$).

Mrp-8/14 levels and the risk of recurrent cardiovascular events

We determined whether Mrp-8/14 levels (plasma and plaque) differed between patients who developed a recurrent cardiovascular event during the three years following their CEA and patients who remained free of events.

Table 2. Hazard ratio (HR) for any CV-events (combined outcome) by 1- unit increase in log Mrp-8/14 plasma or plaque:

Model	HR [95 % CI]	P - value
Plasma Mrp-8/14		
Unadjusted	1.51 [1.02 – 2.23]	0.040
Multivariable adjustment *	1.50 [1.01 – 2.30]	0.046
Multivariable and CRP adjustment	1.57 [1.05 – 2.36]	0.030
Plaque Mrp-8/14		
Unadjusted	1.23 [1.04 – 1.46]	0.018
Multivariable adjustment *#	1.20 [1.01 – 1.44]	0.042
Multivariable and Time between symptom and CEA	1.26 [1.01 – 1.57]	0.038

*Multivariable adjustment for age, male gender, smoking status, hypertension, hypercholesterolemia, diabetes mellitus, history of coronary or peripheral intervention, history of stroke or myocardial infarction, use of statins, plaque characteristics and intraplaque hemorrhage. # Further adjustment for plaque's lipid core size, collagen amount, macrophage and SMCs infiltration and intra plaque hemorrhage.

In a model containing only plasma Mrp-8/14, a 1 unit increase in log Mrp-8/14 was associated with a 51% increase in risk of any recurrent cardiovascular event (HR 1.51, 95% CI [1.02 – 2.23], p = 0.040). Multivariate adjustments for traditional risk factors, did not attenuate the relationship (HR 1.50, 95%CI [1.01 – 2.30], p = 0.046). Addition of serum CRP to the multivariable model lead to similar findings (HR 1.57, 95% CI [1.05 – 2.36], p = 0.030, table 2).
Similar analysis was done for plaque Mrp-8/14: a model containing only plaque Mrp-8/14 showed that a 1 unit increase in log Mrp-8/14 was associated with a 23% increase in risk of any recurrent cardiovascular event (HR 1.23, 95% CI [1.04 – 1.46], p = 0.018). Adjustments for traditional risk factors and plaque characteristics did not change the risk (HR 1.20, 95%CI [1.01 – 1.44], p = 0.042). Further, addition of time between symptom onset and CEA to the multivariate model did not materially affect the relation (HR 1.26, 95% CI [1.01 – 1.57], p = 0.038, table 2).

Discussion

In the present cohort of patients with severe carotid atherosclerotic disease undergoing CEA, we investigated the risk of recurrent cardiovascular events associated with Mrp-8/14 plasma and plaque levels. Both plasma and plaque Mrp-8/14 were elevated in patients who developed a recurrent cardiovascular event compared to those who remained free of events. Both markers were strongly and independently related to an increased risk of recurrent cardiovascular events even after adjustment for traditional risk factors, history of previous events or interventions, medication and plaque characteristics.
The finding that Mrp-8/14 levels measured in plaque relate to an increased risk of recurrent cardiovascular events in any vascular territory, underlies the previously described concept postulating that local plaque protein levels may reflect an individuals constitution to build unstable plaques[266].
We previously showed that after an event (i.e. stroke) until the time of CEA, carotid plaques stabilize at a molecular and cellular level[235]. In the current study, a weak association between plaque Mrp-8/14 levels and the time between symptom onset and CEA was observed, therefore we corrected for this parameter in the model of plaque Mrp-8/14. Plasma Mrp-8/14 levels showed no correlation.
In the past, we reported an association between high Mrp-plaque levels and plaque characteristics: size of lipid core > 40% plaque area, high macrophage infiltration, low collagen and reduced SMC infiltration[237]. Moreover, in the current study an association between plaque Mrp-8/14 levels and the presence of intra plaque hemorrhage was observed. In this context, we decided to correct for the aforementioned plaque characteristics and for intra plaque hemorrhage in the model for plaque Mrp-8/14 although, as recently shown

by our group, the lipid core size and the macrophage numbers in local plaques are not predictive for events originating from other plaques within the vasculature[267].

It would be interesting to know whether Mrp-8/14 plasma levels change once the plaque is removed from the patient. In the present study, we were unable to measure plasma Mrp-8/14 levels after CEA since we only collect blood prior to CEA. Nevertheless, a correlation between plasma and plaque Mrp-8/14 was not seen which may suggest that only a small proportion of the circulating Mrp-8/14 levels originate from atherosclerotic plaque. In addition, we analyzed plasma and plaque Mrp-8/14 levels by the elapsed time from symptom onset (stroke or TIA) to CEA. We found that plaque Mrp-8/14 levels slightly decreased over this time ($r = -0.16$, $p = 0.045$), while plasma Mrp-8/14 levels remained constant ($r = -0.018$; $p = 0.824$). This observation suggests that the local carotid plaque may not be the dominant source of Mrp-8/14 in plasma, however, we cannot rule out plaques as a source. The possibility of other tissue origins of Mrp-8/14 in plasma from our patients remains uncertain.

Two studies, both focusing on coronary events (first or second manifestation) showed that plasma Mrp-8/14 was related to future coronary events. In an ACS cohort, high plasma Mrp-8/14 levels were related to the risk of a first cardiovascular event in apparently healthy postmenopausal women[32]. In this prospective, nested-case control study ($n = 255$ case-control pairs), apparently healthy women, matched for age and smoking, were included and followed for approximately 3 years and any cardiovascular events (nonfatal MI, stroke and cardiovascular death) were documented. Women that developed cardiovascular events during follow-up had higher Mrp-8/14 plasma levels at baseline, than women who remained free of events; the relative risks according to increasing quartiles of Mrp-8/14 were 1.0, 1.7, 1.7 and 2.3 ($p = 0.03$).

Another nested case-control study[31] ($n = 237$ case control pairs) included patients with a first presentation of MI or unstable angina and discharged with moderate/intensive statin treatment; patients were matched for age, gender and smoking status. At 30 days after the ACS, the Mrp-8/14 plasma levels were measured. Patients were then followed for 24 months and endpoints such as cardiovascular death or new MI were documented. The authors found significantly higher Mrp-8/14 plasma levels in patients with endpoints than in patients without; after adjusting for risk factors the relative odds of CV death or MI increased significantly with each increasing quartile of Mrp-8/14: 1.0, 1.3, 1.7 and 2.0 ($p = 0.024$).

In our study, we also investigated the association between plasma Mrp-8/14 and the risk of coronary events and interventions after CEA. We found that a 1 unit increase of log Mrp-8/14 was related to a 108% increase in risk of coronary events or interventions, however, after multivariate adjustments for traditional risk factors the risk decreased to 98% and was not statistically significant (table 3). The present study has a small number of recurrent coronary events and this might diminish the power of the study.

Table 3. Hazard ratio (HR) for different CV-events of 1-unit increase in log Mrp-8/14 plasma or plaque:

Event (follow-up)		Plasma Mrp-8/14 HR [95% CI] (p - value)	Plaque Mrp-8/14 HR [95% CI] (p – value)
Major	Crude:	1.624 [0.950-2.777] (0.076)	1.065 [0.843-1.346] (0.598)
	Adjusted*#:	1.543 [0.906-2.626] (0.110)	0.871 [0.657-1.154] (0.336)
Stroke	Crude:	1.385 [0.620 – 3.092] (0.427)	0.950 [0.664 – 1.361] (0.782)
	Adjusted*#:	1.071 [0.462 – 2.482] (0.872)	0.885 [0.611 – 1.282] (0.519)
Coronary	Crude:	2.086 [1.036 – 4.200] (0.039)	1.255 [0.924 – 1.705] (0.145)
	Adjusted*#:	1.984 [0.970 – 4.061] (0.061)	1.219 [0.782 – 1.900] (0.383
Peripheral intervention	Crude:	1.471 [0.808 – 2.676] (0.206)	1.365 [1.042 – 1.788] (0.024)
	Adjusted*#:	1.432 [0.773 – 2.652] (0.254)	1.470 [0.901 – 2.398] (0.123)
Myocardial infarction	Crude:	1.801 [0.718 – 4.520] (0.210)	1.045 [0.696 – 1.570] (0.830)
	Adjusted*#:	1.800 [0.670 – 4.836] (0.244)	0.955 [0.647 – 1.408] (0.815)

*Adjusted for age, gender, smoking status, hypertension, hypercholesterolemia, diabetes mellitus, history of stroke, myocardial infarction, coronary and peripheral interventions, use of statins, plaque characteristics and intraplaque hemorrhage. # Plaque Mrp-8/14 was further adjusted for plaque's lipid core size, collagen amount, macrophage and SMCs infiltration and intra plaque hemorrhage.

Our cohort is different from those used in the previous studies, which were based on healthy cohorts[32] or cohorts of coronary diseased patients[31]. The current study included patients with recent cerebral ischemia (stroke, TIA). We followed the patients for 3 years after CEA and in comparison with the study of Morrow et al[31], we did not focus on recurrent coronary events but we documented all cardiovascular events in the follow-up. For the first time, we measured Mrp-8/14 levels in plasma and plaque in the same patient and we showed that both are related to recurrent cardiovascular events. Our findings indicate that plasma Mrp-8/14 reflects the patient's instability rather than just the instability of a local plaque.
The findings presented here could have clinical implications. We bring proof to support the concept that high Mrp-8/14 levels measured in plasma are a clinically useful tool in estimating the risk of recurrent cardiovascular events in severely diseased atherosclerotic patients. Furthermore, we show for the first time that high Mrp-8/14 levels measured also locally in plaque are associated with an increased risk of recurrent events. For imaging techniques (e.g. MRI, SPECT) which aim to target plaque proteins and use them to stratify patients at risk of recurrent events, plaque Mrp-8/14 might be a potential target.

