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Gene Expression Profiling of Human Lipoprotein-Loaded Macrophages and Atherosclerotic Lesions with Special Emphasis on ADAMs

ACADEMIC DISSERTATION
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Appreciate every moment of your life

For Tomas, the sunshine in my life

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I Levula M, Jaakkola O, Luomala M, Nikkari S.T and Lehtimäki T. Effects of oxidized low- and high-density lipoproteins on gene expression of human macrophages. Scand J Clin Lab Invest 2006 66:497-508

II Levula M, Oksala N, Airla N, Salenius J-P, Zeitlin R, Järvinen O, Heikkinen M, Partio T, Saarinen J, Somppi T, Suominen V, Virkkunen J, Hautalahti J, Laaksonen R, Kähönen M, Mennander A, Kytömäki L, Soini J.T, Parkkinen J and Lehtimäki T. Several pathways are significantly affected in human advanced atherosclerotic lesions – Tampere Vascular Study. (submitted August 2009)

III Levula M, Airla N, Oksala N, Hernesniemi J.A, Pelto-Huikko M, Salenius J-P, Zeitlin R, Järvinen O, Huovila A-P, Nikkari S.T, Jaakkola O, Ilveskoski E, Mikkelsson J, Perola M, Laaksonen R, Kytömäki L, Soini J.T, Kähönen M, Parkkinen J, Karhunen P.J and Lehtimäki T. ADAM8 and its single nucleotide polymorphism 2662 T/G are associated with advanced atherosclerosis and fatal myocardial infarction – Tampere Vascular Study. Annals of Medicine 2009 Jul 2:1-11

IV Oksala N and Levula M, Airla N, Pelto-Huikko M, Ortiz, R.M, Järvinen O, Salenius, J-P, Ozsait B, Komurcu-Bayrak E, Erginel-Unaltuna N, Huovila A-P, Kytömäki L, Soini J.T, Kähönen M, Karhunen, P.J, Laaksonen R and Lehtimäki T. ADAM-9, ADAM-15 and ADAM-17 are upregulated in macrophages in advanced human atherosclerotic plaques in aorta and carotid

and femoral arteries – Tampere Vascular Study. Annals of Medicine 2009; 41(4):279-90.

The thesis contains also some unpublished data.

ABBREVIATIONS

ABCA	ATP-binding cassette, subfamily A
ACAT	Acyl-CoA cholesterol acyltransferase
ADAM	A Disintegrin and Metalloprotease
AHA	American Heart Association
AMI	acute myocardial infarction
apo	apolipoprotein
CAPG	capping protein
CCL	chemokine ligand
CCR	chemokine receptor
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CHL	cell adhesion molecule with homology to L1CAM
CNS	central nervous system
CSF	colony stimulating factor
CVD	cardiovascular disease
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
EGFR	endothelial growth factor receptor
FABP	fatty acid-binding protein
FLNA	filamin A
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPCR	G-protein-coupled receptor
GSEA	gene set enrichment analysis
GWEA	genome-wide expression analysis
HB	heparin-binding
HDL	high-density lipoprotein
HPETE	hydroperoxyeicosatetraenoicacid

HSDS	Helsinki Sudden Death Study
HSP	heat shock protein
ICAM	intercellular adhesion molecule
IFI	interferon, gamma inducible protein
IFNG	interferon gamma
IFNGR	interferon gamma receptor
Ig	immunoglubulin
IL	interleukin
INOS	inducible nitrix oxide synthase
ITA	internal thoracic artery
ITLN	intelectin
LAD	left anterior descending coronary artery
LCX	left circumflex coronary artery
LGALS	galectin gene
LO	lipoxygenase
LRP	low-density lipoprotein-related protein
LRPAP	LDL receptor-associated protein
LYZ	lysozyme
MCP	monocyte chemotactic protein
M-CSFR	macrophage colony-stimulating factor receptor
MgCl	magnesium chloride
MHC	major histocompatibility complex
MI	myocardial infarction
mm-LDL	minimally-modified low-density lipoprotein
mRNA	messenger ribonucleic acid
NO	nitrix oxide
NPPB	natriuretic peptide precursor B
NRP	neuropilin
Ox-HDL	oxidized high-density lipoprotein
Ox-LDL	oxidized low-density lipoprotein
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PON	paraoxonase