In conclusion, we have expanded the existing evidence by showing that high Mrp-8/14 levels (local (plaque) and systemic (plasma)) are independently related to an increased risk of adverse cardiovascular events following a carotid endarterectomy.

Reference List

1 Pearson TA, Mensah GA, Alexander RW, Anderson JL, Cannon RO, III, Criqui M, Fadl YY, Fortmann SP, Hong Y, Myers GL, Rifai N, Smith SC, Jr., Taubert K, Tracy RP, Vinicor F. Markers of Inflammation and Cardiovascular Disease: Application to Clinical and Public Health Practice: A Statement for Healthcare Professionals From the Centers for Disease Control and Prevention and the American Heart Association. Circulation. 2003;107:499-511.

2 Hessian PA, Edgeworth J, Hogg N. MRP-8 and MRP-14, two abundant Ca(2+)-binding proteins of neutrophils and monocytes. J Leukoc Biol. 1993;53:197-204.

3 Edgeworth J, Gorman M, Bennett R, Freemont P, Hogg N. Identification of p8,14 as a highly abundant heterodimeric calcium binding protein complex of myeloid cells. Journal of Biological Chemistry. 1991;266:7706-7713.

4 Teigelkamp S, Bhardwaj RS, Roth J, Meinardus-Hager G, Karas M, Sorg C. Calcium-dependent complex assembly of the myeloic differentiation proteins MRP-8 and MRP-14. Journal of Biological Chemistry. 1991;266:13462-13467.

5 Nacken W, Roth J, Sorg C, Kerkhoff C. S100A9/S100A8: Myeloid representatives of the S100 protein family as prominent players in innate immunity. Microsc Res Tech. 2003;60:569-580.

6 Croce K, Gao H, Wang Y, Mooroka T, Sakuma M, Shi C, Sukhova GK, Packard RRS, Hogg N, Libby P, Simon DI. Myeloid-Related Protein-8/14 Is Critical for the Biological Response to Vascular Injury. Circulation. 2009;120:427-436.

7 Ionita MG, Vink A, Dijke IE, Laman JD, Peeters W, van der Kraak PH, Moll FL, de Vries JP, Pasterkamp G, de Kleijn DPV. High Levels of Myeloid-Related Protein 14 in Human Atherosclerotic Plaques Correlate With the Characteristics of Rupture-Prone Lesions. Arterioscler Thromb Vasc Biol. 2009;29:1220-1227.

8 Liao H, Wu J, Kuhn E, Chin W, Chang B, Jones MD, O'Neil S, Clauser KR, Karl J, Hasler F, Roubenoff R, Zolg W, Guild BC. Use of mass spectrometry to identify protein biomarkers of disease severity in the synovial fluid and serum of patients with rheumatoid arthritis. Arthritis Rheum. 2004;50:3792-3803.

9 Altwegg LA, Neidhart M, Hersberger M, Muller S, Eberli FR, Corti R, Roffi M, Sutsch G, Gay S, von Eckardstein A, Wischnewsky MB, Luscher TF, Maier W. Myeloid-related protein 8/14 complex is released by monocytes and granulocytes at the site of coronary occlusion: a novel, early, and sensitive marker of acute coronary syndromes. Eur Heart J. 2007;28:941-948.

10 Morrow DA, Wang Y, Croce K, Sakuma M, Sabatine MS, Gao H, Pradhan AD, Healy AM, Buros J, McCabe CH, Libby P, Cannon CP, Braunwald E, Simon DI. Myeloid-related protein 8/14 and the risk of cardiovascular death or myocardial infarction after an acute coronary syndrome in the Pravastatin or Atorvastatin Evaluation and Infection Theraphy: Thrombolysis in Myocardial Infarction (PROVE IT-TIMI 22) trial. American Heart Journal. 2008;155:49-55.

11 Healy AM, Pickard MD, Pradhan AD, Wang Y, Chen Z, Croce K, Sakuma M, Shi C, Zago AC, Garasic J, Damokosh AI, Dowie TL, Poisson L, Lillie J, Libby P, Ridker PM, Simon DI. Platelet Expression Profiling and Clinical Validation of Myeloid-Related Protein-14 as a Novel Determinant of Cardiovascular Events. Circulation. 2006;113:2278-2284.

12 Verhoeven BA, Velema E, Schoneveld AH, de Vries JP, de BP, Seldenrijk CA, de Kleijn DP, Busser E, van der GY, Moll F, Pasterkamp G. Athero-express: differential atherosclerotic plaque expression of mRNA and protein in relation to cardiovascular events and patient characteristics. Rationale and design. Eur J Epidemiol. 2004;19:1127-1133.

13 Peeters W, Hellings WE, de Kleijn DP, de Vries JP, Moll FL, Vink A, Pasterkamp G. Carotid atherosclerotic plaques stabilize after stroke: insights into the natural process of atherosclerotic plaque stabilization. Arterioscler Thromb Vasc Biol. 2009;29:128-133.

14 de Kleijn DPV, Moll FL, Hellings WE, Ozsarlak-Sozer G, de Bruin P, Doevendans PA, Vink A, Catanzariti LM, Schoneveld AH, Algra A, Daemen MJ, Biessen EA, de Jager W, Zhang H, de Vries JP, Falk E, Lim SK, van der Spek PJ, Sze SK, Pasterkamp G. Local Atherosclerotic Plaques Are a Source of Prognostic Biomarkers for Adverse Cardiovascular Events. Arterioscler Thromb Vasc Biol. 2009;ATVBAHA.

15 Hellings WE, Peeters W, Moll FL, Piers SRD, van Setten J, van der Spek PJ, de Vries JP, Seldenrijk KA, De Bruin PC, Vink A, Velema E, de Kleijn DPV, Pasterkamp G. Composition of Carotid Atherosclerotic Plaque Is Associated With Cardiovascular Outcome. A Prognostic Study. Circulation. 2010;CIRCULATIONAHA.

CHAPTER VIII
General discussion

PREFACE

The present doctoral thesis comprises findings with possible implications for the pathobiology of atherosclerosis and its clinical applications. Atherosclerosis, "a disease as old as the mankind", it remains the first malignancy of the Modern World with a high morbidity and mortality in the elderly. Annually, 12 million deaths in the world are caused by the complications of atherosclerotic artery disease (e.g. cerebral stroke and acute myocardial infarction) despite considerably improvements reached in the clinical decisions and treatment of these patients. In the latest decade, the field witnessed a deviation from the standard focus on the pathology of the "vulnerable plaque" towards a broader approach, the identification of the "vulnerable patient" (both terms described in Chapter I). Along with intensive research on the pathobiology of atherosclerosis (basic molecular and animal studies), research strategies are directed towards the discovery of potential (bio) markers for imaging applications or for screening of patients at high risk for CVD. The biobank Athero-Express (described in Chapter I, Study design) is a good and unique research-platform to perform studies on both the vulnerable plaque and the vulnerable patient.

Myeloid-related proteins, markers for the Vulnerable Plaque

It is well known that prevention of adverse cardiovascular events such as myocardial infarction, sudden cardiac death or cerebral stroke is a milestone still to be reached. In spite of the aggressive systemic treatment that patients with cardiovascular disease have to take (e.g. aspirin, cholesterol lowering statins, angiotensin converting enzyme inhibitors), adverse cardiovascular events still occur[1-3]. In addition to the systemic therapies, efforts to identify and treat the local vulnerable plaque are now considered. *What is a vulnerable plaque* is a frequently raised question in the field of atherosclerosis, which remains unanswered. In **Chapter I**, a summary of the current definitions of the "vulnerable plaque" is given. Since most definitions of *the vulnerable plaque* are based on cross-sectional studies of human atherosclerotic specimens (mostly coronary and few carotids), without natural history studies being available, no one really knows what a vulnerable plaque looks like. Each year, new articles are published over the pathobiology, diagnosis, and potential management and the vulnerable plaque is a favourite highlight on most international symposia. Despite all efforts, until now no consensus has been reached regarding the criteria to identify and treat such plaques.

Overall in this thesis, *the vulnerable plaque* is referred to as a rupture-prone plaque with a large lipid core, high influx of macrophages, reduced collagen amount and smooth muscle cell content, high density of plaque micro-vessels, and high levels of cytokines (e.g. IL-6, IL-8) and MMPs (e.g. MMP-8, MMP-9). These characteristics are derived from cross-sectional studies on coronary lesions which also apply to carotid lesions[4].

In these rupture-prone plaques (with the characteristics mentioned above) the levels of Mrp-8, Mrp-14 and Mrp-8/14 complex are considerably higher than in stable plaques (as presented in **Chapter III**). Therefore, Mrp-8, Mrp-14 and Mrp-8/14 should be considered markers for the high risk, vulnerable plaque.

Are such markers (proteins) important? What would be their clinical utility? For a local therapy to be completely effective, all vulnerable plaques that might cause adverse events should be identified. With regards to *in vivo* detection of the vulnerable plaque, invasive (e.g. angioscopy, intravascular ultrasound, molecular imaging, thermography etc.) and non-invasive (e.g. magnetic resonance imaging, computed tomography, etc.) devices are used. These include devices that can identify a lipid-rich necrotic core, the thin fibrous cap, plaque inflammation (e.g. macrophages) and the vasa vasorum. The availability of all these techniques rarely translates into the clinical utility because the question *what is a vulnerable plaque?* still applies. If one should rely on the assumption that a vulnerable plaque is a plaque with the previously mentioned morphological characteristics, then how thin should the fibrous cap be, how large should the lipid core be, and how much inflammation should be present to make it vulnerable at the moment when is found and lead to a rupture resulting in a cardiovascular event[5]? At this point, markers (proteins) might prove helpful; markers such as Mrp-8, Mrp-14 and Mrp-8/14. Since the levels of

Mrps are higher in rupture-prone plaques than in stable ones (**Chapter III**), they might constitute prime candidates for *in vivo* the detection of high risk plaques. Such proteins could be targeted with specific antibodies (administered locally or systemically) and visualized inside the lesions across the vasculature by, for example, non-invasive magnetic resonance imaging. Those lesions which show a high intensity (translated into high levels of Mrp-8, -14 or -8/14) should be regarded as the most vulnerable ones. As appealing as such a technique might sound, at the present researchers perform the first tests in animal models and it may take years until it will reach clinical application. Even so, simply identifying the vulnerable plaques won't be enough; an efficient interventional therapy to combat these plaques should be available. For this purpose, proteins identified as markers for the dangerous lesions could prove helpful.