PPARG	peroxisome proliferator-activated receptor gamma
QRT-PCR	quantitative reverse-transcriptase polymerase chain reaction
RCA	righ coronary artery
RECK	reversion-inducing cysteine-rich protein
RGD	Arg-Gly-Asp RGD adhesion sequence
RGS	regulator of G-protein signaling
RNA	ribonucleic acid
ROS	reactive oxygen species
RPS	ribosomal protein
SCD	sudden cardiac death
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH3	SRC homology 3 domain
SH3PX1	SH3-andphox homology (PX) domain-containing protein
SLAMF	signaling lymphocyte activation molecule family member
SMC	smooth muscle cell
SR-A	scavenger receptor A
SREB	sterol-regulatory binding element
SVMP	snake venom metalloprotease
SYBR	asymmetrical cyanine dye
TACE	tumor necrosis alpha converting enzyme
TBST	tris-buffered saline tween
TGFB	tumor growth factor beta
Th	helper T cells
THBS	thrombospondin
TIMP	tissue inhibitor of matrix metalloprotease
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
Treg	regulatory T cell
TVS	Tampere Vascular Study
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VLCAD	very long chain acyl-CoA dehydrogenase
VLDL	very low-density lipoprotein

ABSTRACT

Background. Atherosclerosis is the most important cause of cardiovascular diseases and globally, the major cause of death. Generally, atherosclerosis can be considered to be a form of chronic inflammation resulting from interaction between modified lipoproteins, monocyte-derived macrophages, T cells, and the normal cellular elements of the arterial wall.

Objectives. The objectives of the thesis were to 1) study the gene expression changes induced by oxidized low-density lipoprotein (ox-LDL) and oxidized high-density lipoprotein (ox-HDL) molecules in cultured human monocyte-macrophages, 2) study the gene expression changes that prevail in advanced human atherosclerotic arteries, 3) define the expression of ADAM8 mRNA and protein in atherosclerotic arteries and study if there is an association of its 2662 T/G allelic variant (rs2995300) with atherosclerosis and myocardial infarction (MI) and 4) define the expression of ADAM9, -15 and -17 mRNA and protein in human atherosclerotic plaques and identify their catalytically active forms in the plaques.

Subjects and Methods. The gene expression changes characterizing early atherosclerotic lesion formation were studied with cDNA microarray and quantitative RT-PCR (QRT-PCR) using cultured human monocyte-macrophages obtained from leukocyte-rich buffy coats collected from healthy blood donors. The Tampere Vascular Study (TVS) material was used to evaluate the gene expression changes prevailing in advanced human atherosclerotic arteries using genome-wide oligonucleotide array. The TVS study material consisted altogether of 24 atherosclerotic arteries and six non atherosclerotic control arteries. Atherosclerotic arteries were collected from carotid and femoral arteries as well as abdominal aortas. Internal thoracic arteries were used as controls. Gene expression changes were verified with QRT-PCR and the localization of the ADAM proteins studied was studied with immunohistochemistry. The association of ADAM8 allelic variant with atherosclerosis and MI was analyzed with TaqMan 5´exonuclease assay and fluorescent allele-specific TagMan probes using the Helsinki Sudden Death Study (HSDS) material.

Results. 1) Ox-LDL and ox-HDL significantly affected the gene expression profiles of monocyte-macrophages. Lipoprotein treatments (LDL vs. HDL) mainly induced opposite expression in the gene expression of monocyte-macrophages but in addition, a significant number of genes was found to respond similarly to lipoprotein treatments. Several new candidate genes for foam cell formation were found. 2) Using genome-wide gene expression array (GWEA), we characterized the generally most up- and down-regulated genes in atherosclerotic plaques and found eight genes specific for aortic plaques and three genes for femoral plaques. In addition, a total of 28 pathways dysregulated (20 up- and 8 down-regulated compared to non-atherosclerotic controls) in plaques were defined with special emphasis on a T cell chemokine pathway. 3-4) The expression of ADAM8, -9, -15 and -17 were found to be significantly induced in the atherosclerotic plaques and the allelic variant of ADAM8 (rs2995300) was significantly associated with the area of complicated atherosclerotic plaques and fatal MI in HSDS material.

Conclusions. Microarray technology was found to be applicable in the screening of gene expression changes in lipoprotein-loaded monocyte-macrophages as well as in advanced human atherosclerotic arteries. Several novel candidate genes and pathways potentially involved in the development of atherosclerosis were found. The pronounced expression of ADAM8, -9, -15, and -17 in the atherosclerotic plaque and the association of ADAM8 allelic variant with the areas of complicated atherosclerotic plaques and fatal myocardial infarct support the involvement of ADAMs in atherosclerosis.