Food for Thought

The vulnerable plaque is the underlining condition of acute coronary syndromes and cerebral strokes. The identification of the vulnerable plaque in vivo is a milestone to be reached. Current strategies involve the use of the classical features: size of the lipid core, fibrous cap thickness and amount of inflammation. New, reliable, and better accessible markers are desirable. Mrp-8, Mrp-14 and Mrp-8/14 levels are higher in vulnerable plaques than in stable ones, making these proteins markers for the high risk, rupture prone plaque and appealing targets for in vivo detection of these lesions.

Myeloid-related proteins are expressed by inflammatory cells in atherosclerotic plaques

Virchow's view that atheroma is the result of chronic inflammatory disease of the intima persisted throughout the nineteenth century and is consistent with the current views of the pathogenesis of atherosclerosis. There is a vast body of literature on the involvement and role of inflammatory cell types (i.e. macrophages, lymphocytes, and less numerous cells like mast cells and neutrophils) in atherogenesis (reviewed in **Chapter I**). Among all cell types present in a plaque, macrophages are the most numerous and their capacity to phagocyte modified lipids and transform into foam cells, made them a "hallmark"of the disease. In consequence, plaque macrophages gained enormous interest and investigators aimed to unravel all sides of macrophage biology in plaques. Due to these efforts, we know today that macrophages are beneficial but also detrimental for the plaque: their beneficial side relies on their phagocytic ability to engulf and process modified lipids and thus, to clean up the intima; their detrimental side is that once activated inside the intima (by various agents), they produce/secrete cytokines and matrix degrading/synthesising

proteases, inducing the atherosclerotic changes of the intima. Plaque macrophages express a large variety of proteins, among which calgranulins (Mrp-8, Mrp-14 and Mrp-8/14). Mrp-8 and Mrp-14 have been described in plaque macrophages, for instance in the study of McCormick et al.[6]; interestingly, the authors report that only lesion macrophages express Mrps, while the macrophages residing in the lesion-free arterial intima lack these proteins. In addition, the study of McCormick et al. reports the existence of a subset of plaque-macrophages expressing Mrps (not all CD68-positive macrophages are Mrp-positive; observations derived from 11 carotid and 9 aortic plaques).

Consistent with this observation, in **Chapter III** we report the expression of Mrp-8 and Mrp-14 in a subset of CD68-positive plaque macrophages (90 carotid plaques were stained and analyzed). A closer microscopy investigation revealed that the Mrp-expressing macrophages were not foam cells. Plaque foam macrophages, identified based on the CD68-expression correlated with the typical foam-cell morphology (intracellular fat, nucleus pushed to the side of the cytoplasm) were Mrp-8/14 negative. Interestingly, in rupture-prone, vulnerable plaques the percentage of non-foam macrophages expressing Mrps was higher than in stable plaques. In other words, substantially more non-foam macrophages in rupture-prone plaques bring Mrps to expression. One might argue that this observation is not surprising since rupture-prone plaques usually contain more macrophages and presumably more non-foam macrophages. It is true that one of the features that separate these plaques from the stable ones is the abundance of macrophages. However, the fact that the micro-environment of rupture-prone lesions attracts more Mrp-expressing macrophages or induces Mrp-expression in more macrophages is quite intriguing.

This led us to the next investigation, presented in **Chapter III**. We hypothesised that macrophages expressing Mrp-8, -14 and -8/14 will not acquire a foamy-phenotype when fed human modified lipids (in this study represented by oxLDL). Using an *in vitro* system to generate human monocyte-derived macrophages from healthy blood monocytes, we could prove that macrophages expressing Mrps (Mrp-8, -14 and -8/14) do not phagocyte human oxLDL and do not acquire a foamy-phenotype while those macrophages lacking Mrps do. Moreover, with this system we confirmed that a subset of human macrophages is Mrp-positive, since at the time of monocyte maturation into macrophages (after 7 days of culture under non-adherent conditions and without any further cytokine stimulation) only half of the population showed Mrp-8/14 expression. Corroborating these observations with those obtained from the histological analyses on human plaques, the following conclusion can be drawn: that rupture-prone plaques contain more Mrp-expressing non-foam macrophages than stable plaques, and these Mrp-macrophages are protected against foam-cell development. *How came that Mrp-8/14 expressing macrophages do not phagocyte oxLDL as the other plaque macrophages do? What is their role within complex plaque environment?*

We addressed these important questions by performing additional investigations in the *in vitro* system of monocyte-derived macrophages and foam cell formation (described in **chapter III** and **IV**). The findings are presented in **Chapter IV**. That Mrp-8/14 expressing monocyte-derived macrophages did not uptake lipids might be explained by reduction in expression of scavenger receptors on these cells; therefore we assessed the expression of CD36 and CD68, well known lipids-scavenger receptors and major mediators of foam cell formation and promoters of atherosclerosis in general[7]. Interestingly, the Mrp-8/14 expressing monocyte-derived macrophages showed either similar (for CD36) or increased (for CD68) expression of these scavenger receptors, compared with monocyte-derived macrophages lacking Mrp-8/14. Moreover, their overall phagocytic capacity was not altered (they ingested sugar dextran). In addition, expression of two scavenger receptors, CD163 and CD206 (also known as the mannose receptor) was increased on Mrp-8/14 expressing monocyte-derived macrophages compared to cells lacking Mrp-8/14. Macrophages use CD163 to bind and clear haemoglobin-haptoglobin complexes from the vessel wall; in human atherosclerotic lesions[8], $CD163^{high}$ macrophages secrete anti-oxidant and anti-inflammatory molecules (e.g. IL-10) in likely attempt to counter the atherogenic effects of plaque haemorrhage. The expression of CD163 is upregulated in vulnerable/unstable plaque regions as compared to stable areas[9] and thus CD163 might be a marker for high-risk atherosclerotic plaques. Similarly, Mrp-8/14 levels were high in the rupture-prone human plaques suggesting Mrp-8/14 as a marker for these plaques (**chapter III**). Because the macrophage subset expressing Mrp-8/14 also expressed high CD163, at least in our *in vitro* system, imply that *in vivo* these macrophages exert atheroprotective functions within the environment of a high-risk plaque. That Mrp-8/14 expressing macrophages might be atheroprotective is further supported by the observation that *in vitro* these cells secreted, in the presence of lipids, high amounts of anti-inflammatory molecules (e.g. IL-10 and TGF-beta) compared to macrophages lacking Mrp-8/14 (data shown in **chapter IV**). In contrast, macrophages lacking Mrp-8/14 secreted pro-inflammatory cytokines, such as IL-6.

Taken together, we propose that macrophages lacking and macrophages expressing Mrp-8/14 follow the dichotomy of classically (also known as M1) and alternatively (or M2) activated macrophages proposed in literature. Similar to the alternatively activated (M2) macrophages, Mrp-8/14 expressing macrophages, *in vitro*, have an increased scavenger receptor (CD68, CD163 and CD206) expression and secrete the anti-inflammatory, anti-atherogenic cytokines IL-10 and TGF-beta[10, 11]. Macrophages lacking Mrp-8/14 have increased CD16 surface expression and predominantly secrete the pro-inflammatory cytokine IL-6 *in vitro*, therefore resembling the classically activated (M1) macrophage subset[12]. Both populations of polarized macrophages, M1 and M2 have been found in human atherosclerotic plaques where the M2 macrophages populate more stable plaque regions situated distant from the lipid core[13]. The general concept is that M1 macrophages

promote atherosclerosis while the M2 macrophages inhibit plaque growth and mediate plaque stability. In line with this concept, in rupture-prone plaques macrophages lacking Mrp-8/14 expression might promote inflammation while macrophages expressing Mrp-8/14 might suppress the inflammatory reaction induced by the former. Within the complex environment of a rupture-prone, high-risk plaque the atheroprotective effects of Mrp-8/14 expressing macrophages might be counteracted by the proatherogenic effects of macrophages lacking Mrp-8/14.

Next to macrophages, neutrophils were identified as another cellular source for Mrp-8 and Mrp-14 within plaques (**Chapter V**). Neutrophils were identified based on a specific membrane marker CD66b and scored in a large number of human carotid plaques. Although less numerous than macrophages, neutrophils are part of the plaque's cellular compartment. Neutrophils are known to migrate to and infiltrate into inflamed tissues, such as plaques, where they respond to inflammatory stimuli (e.g. IL-8) by releasing their granules filled with tissue destructive proteins (e.g. MMPs, elastases, etc). In the study presented in chapter V, all neutrophils infiltrated into plaques expressed Mrp-8 and Mrp-14, and no negative cells were observed. Moreover, the number of neutrophils was significantly higher in rupture-prone plaques than in stable ones. The distribution of neutrophils within plaques proved to be heterogeneous: these cells were seen underneath the luminal endothelium, around micro-vessels within the cap and shoulder regions, and in areas with intraplaque haemorrhage. Thus, neutrophils infiltrate into plaques through the damaged endothelium, through the plaque vasa vasorum and via plaque bleeding. Whether this is an active phenomenon, it is a matter of debate. Here some evidence supporting the idea of active infiltration. First, the levels of IL-8 (a powerful neutrophil chemo-attractant) are high in rupture-prone plaques and associate with high numbers of neutrophils (data shown in **Chapter V**). So, the signal required to attract the neutrophils in plaques is present; IL-8 is synthesized and expressed by plaque macrophages, T-lymphocytes and endothelial cells. Secondly, the luminal endothelium and the endothelium of the vasa vasorum express adhesion molecules like P-selectin, a neutrophil adhesion molecule required for their diapedesis[14], which facilitate the firm adhesion of neutrophils to the endothelium and induce subsequent migration into the plaque. Once infiltrated into plaques, neutrophils could be further activated and stimulated by, for example IL-8. IL-8 is also known as a neutrophil stimulator, inducing the release of MMP-8 and MMP-9 from neutrophil tertiary granules[15]. In support of this idea, high levels of plaque IL-8 were associated with high levels of plaque MMP-8 and MMP-9 (data shown in **chapter V**) and, as mentioned above, with high numbers of neutrophils in plaque. In turn, high number of neutrophils correlated with high MMP-8 and MMP-9 levels. Based on these observations, we propose the following interrelation for rupture-prone plaques: IL-8 (in high levels) attracts high numbers of neutrophils and stimulates them to express/secrete MMP-8 and MMP-9 (figure 1).

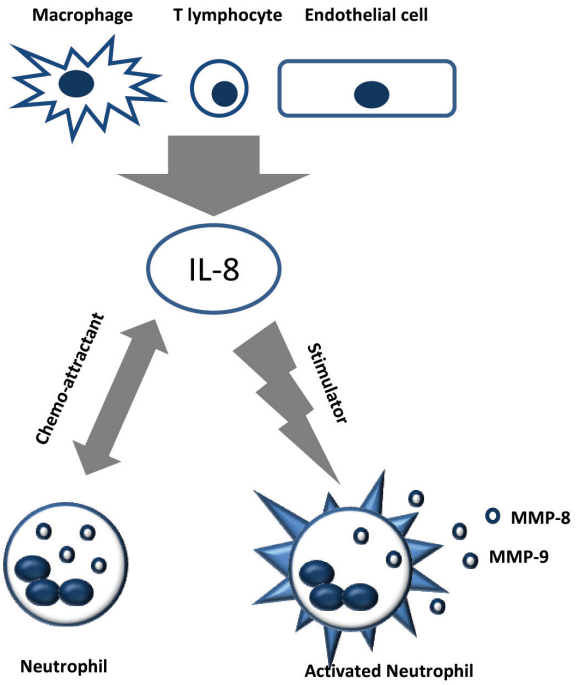


Figure 1. Proposed interrelation between IL-8 (as chemo-attractant and stimulant), neutrophils (as target cells) and MMP-8 and MMP-9 (as neutrophilic products) in human carotid plaques.

MMP-8 and MMP-9 are collagen degrading proteases highly expressed in rupture-prone plaques[16] with a heterogeneous cellular source (vascular endothelial cells, smooth muscle cells and macrophages). Although neutrophils can be counted as a cellular source, they are definitely not the only cells to produce/secrete MMP-8 and MMP-9 in plaques.

The interrelation between IL-8, Mrps and neutrophils/macrophages is quite interesting. In chapter III, a strong association between high IL-8 levels and high Mrp-8, Mrp-14 and Mrp-8/14 levels is shown in plaques. The role of Mrps in neutrophil biology has been investigated in Mrp-14 knockout mice. In these mice, circulating neutrophils also lack Mrp-8, due to the instability of the Mrp-8 in the absence of Mrp-14 (at the protein level only, since the transcription of Mrp-8 is not altered). Neutrophils lacking Mrp-8 and Mrp-14 exhibited a more organized microfilament system and a reduction of IL-8 mediated CD11b surface up-regulation, resulting in a diminished potential to respond to chemo-attractant molecules *in vitro*. Nonetheless, in an inflammatory in vivo model of sterile peritonitis induced by intraperitoneal injection of thioglycolate, no abnormal neutrophil emigration that was observed, suggesting that Mrp-8 and Mrp-14 molecules may be dispensable, at least for the inflammatory model investigated in that study.

The Mrp-expressing macrophages and neutrophils in plaques originate from blood leukocytes

Macrophages as well as neutrophils expressing Mrp-8 and Mrp-14 are found in human atherosclerotic plaques, as described in the above paragraphs. What is the origin of these cells? The answer to this question is found in the blood circulating leukocytes, namely monocytes and neutrophils. Monocytes and neutrophils express Mrp-8 and Mrp-14; these proteins account for 40 to 60% of the cytosolic proteins in circulating neutrophils and 20% in monocytes REFERENCE. Upon leukocyte activation, the Mrp-8/14 heterodimer is translocated to the cell surface membrane. Although the exact mechanism by which Mrp-8/14 gets access to the extracellular compartment is not completely understood, there is good evidence that this is an active process (described in **Chapter I**). In regard to Mrp-8/14 expression, monocytes divide into two subgroups: those who lack and those who express the heterodimer on their cell surface. The Mrp-8/14 positive subset of circulating monocytes represents up to 10% of the monocytes within a healthy individual; the percentage of Mrp-8/14 positive neutrophils within healthy individuals represents up to 15% of total neutrophils REFERENCE.

In the diseased, the expression of Mrp-8/14 on monocyte's surface is increased: in type 1 diabetics, monocytes are able to express and secrete more Mrp-8/14 as compared to healthy control monocytes or monocytes from type 2 diabetics[17].

The expression of Mrp-8/14 on leukocyte's cell surface membrane is not accidental. First, as mentioned above, the translocation of the Mrp-8/14 heterodimer to the cell surface is an active process. Second, the monocytes caring membrane bound Mrp-8/14 have an enhanced adhesive capability and are the preferentially migrating subpopulation of monocytes through the macrovascular endothelium[18]. In addition, this subset of monocytes expresses more CD11b than the Mrp-8/14 cell surface-negative monocytes[19]. The transendothelial migration of Mrp-8/14 cell surface-positive monocytes was inhibited by an anti-ICAM-1 antibody; thus, Mrp-8/14 could function as a regulator enhancing ICAM-1 ligand binding to CD11b (a β2-integrin). The membrane bound Mrp-8/14 as well as the secreted Mrp-8/14 act as modulators of monocyte transendothelial migration. Concomitant with membrane expression, the Mrp-8/14 heterodimer is actively secreted during leukocyte activation. Mrp-8/14 binds specifically to human endothelial cells[20] by a mechanism involving heparin sulfate proteoglycans and novel carboxylated glycans[21, 22]. Mrp-8/14 induces a thrombogenic and a proinflammatory response in human endothelial cells, characterized by induction of cytokines and adhesion molecules[23]. *In vitro* stimulation of endothelial monolayer with Mrp-8/14 resulted in the loss of cell-cell contacts and in increased permeability. Mrp-8/14 heterodimer is circulating in plasma of patients with atherosclerosis (data shown in Chapter VI, and reference 24).

A recent study (Healy et al[24]) shows that human circulating platelets are able to synthesize Mrp-8 and Mrp-14. The authors profiled platelet mRNA (by microarray) from patients

with documented cardiovascular disease and performed RT-PCR to confirm the presence of, among others, Mrp-8 and Mrp-14 in platelets. Although, to our knowledge, this is the only study showing Mrp-expression in human platelets, it suggests that platelets might be an additional source for the circulating Mrp-8/14 plasma levels.

Food for Thought

Blood circulating monocytes and neutrophils express Mrp-8, Mrp-14 and Mrp-8/14. A subset of monocytes and neutrophils expresses Mrp-8/14 on their cell surface; this subset is far more numerous in atherosclerotic disease patients than in healthy individuals. Next to being exposed on monocyte's and neutrophil's cell surface, Mrp-8/14 heterodimer might be also released in blood from these cells. The membrane bound Mrp-8/14 as well as the secreted Mrp-8/14 bind to vessel's endothelium and facilitate the migration of monocytes and neutrophils into the atherosclerotic plaque. The migration of Mrp-positive neutrophils might be facilitated by IL-8. Infiltrated Mrp-positive neutrophils could be further stimulated by IL-8 (present in high amounts in the vulnerable plaque) to secrete MMP-8 and MMP-9. Rupture-prone, vulnerable plaques contain more Mrp-expressing cells than stable lesions. Within plaques, Mrp-8/14 positive monocytes/macrophages do not phagocyte modified lipids and do not transform into foam cells, in comparison with Mrp-negative macrophages. These Mrp-8/14 expressing macrophages are anti-inflammatory thus atheroprotective.

Myeloid-related proteins, markers for Vulnerable Patient

Since atherosclerosis is a systemic, multifactorial disorder, no wonder that the concept of the "vulnerable patient" (described in **Chapter I**) is appealing for both clinicians and researchers in the field of cardiovascular disease. For the identification of the *vulnerable patient*, (bio) markers are a desirable tool; (bio) markers, which are usually related to the disease or disease severity, should help the clinician to optimally manage the patient[25].

Long before being related to cardiovascular diseases (e.g. atherosclerosis, cardiac ischemia), Mrp-8/14 heterodimer was coined as a biomarker of chronic inflammatory diseases such as rheumatoid arthritis, juvenile idiopathic arthritis and inflammatory bowel disease. Indeed, in patients suffering from these diseases the serum Mrp-8/14 levels correlate better with disease activity than classical markers of inflammation such as erythrocyte sedimentation rate and CRP[26]. In addition, Mrp-8/14 proved to be actively involved in the pathogenesis of these inflammatory, autoimmune disorders and therefore it became a disease modulator and not just a biomarker.