TIIVISTELMÄ

Tausta. Ateroskleroosi on monitekijäinen sairaus, jonka kehittymiseen vaikuttavat perimä ja ympäristötekijät. Sairaus aiheuttaa vuosikymmenien saatossa valtimoiden tukkeutumista, mikä johtaa verenkiertohäiriöihin sydämessä, aivoissa ja alaraajoissa. Ateroskleroosia voidaan tarkastella kroonisena tulehdussairautena, jonka etenemisessä etenkin hapettuneilla lipoproteiineilla, monosyyteistä erilaistuneilla makrofageilla ja T soluilla on merkittävä rooli. Ateroskleroosin syntymiseen ja etenemiseen vaikuttavia tekijöitä voidaan tutkia selvittämällä sairaudessa ilmentyviä geenejä tai usean geenin muodostamia toiminnallisia kokonaisuuksia. ADAM- (A Disintegrin And Metalloprotease) metalloproteaaseilla on useita ateroskleroosin kannalta mielenkiintoisia ominaisuuksia, esimerkiksi sytokiinien ja kasvutekijöiden aktivointi tai inaktivointi.

Tavoiteet. Väitöskirjatyön tavoitteena oli 1) tutkia hapettuneiden LDL- ja HDL-partikkeleiden aiheuttamia geenien ilmentymisen muutoksia ihmisten monosyytti-makrofageissa, 2) selvittää ihmisten ateroskleroottisille valtimoille tyypillisiä geenien ilmentymisen muutoksia, 3) määrittää ADAM8 mRNA:n ja proteiinin ilmentyminen ateroskleoottisissa valtimoissa sekä selvittää sen 2662 T/G (rs2995300) polymorfismin yhteyttä ateroskleroosiin ja sydäninfarktiin ja 4) määrittää ADAM9, -15 ja -17 mRNA:n ja niiden katalyyttisesti aktiivisten proteiinien ilmentyminen ateroskleroottisissa valtimoissa.

Aineisto ja menetelmät. Hapettuneiden lipoproteiinien vaikutuksia tutkittiin Suomen Punaiselta Ristiltä saaduista ”Buffy-Coat” valkosoluvalmisteista eristetyillä monosyytti-makrofageilla cDNA mikroarray-menetelmällä ja QRT-PCR-menetelmällä (QRT-PCR). Ihmisen terveissä rintakehän seinämävaltimoissa ja ateroskleroottisissa kaula- ja reisivaltimoissa sekä aortoissa ilmentyviä geenejä, ADAM-geenit mukaanlukien, tutkittiin Tampereen yliopistollisessa sairaalassa kerätystä TVS-materiaalista koko genomin laajuisella mikroarray-menetelmällä ja QRT-PCR:llä. ADAM8, -9, -15 ja -17 proteiinien lokalisaatiota ateroskleroottisissa valtimoissa tutkittiin immunohistokemiallisin menetelmin ja ADAM9, -15 ja-17 proteiinien katalyyttisesti aktiivisisten muotojen olemassaolo varmistettiin Western-

blot menetelmällä. ADAM8 polymorfismin yhteyttä ateroskleroosiin ja sydäninfarktiin tutkittiin HSDS-materiaalilla käyttäen polymorfismille spesifejä TaqMan koettimia ja 5´eksonukleaasi-aktiivisuuteen perustuvaa menetelmää.

Tulokset. 1) Mikroarray-menetelmä havaittiin toimivaksi kartoitettaessa hapettuneiden LDL- ja HDL-partikkeleiden vaikutuksia ihmisen monosyytti-makrofagien geenien imentymiseen. Hapettuneilla lipoproteiineilla (ox-LDL ja ox-HDL) havaittiin olevan pääasiallisesti vastakkainen vaikutus geenien ilmentymiseen, vaikkakin huomattava osa geeneistä käyttäytyi samansuuntaisesti lipoproteiinikäsittelyiden jälkeen. Tutkimuksessa löydettiin useita uusia mahdollisesti vaahtosolujen muodostumiseen osallistuvia geenejä. 2) Koko genomin laajuisella mikroarray-menetelmällä selvitettin ateroskleroottisissa valtimoissa eniten ja vähiten ilmentyvät yksittäiset geenit, sairaudessa muuntuneet geenien toiminnalliset kokonaisuudet ja signaalinvälitysreitit (yhteensä 28 kpl, joista 20 säädelty ylös- ja 8 alaspäin verrattuna terveisiin valtimoihin), sekä karakterisoitiin ateroskleroottisille aortta- ja reisivaltimoille ominaiset geenien ilmentymisen muutokset. 3) Tutkimuksessa havaittiin ensimmäisenä maailmassa ADAM8 mRNA:n ja proteiinin ilmentymisen voimistuneen ateroskleroosissa ja sen rs2995300 polymorfismin liittyvän komplisoituneiden ateroskleroottisten plakkien pinta-alaan ja sydänakkikuolemaan. 4) ADAM9, -15 ja -17 mRNA:n ja proteiinien ilmentymisen havaittiin lisääntyneen ja varmistettiin niiden aktiivisten muotojen esiintyminen ateroskleroottisissa valtimoissa .