During the last 5 years, the Mrp-8/14 heterodimer was shown to have promise as a biomarker for cardiovascular diseases. A first prospective, nested case-control study

documents that plasma Mrp-8/14 levels predict the risk of a first cardiovascular event in apparently healthy individuals[27]. A second study performed in coronary disease patients, shows that Mrp-8/14 plasma levels are elevated in patients with ACS compared to patients with stable or no CAD; moreover, the plasma Mrp-8/14 levels are elevated prior to necrosis markers (i.e. myoglobin, CK-MB, and troponin)[28]. A third study including patients with latest manifestation of MI and discharged with intensive statin therapy, shows that the plasma Mrp-8/14 levels are higher in patients who subsequently developed a new MI or died of CVD than in patients who remained free of recurrent events[29]. A more recent study, documents in AMI patients who had undergone successful primary angioplasty, a transient increase in serum Mrp-8/14 levels with a peak value at 3-5 days after the onset of AMI[30]; the Mrp-8/14 levels were considerably higher than those in UAP patients.

Based on the above mentioned findings, we can now claim that "a person is prone to [a cardiovascular event] heart attack if Mrp-8/14 is present at a high level" (according to Daniel Simon, Harvard Medical School).

The question arises whether Mrp-8/14 is simply a biomarker of cardiovascular disease or whether it contributes to the pathological responses in atherosclerosis and cardiac ischemia. In a recent editorial, the same cardiologist from Harvard Medical School is challenged by the question "*Mrp-8/14 (S100A8/A9) complex, more than just a biomarker of cardiovascular risk?*" and creates a summary of the main findings on the subject, concluding that "*It is interestingly evident that in addition to being a biomarker of inflammation and CV risk, Mrp-8/14 is also an important regulator of atherogenic processes*"[31]. We can't agree more with his conclusion, based on the findings in both mice and humans described also in this chapter.

What makes Mrp-8/14 an attractive new biomarker for CVD? Is there clinical perspective?

In this dynamic field of biomarkers, in which the number of new candidates is expected to exponentially increase in the coming years, it is important to underline the advantage(s) that Mrp-8/14 might have. One of the appealing properties of Mrp-8/14 as biomarker of cardiovascular risk is its availability in the serum/plasma of patients suffering from a heart attack hours before the commonly used markers reach detectable levels (study of Altwegg et al[32]). In the hospital, at the emergency room it can take up to eight to twelve hours for current tests to confirm or infirm a heart attack. A simple blood test to measure the Mrp-8/14 levels could provide the answer within minutes. This idea is supported by a group of cardiologists at Harvard's Medical School's Brigham and Women's Hospital, who estimates that the Mrp-8/14 test will be available for clinicians use in the emergency room within the next two years (interview in the magazine of Case Western Reserve University School of Medicine, winter 2009 Edition). Such a diagnostic test may prove helpful also for the general practitioner to screen the individuals at high risk of cardiovascular events, after

the routine cholesterol check. This idea is supported by the study of Healy et al[33]; in apparently healthy women with elevated cholesterol levels, high blood pressure and other risk factors, high circulating Mrp-8/14 levels could separate those women who went on to have MI from those who remained free of any cardiovascular event. The elevated Mrp-8/14 levels were associated with more than double risk of MI. The elevated Mrp-8/14 plasma levels are also useful in predicting the risk of recurrent cardiovascular events. As previously described in this section, two studies show the predictive power of Mrp-8/14 levels for recurrent ACSs in patients with previous coronary heart disease. In a different cohort (carotid stenosis with previous CVD), we show that elevated Mrp-8/14 plasma levels predict the risk of any recurrent CV-event. Therefore, Mrp-8/14 could serve as a marker of CV-risk.

As a follow-up of these investigations, a study, initiated in 2008 is running at the University Hospital Zürich under the name "*Study of Myeloid-Related Protein 8/14 and Additional Biomarkers (Multi Marker Approach) for Early Diagnosis and Risk Stratification in Patients Presenting with Acute Chest Pain at the Emergency Department*". In this study, all patients (18 to 90 years old) presenting at the emergency department with measurement of troponin during the routine diagnostic evaluation, are included. The endpoints are as follows: acute coronary syndrome, coronary occlusion, coronary thrombosis, myocardial infarction and myocardial ischemia. The medical market is also taking initiative; Roche Diagnostics applied for a patent ("*Use of MRP 8/14 levels for discrimination of individuals at risk of acute coronary syndromes*") for a diagnostic tool to measure Mrp-8/14 (plasma or serum) levels in patients presenting with chest pain and/or breathlessness. The proposed cut-off point is 8 mg/L.

Several issues merit consideration while regarding Mrp-8/14 plasma as a biomarker of CVD. One issue is the fact that Mrp-8/14 levels are measured in plasma or serum of patients without knowing the exact origin of this protein in plasma/serum. Is the circulating Mrp-8/14 secreted from the underlining atherosclerotic plaque(s), is it secreted by the circulating leukocytes sensing the danger of the thrombotic events preceding an ischemic event? Or is it partially originating from other inflamed tissues (for example the joints affected by arthritis in RA, etc.) therefore from patient's co-morbidities? A source of the circulating Mrp-8/14 in patients with CVD is surely the vulnerable atherosclerotic plaque since this lesion has high levels of Mrp-8, Mrp-14 and Mrp-8/14 (as shown in this thesis, **Chapter III**). From plaques, Mrps can be expulsed into the blood stream. Since no correlation between plaque and plasma Mrp-8/14 levels was seen (as mentioned in **Chapter VII**), the atherosclerotic plaque isn't the only source for the circulating Mrp-8/14. Another source could be the blood circulating leukocytes. At the site of coronary occlusion, in culprit lesions, monocytes and neutrophils were found to express and release Mrp-8/14[34]. Therefore, the circulating monocytes and neutrophils represent a plausible source. Another

source might be the infracted myocardium. In AMI patients, the peak level of serum Mrp-8/14 correlates with the peak of CK-MB levels, suggestive for secretion of Mrp-8/14 from the activated macrophages present in the areas of myocardial infarction[35]. The existence of non-CV related co-morbidities (such as RA, inflammatory bowel disease, etc.) in CVD patients may account for a different source for the circulating Mrp-8/14 levels. None of the studies documenting the predictive value of plasma Mrp-8/14 and mentioned previously in this section, report any information regarding existence of relevant co-morbidities in the investigated patients; except, of course, for the validation study of Healy et al. who included only apparently healthy women. No study reports the use of chronic medications, others than lipid-lowering drugs, blood pressure lowering drugs, and anti-coagulants. In our study presented in **chapter VII**, the existence of relevant co-morbidities is not documented in the patient's file from the Athero-Express biobank. Therefore, we cannot exclude other inflamed tissues as a source for the circulating Mrp-8/14 levels.
Another issue is the timing of the Mrp-8/14 appearance in blood. Are Mrp-8/14 complexes released before or after the onset of an ischemic event? It's a "chicken-and-egg" problem. Which came first? At this point, without conclusive studies, it is impossible to answer it. What is the optimal time interval after an ischemic event to measure the Mrp-8/14 in blood? Katashima et al[36], tried to answer this question by measuring serum Mrp-8/14 levels in patients with AMI and UAP, during the acute period of 8 days. In UAP patients, with vulnerable coronary plaques, the Mrp-8/14 levels remained unchanged for one week while in AMI patients, the levels reached a peak (50% increase) at 3-5 days after the onset of AMI and showed still a high level at 6-8 days after (all compared to day 1, considered the baseline value). It seems that the highest values are reached between days 3 and 5 after an AMI, however at day 8 the levels of Mrp-8/14 in blood were still higher compared to day 1; the measurements stopped at day 8. For how long after the onset of an ischemic attack the Mrp-8/14 levels persist in blood? In Chapter VI, we show that the plasma Mrp-8/14 levels remain constant in time following a stroke or a TIA (up to 180 days and more) until the CEA. During this time interval the levels stayed higher than the Mrp-8/14 levels measured in asymptomatic patients (patients with carotid stenosis greater than 75% but without clinical complains). This suggests that the Mrp-8/14 levels remain unchanged for months in blood, after the onset of an ischemic event. This characteristic is, for a good biomarker, desirable.

Another approach could be to assess the plaque Mrp-8/14 levels and use it as a biomarker for the CV-risk. In **chapter VII**, we show that Mrp-8/14 measured in high levels in carotid plaques are related to an increased risk of any recurrent CV-event. This finding confirms a previously postulated concept by our research group, namely that plaque proteins contain information regarding the instability of the entire vasculature[37]. What concerns the clinical applicability of such a concept, it might prove costly and inefficient when opposed to a

simple blood measurement. The atherosclerotic tissue surgically removed could be sent to the laboratories for protein extraction and subsequent Mrp-8/14 quantification with already available and largely used assays (such as the Buhlmann ELISA kit used by clinicians to asses Mrp-8/14 levels in plasma); those patients which have high Mrp-8/14 levels in their plaques should be considered at high risk for an adverse CV-event.

Food for Thought

In the field of cardiovascular prevention, during the last five years Mrp-8/14 (plasma or serum) has shown promise and it is gaining more and more interest. It is expected that the circulating Mrp-8/14 levels will help the cardiologist to differentiate, among patients presenting at the emergency room with acute severe chest pain, those patients with an acute myocardial infarction from those with unstable angina. It is expected to facilitate the identification of patients with chest pain of an ischemic etiology, in patients presenting with chronic or atypical chest pain. Mrp-8/14 has the potential of a diagnostic biomarker for MI: it is rapidly elevated after a MI, has tissue specificity (plaque and myocardial origin), it is released proportional to disease extent (peak value at peak CK-MB), it is easily accessible (blood measurement), it is cost efficient, has predictive value. In other diseases (e.g. RA), Mrp-8/14 is used as a biomarker to monitor disease activity and response to therapy.

Reference List

1 MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomised placebo-controlled trial. Lancet 2002 July 6;360(9326):7-22.

2 Cannon CP, Braunwald E, McCabe CH, Rader DJ, Rouleau JL, Belder R, Joyal SV, Hill KA, Pfeffer MA, Skene AM. Intensive versus moderate lipid lowering with statins after acute coronary syndromes. N Engl J Med 2004 April 8;350(15):1495-504.

3 LaRosa JC, Grundy SM, Waters DD, Shear C, Barter P, Fruchart JC, Gotto AM, Greten H, Kastelein JJ, Shepherd J, Wenger NK. Intensive lipid lowering with atorvastatin in patients with stable coronary disease. N Engl J Med 2005 April 7;352(14):1425-35.

4 Redgrave JN, Lovett JK, Gallagher PJ, Rothwell PM. Histological assessment of 526 symptomatic carotid plaques in relation to the nature and timing of ischemic symptoms: the Oxford plaque study. Circulation 2006 May 16;113(19):2320-8.

5 John AA, Sundararajan S. Vulnerable Plaques and Patients: Improving Prediction of Future Coronary Events. The American journal of medicine 123[1], 10-16. 1-1-2010. Ref Type: Abstract

6 McCormick MM, Rahimi F, Bobryshev YV, Gaus K, Zreiqat H, Cai H, Lord RSA, Geczy CL. S100A8 and S100A9 in Human Arterial Wall: IMPLICATIONS FOR ATHEROGENESIS. J Biol Chem 2005 December 16;280(50):41521-9.

7 Stoger JL, Goossens P, de Winther MP. Macrophage heterogeneity: relevance and functional implications in atherosclerosis. Curr Vasc Pharmacol 2010 March;8(2):233-48.

8 Boyle JJ, Harrington HA, Piper E, Elderfield K, Stark J, Landis RC, Haskard DO. Coronary intraplaque hemorrhage evokes a novel atheroprotective macrophage phenotype. Am J Pathol 2009 March;174(3):1097-108.

9 Papaspyridonos M, Smith A, Burnand KG, Taylor P, Padayachee S, Suckling KE, James CH, Greaves DR, Patel L. Novel candidate genes in unstable areas of human atherosclerotic plaques. Arterioscler Thromb Vasc Biol 2006 August;26(8):1837-44.

10 Stoger JL, Goossens P, de Winther MP. Macrophage heterogeneity: relevance and functional implications in atherosclerosis. Curr Vasc Pharmacol 2010 March;8(2):233-48.

11 Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. Immunity 2010 May 28;32(5):593-604.

12 Stoger JL, Goossens P, de Winther MP. Macrophage heterogeneity: relevance and functional implications in atherosclerosis. Curr Vasc Pharmacol 2010 March;8(2):233-48.

13 Bouhlel MA, Derudas B, Rigamonti E, Dievart R, Brozek J, Haulon S, Zawadzki C, Jude B, Torpier G, Marx N, Staels B, Chinetti-Gbaguidi G. PPARgamma activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. Cell Metab 2007 August;6(2):137-43.

14 Leclercq A, Houard X, Philippe M, Ollivier V, Sebbag U, Meilhac O, Michel JB. Involvement of intraplaque hemorrhage in atherothrombosis evolution via neutrophil protease enrichment. J Leukoc Biol 2007 December 1;82(6):1420-9.

15 Chakrabarti S, Patel KD. Regulation of matrix metalloproteinase-9 release from IL-8-stimulated human neutrophils. J Leukoc Biol 2005 July 1;78(1):279-88.

16 Galis ZS, Sukhova GK, Lark MW, Libby P. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. J Clin Invest 1994 December;94(6):2493-503.

17 Bouma G, Coppens JM, Lam-Tse WK, Luini W, Sintnicolaas K, Levering WH, Sozzani S, Drexhage HA, Versnel MA. An increased MRP8/14 expression and adhesion, but a decreased migration towards proinflammatory chemokines of type 1 diabetes monocytes. Clin Exp Immunol 2005 September;141(3):509-17.

18 Nacken W, Roth J, Sorg C, Kerkhoff C. S100A9/S100A8: Myeloid representatives of the S100 protein family as prominent players in innate immunity. Microsc Res Tech 2003 April 15;60(6):569-80.

19 Eue I, Pietz B, Storck J, Klempt M, Sorg C. Transendothelial migration of 27E10+ human monocytes. Int Immunol 2000 November 1;12(11):1593-604.

20 Eue I, Konig S, Pior J, Sorg C. S100A8, S100A9 and the S100A8/A9 heterodimer complex specifically bind to human endothelial cells: identification and characterization of ligands for the myeloid-related proteins S100A9 and S100A8/A9 on human dermal microvascular endothelial cell line-1 cells. Int Immunol 2002 March 1;14(3):287-97.

21 Robinson MJ, Tessier P, Poulsom R, Hogg N. The S100 family heterodimer, MRP-8/14, binds with high affinity to heparin and heparan sulfate glycosaminoglycans on endothelial cells. J Biol Chem 2002 February 1;277(5):3658-65.
22 Srikrishna G, Panneerselvam K, Westphal V, Abraham V, Varki A, Freeze HH. Two proteins modulating transendothelial migration of leukocytes recognize novel carboxylated glycans on endothelial cells. J Immunol 2001 April 1;166(7):4678-88.
23 Viemann D, Strey A, Janning A, Jurk K, Klimmek K, Vogl T, Hirono K, Ichida F, Foell D, Kehrel B, Gerke V, Sorg C, Roth J. Myeloid-related proteins 8 and 14 induce a specific inflammatory response in human microvascular endothelial cells. Blood 2005 April 1;105(7):2955-62.
24 Healy AM, Pickard MD, Pradhan AD, Wang Y, Chen Z, Croce K, Sakuma M, Shi C, Zago AC, Garasic J, Damokosh AI, Dowie TL, Poisson L, Lillie J, Libby P, Ridker PM, Simon DI. Platelet Expression Profiling and Clinical Validation of Myeloid-Related Protein-14 as a Novel Determinant of Cardiovascular Events. Circulation 2006 May 16;113(19):2278-84.
25 Vasan RS. Biomarkers of Cardiovascular Disease: Molecular Basis and Practical Considerations. Circulation 2006 May 16;113(19):2335-62.
26 Ehrchen JM, Sunderkotter C, Foell D, Vogl T, Roth J. The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. J Leukoc Biol 2009 September;86(3):557-66.
27 Healy AM, Pickard MD, Pradhan AD, Wang Y, Chen Z, Croce K, Sakuma M, Shi C, Zago AC, Garasic J, Damokosh AI, Dowie TL, Poisson L, Lillie J, Libby P, Ridker PM, Simon DI. Platelet Expression Profiling and Clinical Validation of Myeloid-Related Protein-14 as a Novel Determinant of Cardiovascular Events. Circulation 2006 May 16;113(19):2278-84.
28 Altwegg LA, Neidhart M, Hersberger M, Muller S, Eberli FR, Corti R, Roffi M, Sutsch G, Gay S, von Eckardstein A, Wischnewsky MB, Luscher TF, Maier W. Myeloid-related protein 8/14 complex is released by monocytes and granulocytes at the site of coronary occlusion: a novel, early, and sensitive marker of acute coronary syndromes. Eur Heart J 2007 April 2;28(8):941-8.
29 Morrow DA, Wang Y, Croce K, Sakuma M, Sabatine MS, Gao H, Pradhan AD, Healy AM, Buros J, McCabe CH, Libby P, Cannon CP, Braunwald E, Simon DI. Myeloid-related protein 8/14 and the risk of cardiovascular death or myocardial infarction after an acute coronary syndrome in the Pravastatin or Atorvastatin Evaluation and Infection Theraphy: Thrombolysis in Myocardial Infarction (PROVE IT-TIMI 22) trial. American Heart Journal 2008 January;155(1):49-55.
30 Katashima T, Naruko T, Terasaki F, Fujita M, Otsuka K, Murakami S, Sato A, Hiroe M, Ikura Y, Ueda M, Ikemoto M, Kitaura Y. Enhanced expression of the S100A8/A9 complex in acute myocardial infarction patients. Circ J 2010 March 25;74(4):741-8.
31 Croce K. S100A8/A9 Complex– More Than Just a Biomarker of Cardiovascular Risk? &ndash. Circulation Journal 2010;74(4):626-7.
32 Altwegg LA, Neidhart M, Hersberger M, Muller S, Eberli FR, Corti R, Roffi M, Sutsch G, Gay S, von Eckardstein A, Wischnewsky MB, Luscher TF, Maier W. Myeloid-related protein 8/14 complex is released by monocytes and granulocytes at the site of coronary occlusion: a novel, early, and sensitive marker of acute coronary syndromes. Eur Heart J 2007 April 2;28(8):941-8.
33 Healy AM, Pickard MD, Pradhan AD, Wang Y, Chen Z, Croce K, Sakuma M, Shi C, Zago AC, Garasic J, Damokosh AI, Dowie TL, Poisson L, Lillie J, Libby P, Ridker PM, Simon DI. Platelet Expression Profiling and Clinical Validation of Myeloid-Related Protein-14 as a Novel Determinant of Cardiovascular Events. Circulation 2006 May 16;113(19):2278-84.
34 Altwegg LA, Neidhart M, Hersberger M, Muller S, Eberli FR, Corti R, Roffi M, Sutsch G, Gay S, von Eckardstein A, Wischnewsky MB, Luscher TF, Maier W. Myeloid-related protein 8/14 complex is released by monocytes and granulocytes at the site of coronary occlusion: a novel, early, and sensitive marker of acute coronary syndromes. Eur Heart J 2007 April 2;28(8):941-8.
35 Katashima T, Naruko T, Terasaki F, Fujita M, Otsuka K, Murakami S, Sato A, Hiroe M, Ikura Y, Ueda M, Ikemoto M, Kitaura Y. Enhanced expression of the S100A8/A9 complex in acute myocardial infarction patients. Circ J 2010 March 25;74(4):741-8.
36 Katashima T, Naruko T, Terasaki F, Fujita M, Otsuka K, Murakami S, Sato A, Hiroe M, Ikura Y, Ueda M, Ikemoto M, Kitaura Y. Enhanced expression of the S100A8/A9 complex in acute myocardial infarction patients. Circ J 2010 March 25;74(4):741-8.