Johtopäätökset. Mikroarray-menetelmän havaittiin olevan toimiva analysoitaessa niin lipoproteiinikäsittelyiden vaikutuksia monosyytti-makrofagien geenien ilmentymiseen, kuin myös ihmisen ateroskleroottisissa valtimoissa ilmentyviä geenejä. Tutkimuksessa löydettiin useita uusia kandidaattigeenejä, jotka mahdollisesti vaikuttavat ateroskleroosin syntymiseen ja etenemiseen. ADAM8, -9, -15 ja -17 ilmentymisen havaittiin lisääntyneen ateroskleroottisissa valtimoissa ja ADAM8 rs2995300 polymorfismin liittyvän komplisoituneiden ateroskleroottisten plakkien pinta-alaan ja sydänakkikuolemaan.

INTRODUCTION

Atherosclerosis is a systematic, multifactorial disease that is the most prevalent of all diseases and a major cause of death in both industrialized and developing nations. Atherosclerosis can cause stenosis or occlusion of arteries, the principal manifestations being heart attack, stroke and lower limb ischemia.

Atherosclerosis is a chronic inflammatory disease disease (Ross 1999; Hansson et al. 2006) that may persist for many years before clinical manifestations become evident. The early lesions of atherosclerosis, fatty streaks, consist mainly of cholesterol-enriched macrophages (Stary et al. 1994), usually found in the aorta in the first decade of life, the coronary arteries in the second decade and the cerebral arteries in the third and fourth decades (Lusis 2000). Fatty streaks are not clinically relevant, but may proceed to more advanced lesions that are characterized by the accumulation of lipid-rich necrotic debris and smooth muscle cells. The atherosclerotic process can evolve into complex phenotype characterized by plaque rupture and thrombosis (Stary et al. 1995).

While several classical risk factors (elevated plasma LDL, cigarette smoking, hypertension, diabetes mellitus, male sex) for atherosclerosis have already been identified that confer a high probability of future pathogenic events, additional prognostic markers are required for a more accurate prediction of the risk. In addition, clarifying the inflammatory mechanisms in the atherosclerotic plaque formation may result in pharmacological and possibly gene therapy applications for disease prevention or treatment.

The microarray method is a transcendent in screening method that can be used to study the gene expression of multiple genes, even all human genes in diseases (Duggan et al. 1999; Lockhart and Winzeler 2000; Hiltunen et al. 2002). The microarray method is particularly well suited to atherosclerosis studies since atherosclerosis is a disease with multiple genes influencing its progression. With the microarray method it is possible to screen the gene expression changes typical for diseases widely and find new genes potentially involved in disease progression.

In this study, the involvement of ADAMs was established in a GWEA of human atherosclerotic plaques. ADAMs are a family of transmembrane and secreted proteins that function in cell adhesion and proteolytic processing of diverse cell surface receptors and signaling molecules (Huovila et al. 2005; Edwards et al. 2008). Since inflammatory activation is a key element in atherosclerosis, ADAMs are of particular interest due to their capability to cleave various subtrates that affect leukocyte recruitment, cell adhesion and proliferation (Herren 2002; Canault et al. 2006; Gomez-Gaviro et al. 2007).

The purposes of this study were to 1) investigate the effect of oxidized low-density and high-density lipoproteins on the gene expression of healthy human monocyte-macrophages and reveal the genes involved in the early phases of atherosclerosis, 2) investigate the typical gene expression changes in advanced human atherosclerotic ateries and 3) study the involvement of ADAM family members 8, 9, 15, and 17 in more detail that were found to be affected in atherosclerosis.