37 de Kleijn DPV, Moll FL, Hellings WE, Ozsarlak-Sozer G, de Bruin P, Doevendans PA, Vink A, Catanzariti LM, Schoneveld AH, Algra A, Daemen MJ, Biessen EA, de Jager W, Zhang H, de Vries JP, Falk E, Lim SK, van der Spek PJ, Sze SK, Pasterkamp G. Local Atherosclerotic Plaques Are a Source of Prognostic Biomarkers for Adverse Cardiovascular Events. Arterioscler Thromb Vasc Biol 2009 December 17;ATVBAHA.

CHAPTER IX
Summary
Summary in Dutch

The current thesis describes the association of myeloid-related protein (Mrp) -8, -14 and -8/14 levels in plaque and plasma with both the atherosclerotic plaque phenotype and secondary cardiovascular events after carotid atherectomy. For this the AtheroExpress biobank was used, collecting carotid atherectomy specimen with follow-up for cardiovascular events after operation.

Mrp -8 and -14 are two calcium binding proteins mainly expressed in cells of myeloid origin, particularly in monocytes and neutrophils. They were identified as highly expressed proteins in atherosclerotic plaques of patients with recurrent cardiovascular events during three years follow-up after their carotid endarterectomy when compared with patients free of events (age and gender matched). In Chapter III: the association between Mrp-8, -14 and -8/14 and the plaque phenotype was investigated. High Mrp-8, -8/14 but mainly Mrp-14 levels were found in those plaques with features of "vulnerability": larger lipid pools, reduced collagen content, high macrophage and low smooth muscle cells infiltration. These plaques also contained high levels of cytokines and tissue degrading proteases.

Mrp-8, -14 and Mrp-8/14 are expressed by subset of non-foam macrophages and neutrophils. Foam macrophages lack Mrp-expression while the Mrp-expressing macrophages lack the morphology of foam cells (increased cell size, lipid droplets in the cytoplasm and nucleus pushed to the membrane side of the cytoplasm). This *in vivo* observation was further supported by *in vitro* proof that Mrp-8/14 expressing monocyte-derived macrophages did not phagocyte lipids and therefore did not acquire foam morphology while monocyte-derived macrophages lacking Mrp-8/14 did.

In Chapter IV we further investigated this finding and showed that both monocyte-derived macrophage populations have similar levels of lipids-scavenger receptors. In the presence of lipids, the Mrp-8/14 expressing monocyte-derived macrophages secreted higher levels of anti-inflammatory cytokines compared to the cells lacking Mrp-8/14; whereas the latter secreted pro-inflammatory cytokines. Moreover, the Mrp-8/14 expressing monocyte-derived macrophages expressed at high levels the mannose receptor and the CD163 receptor. Based on this evidence, monocyte-derived macrophages expressing Mrp-8/14 were identified as an alternatively activated macrophage subset (also known as the M2-subset).

In chapter V we showed that high numbers of plaque neutrophils express Mrp-8 and Mrp-14 and are associated with high interleukin (IL)-8 and matrix metalloproteinase -8 and -9 plaque levels. In chapter VI, patients with symptomatic carotid atherosclerosis, had higher Mrp-8, -14 and -8/14 plaque and plasma levels than the asymptomatic patients; plaque Mrp-8/14 levels were high shortly after a cerebrovascular event, decreased at latter time

points and increased again from 180 days post event while plasma Mrp-8/14 levels were high after an event and remained constant over time. In chapter VII, the association between plaque and plasma Mrp-8, -14 and -8/14 and the "vulnerable" patient was assessed. Patients who experienced any adverse cardiovascular event one year after the cerebrovascular event leading to the carotid endarterectomy, had higher plasma and plaque Mrp-8/14 levels compared to patients free of events. Both plaque and plasma Mrp-8/14 levels are related to an increased risk of adverse cardiovascular events following a carotid endarterectomy, independent of traditional cardiovascular risk factors. In Chapter VIII, the data presented in the current doctoral thesis are discussed in the perspective of clinical appliance and usefulness. In Chapter IX, all data are summarized.

Main findings described in this thesis:	**Chapter**
Mrp-8, -14 and -8/14 are markers for the Vulnerable plaques	III, IV, V
Mrp-8, -14 and -8/14 are expressed by non-foam plaque macrophages	III
Mrp-8/14 expressing macrophages do not phagocyte lipids AND resemble the M2-macrophage subset	III, IV
Mrp-8, -14 and -8/14 are expressed by plaque neutrophils	V
Mrp-8, -14 and -8/14 are markers for the Vulnerable patient	VI, VII
Mrp-8/14 plaque and plasma predict the risk of adverse cardiovascular events	VII

Elk jaar sterven wereldwijd vele mensen aan hartinfarct en beroerte, de gevolgen van atherosclerose (aderverkalking). Atherosclerose is een ziekte die leidt tot vorming van afzettingen in de slagaders (atherosclerotische plaques) en daarmee het dichtslibben van slagaders. Atherosclerose is een systeemziekte, wat inhoud dat de ziekte in de slagaderen van het hele lichaam voorkomt en niet op een bepaalde locatie. Gevolgen, door de cellulaire en moleculaire factoren van een lokale plaque te bestuderen, kan men iets zegen over het stadium van atherosclerose - ontwikkeling in het hele lichaam. Risicofactoren zijn hoge bloeddruk (hypertensie), diabetes, roken en hoog cholesterol en die zijn geassocieerd met een slechte prognose. De voorspelde waarde voor het krijgen van een hartinfarct of beroerte van deze factoren is beperkt.

Myeloid- gerelateerd eiwitten (Mrp) 8 en 14 zijn twee eiwitten die komen tot expressie vooral in monocyten en neutrofielen granulocyten. De expressie van deze twee eiwitten is hoger in de atherosclerotische plaques van de halsslagader van patiënten met uitingen (vasculaire events zoals hartinfarct en beroerte) en is lager in de plaques van patiënten zonder uitingen.

In hoofdstuk III, is de associatie tussen de Mrp-8, -14 en -8/14 complex in humane atherosclerotische plaques en de plaque fenotype onderzocht. Een hoog Mrp-8, -8/14 complex maar vooraal Mrp-14 expressie in zogenaamde “kwetsbare “ plaques was gevonden. Deze “kwetsbare” plaques kenmerken zich door een grote vetrijke plaquekern en een dunne fibreuze kap met ontstekingscellen zoals macrofagen, neutrofielen en lymfocyten.

Het doel van hoofdstuk IV was om inzicht te krijgen in de associatie tussen Mrp-8, -14 en -8/14 en plaque macrofagen. Er is een associatie gevonden tussen de Mrp - eiwitten en plaque macrofagen die geen vet bevatten (non-foam macrophages). In andere worden in atherosclerotische plaques bestaan twee soorten macrofagen: vet- geladen macrofagen en macrofagen zonder vet. De tweede soort cellen zijn Mrp- positief. Vervolgens hebben we bepaald dat de Mrp- positieve macrofagen een anti- inflammatoir fenotype hebben. In plaques de expressie van Mrp- eiwitten is ook in neutrofielen granulocyten ontdekt, zoals in hoofdstuk V beschreven.

In hoofdstuk VI bestudeerden we de fluctuatie in de concentraties van Mrp-eiwitten in atherosclerotische plaques en in de bloed van patiënten met en zonder een beroerte. Eerder hebben we beschreven dat de duur van het tijdsinterval tussen een beroerte en de operatieve ingreep verband houdt met wisselende eiwitten-concentraties in de atherosclerotische plaques van de halsslagader. Naarmate het tijdsinterval langer wordt, kan de concentratie van de eiwitten afnemen. Dat geld ook voor de concentraties van de Mrp- eiwitten (hoofdstuk VI).

Hoofdstuk VII beschrijft de studie naar Mrp- eiwitten in relatie tot het optreden van vasculaire events (zoals hartinfarct of beroerte). Uit deze studie blijken patiënten met een verhoogde Mrp-8/14 concentratie in de plaque en bloed, een hoog risico te hebben op een vasculair event binnen drie jaar na halsslagader operatie in vergelijken tot patiënten met een laag Mrp-8/14 concentratie.

CHAPTER X
Appendix

List of abbreviations
Authors and affiliations
Review committee
Acknowledgements
Curriculum vitae

Abreviation	Explanation
ACS,	Acute Coronary Syndrome
AMI,	Acute Myocardial Infarction
ApoE,	Apolipoprotein E
CV(D),	Cardio – Vascular (Disease)
CRP,	C- Reactive Protein
CEA,	Carotid Endarterectomy
CD,	Cluster of Differentiation
CK,	Creatin Kinase
EC,	Endothelial Cell
EDA,	Extra Domain A of fibronectin
FFA,	Free Fatty Acid
FACS,	Fluorescent Activated Cell Sorter
FC,	Flow Cytometry
HDL,	High Density Lipoprotein
HSP,	Heat Shock Protein
HMGB,	High Mobility Group Box
IL,	Interleukin
LDL,	Low Density Lipoprotein
LPS,	Lipopolysaccharide
MI,	Myocardial Infarction
MCP,	Monocyte Chemoattractant Protein
MRP,	Myeloid Related Protein
MDM,	Monocyte Derived Macrophage
MMP,	Matrix MetalloProteinase
MAPK,	Mitogen-Activated Protein Kinase
oxLDL,	oxidized Low Density Lipoprotein
ORO,	Oil-Red-O
PG,	Peptidoglycan
RAGE,	Receptor for Advanced Glycation Endproducts
SMC,	Smooth Muscle Cell
SR,	Scavenger Receptor
TIA,	Transient Ischemic Attack
TLR,	Toll-Like Receptor
TG,	Tryglicerides
TNF,	Tumor Necrosis Factor
TGF,	Tissue Growth Factor

Authors and affiliations

University Medical Center Utrecht, the Netherlands
Department of Vascular surgery:
Prof. dr. F.L.Moll, Dr. W. Peeters

Department of Cardiology, Experimental Cardiology Laboratory:
Prof. dr. D.P.V. de Kleijn, Prof. dr. G. Pasterkamp, Drs. G. M. Ionita, Dr. Fatih Arslan, Dr. W. Peeters, Msc. P. van den Borne, Msc. L. M. Catanzariti, Bsc. C. Strijder

Department of Pathology: Dr. A. Vink, Bsc. P. Homoet-van der Kraak
Julius Center of Clinical Epidemiology: Prof. dr. M. Bot

St. Antonius Hospital Nieuwegein, the Netherlands
Department of Vascular Surgery: Dr. J.P.P.M. de Vries

Erasmus Medical Center, Rotterdam, the Netherlands
Department of Immunology, Multiple Sclerosis Center: Prof. dr. J. Laman, Dr. E.I. Dijke, Bsc. M. van Meurs

Nanyang Technological University, Singapore
Dr. Siu Kwan Sze

Review committe

Prof. dr. Marianne Verhaar
Department of Nephrology, University Medical Centre Utrecht, the Netherlands

Prof. dr. Michiel Bots
Julius Center, Department of Epidemiology, University Medical Centre Utrecht, the Netherlands

Prof. dr. Frans L. Moll
Department of vascular surgery, University Medical Center Utrecht, the Netherlands

Prof. dr. Jon Laman
Department of Immunology, Erasmus Medical Center, Rotterdam, the Netherlands

Acknowledgements

"Gratitude is the memory of the heart" Jean Baptiste Massieu

I always believed it is more in this life for me and I never stopped chasing that believes. Many wonderful and exiting events happened in the last four years, since I've moved to the Netherlands and until today when I'm closing another chapter in my life. I moved to a foreign country and learned/still learning its language, tasted the sweetness of research, got married, bought a house and since a few months started the clinical training to become a pathologist. All this being possible thanks to many people and therefore I would take the next lines to express my gratitude for them.

Dear promoter, prof. dr. Gerard Pasterkamp and prof. dr. Dominique de Kleijn, dear Gerard and Dominique. I wouldn't be were I am today without your help! And these are not just words, this is the truth. You open the doors of your lab for me, helped me with the formalities to move to the Netherlands, introduced me to the world of science, trusted me with a Ph.D. project and so gave me the chance to develop. Along the road, you encouraged me to come with new ideas and I guess the famous words "think outside the box" will continue to come in my mind whenever I think about you, Gerard. I enjoyed the freedom of choosing and planning experiments, of working with different research groups from Rotterdam and London and grow in a healthy and inspirational research environment. Dear Dominique, you were also a friend in my moments of uncertainty and lost and always knew to find the right words to put me back on the right track. You both helped me in my efforts of learning Dutch and that opened new avenues for me here in the Netherlands. My gratitude for you is forever!

Dear co-promoter, dr. Aryan Vink, dear Aryan. When I think about you one word comes as first in my mind: perfection. In your work you are always reaching for it and you encourage your co-workers to do the same. I admire you for that! Now and for the coming four years you being my mentor at pathology, I can only hope to assimilate as much as possible from your manner of work. Thank you for your help and support! I am looking forward to what feature will bring me next to you at the pathology department!

My dear aios/oios colleagues, Daphne, Ellen, Fatih and René with whom I've shared the aios-room. Guys, thanks a lot for your good ideas, discussions and constructive critic en most important, for the warm and friendly atmosphere in and outside our small working room! I miss René's lekkere koffie! Veel succes met afronden van jullie onderzoekprojecten en ik kijk eruit naar jullie boeken en promotiefeesten! The "toren" gusy, Wouter, Willem, Rob, Vincent, Dave and Sander, thanks for your help and fun

in and outside the lab. Succes met jullie proefschriften en in jullie carrière als vaatchirurgen.

A special thank also to all my colleagues from the lab of Experimental Cardiology. Dear Chaylendra, thanks for my first steps in the lab, for teaching me first the basic and afterwards, more complex techniques. I still remember my first week in the lab which was also the first week working side by side with you, what a week that was: after learning how to use a pippet, learning how to plan experiments, learning (immune) histology, PCR, cloning, sequencing and to analyze data! Your precise and organized manner of work is inspiring! Dear Aryan, working beside you was a real pleasure; always cheerful and always making jokes about the dutch and the romanians! Not to forget your devote to the lab and your invaluable effort to keep it ongoing. Dear Sander, Loes, Marjolijn, Corina, Els, Mirjam, Karlijn, Imo, Willy, Sanne, Pleunie, Joost, Marish, Krijn, Alain, Angelique, Marie-Jose, Anke, Sophie, Piet, Leo, Pieter (an those unintentionally forget) many thanks for your help, advices and for making the work space so pleasant and nice. Dear Sebastian Grundmann, I am grateful to you for trusting me and supporting me to make the next step towards a ph.d.- training. Colleagues from the GDL, Ben, Maringa, Merel also I didn't had the opportunity to closely work with you, thanks for the nice moments outside the lab. Colleagues from the Vascular Biology and Nephrology labs, thank you all for your help, for the advices on different protocols and for the nice chats during the coffee breaks.

Dear Astrid, Evelyn and Louise, the ladies behind the Athero-Express administration. Dear Astrid and Evelyn, thanks for your valuable help with the patient data and material and for the nice conversations we had during my short visits in your work-nest. Dear Louise, our brainstorms around the patient data and statistical analysis luckily resulted in two nice articles. And not to forget, I am thankful to you for the delicious "salmon-deep" receipt which became one of our favourite's appetizers.

Dear colleagues, foreigners coming from different countries around the world, Eissa, Pradeep, Sridevi, Jia, Louise, Marta, Gonen thank you for the wonderful drinks, dinners and more importantly for your friendship. Eissa, dear friend, I am looking forward to your ad-hoc parties and to your big promotion-party! Dear Muriel and Eneda, I am grateful for your friendship, sometimes is that enough for help. Eneda, it's amazing how you manage to group all of us for a movie, a dinner or an art event whenever you return from London; good luck with your thesis! Muriel, I am curious what other adventures the future will bring you "here in Gouda land" and I can't wait to taste your delicious meals again! Friends know why!

Dear dr. Claudia Monaco, thank you for giving me the opportunity to come to your lab in London. Although our attempts to isolate and culture living macrophages from human plaques didn't provided us the desired results, those days spend in your lab stimulated my interest in working with macrophages. I am thankful and honoured that you accepted the invitation to be a member of the opposing committee at my thesis-defence. Greetings and thanks to your Ph.D.-students who made my work in London really nice and who took me to lunch in an unexpected place, in the cemetery next to the institute.

Dear colleagues from Erasmus MC, dear prof. dr. Jon Laman, dear dr. Esmé Dijke. Dear Jon, it was an honour and a pleasure to meet you and work under your supervision. In my eyes, you are the perfect example of a scientist, always eager to investigate and know everything in the smallest detail. I learned enormous from you and your group and I hope our combined efforts will soon result in a good publication. I am honoured you accepted the invitation to be part of the evaluation and the opposing committees of my thesis. Dear Esmé, we spend long days, many weekends and weeks isolating, culturing, sorting and "facs"-ing monocytes and macrophages. We generated large amounts of data and we had so much fun along! I'm sure they miss our singing-duets in the culture room at the 8th floor! I hope we keep in touch although you are now so far away!

Dear Wendy Schoneveld, thank you for your patience and work on the lay-out of this book.

To end in a personal note, I thank my family who supported me along all these four years. I realize that sometimes the distance is difficult and painful; you have all my love and respect! Dragă mamă, dragă tată, vă sunt adânc recunoscătoare pentru sprijinul si increderea acordate de-a lungul acestor aproape patru ani, culminati astazi cu aceasta teza de doctorat. Stiu ca departarea a fost si inca este de multe ori greu de suportat, insa sper ca viitorul sa ne gaseasca mult mai des impreuna. Dragii mei "Dica" si "Gicu", va multumesc din suflet pentru ca mi-ati fost mereu aproape cu o vorba de incurajare si cu multa caldura. Augustin, the most important person in my life, it is not possible to thank you in a few words therefore I will just say that your love and support were my reasons to keep going. I love you!

Thank you! Dank jullie wel! Mulțumesc!

Mihaela

Curriculum Vitae

Gabriela Mihaela Raicu (-Ionita) was born on May 26, 1981 in Buzau, Romania. After graduating from secondary school in 1996, she studied computer sciences at the "B.P. Hasdeu" high school Buzau. From 2000 until 2006 she studied medicine at the "Carol Davila" University in Bucharest. During this period she was an active member of the aerobics gymnastics group and participated in local interuniversity competitions. In October 2006 she moved to Utrecht, the Netherlands for a six months research internship in the Laboratory of Experimental Cardiology, University Medical Centre Utrecht. During this internship she discovered the amazing world of research and, from April 2007 she started a PhD training ship in the same laboratory, which led to this thesis. After finishing her PhD training she started her residency in Pathology at the University Medical Centre Utrecht.