1. Atherosclerosis

1.1 Prevalence and risk factors

Cardiovascular disease (CVD) is the most prevalent of all diseases in both industrialized and developing nations. The principal manifestations of CVD are heart attack, stroke amd lower limb ischemia. Despite a reported 60% decrease in the age-adjusted CVD mortality rate over the past 30 years, disease prevalence remains largely unchanged. According to the World Health Organization (WHO), CVD was the number one cause of death, with 16.7 million people dying due to CVD in 2003, representing 30% of deaths worldwide. In Europe, CVD was a direct cause of over 4.35 million deaths and accounted for 43% of all deaths in men and 55% in women of all ages (including 40% of all deaths under the age of 75 years) (Cifkova et al. 2008). According to Statistics Finland, coronary artery disease was a leading cause of death causing ca. 30% of all deaths. These grave statistics attest that in addition to being the leading cause of death, CVD causes impaired quality of life, disability, lost productivity and economic loss (Murray and Lopez 1997).

Atherosclerosis is a systematic, multifactorial process, the complexity of which makes it difficult to clearly to define the risk as attributable to any one risk factor. The prevalence and severity of atherosclerosis are related to several “old” and “new” risk factors. According to the American Heart Association (AHA), risk factors can be categorized as the 1) traditional and conventional, 2) predisposing, and conditional risk factors. 1) A conventional risk factor appears to have a direct causal role in atherogenesis. The four major risk factors for vascular disease are smoking, diabetes mellitus, dyslipidemia and hypertension. 2) Predisposing risk factors confer their risk through conventional factors and through potentially independent ways. Predisposing risk factors are divided into nonmodifiable and modifiable. Nonmodifiable risk factors include advanced age, gender (male sex,

postmenopausal women), family history and genetics, race (black), and ethnicity (e.g. non-Hispanic black) whereas modifiable risk factors include overweight and obesity, physical inactivity, insulin resistance and socioeconomic-behavioral factors. 3) Conditional risk factors are found to be associated with an increased risk of CVDs, although their independent contribution is not well documented. Conditional risk factors include homocysteine, C-reactive protein, fibrinogen, lipoprotein (a) and hypertriglyceridemia. In addition, there are numerous of emerging risk factors, e.g., inflammatory markers, vascular calcification markers, hemostatic factors, adipokines etc., of which clinical value needs to be ascertained in the future (Liapis et al. 2009).

1.2 Hypotheses on atherogenesis

In the past, several theories have been presented about the development of atherosclerosis. Although each hypothesis provides a different perspective on the initiation of atherosclerosis, there are many common features among them, e.g., inflammation and the involvement of low-density lipoprotein (LDL). At present, atherosclerosis is explained as an inflammatory response elicited by lipoprotein retention in the arterial intima.

The hypothesis of monoclonal cell growth. In 1973, Benditt and Benditt proposed that the smooth muscle cells (SMCs) of an atherosclerotic plaque were of monoclonal origin (Benditt and Benditt 1973). Atherosclerotic lesions would thus be analogous to neoplastic alterations of the vascular wall. This hypothesis has not, however, been supported later.

The response-to-injury hypothesis. Early hypotheses included the "organized thrombus hypothesis" of Rokitansky, who suggested that intimal thickening was due to arterial fibrin deposition (Rokitansky 1852) and a "lipid transudation" hypothesis offered by Virchow in 1858, describing atherosclerosis as a disease caused by lipid complexes with mucopolysaccharides (Virchow 1989). These hypotheses shared the idea that atherosclerosis is a passive phenomenon of deposition rather than an active cellular process. These theories were accompanied by the response-to-injury hypothesis, originally proposed by Ross and Glomset (Ross and Glomset 1973). According to this hypothesis, the proposed initial step in atherogenesis is that

endothelial erosion leads to a number of compensatory responses that alter the normal vascular properties.

The response-to-retention hypothesis. According to the response-to-retention hypothesis of atherosclerosis, mild to moderate hyperlipidemia causes lesion development only in specific sites within the arterial tree characterized by local synthesis of apolipoprotein B-retentive molecules such as biglycan and decorin (Williams and Tabas 1995). Accumulation is thought to result from apolipoprotein B-100 motifs and arterial factors, e.g., secretory sphingomyelinase, that facilitate lipoprotein aggregation (Williams and Tabas 1998).

The oxidative modification hypothesis. Brown and Goldstein observed that chemical modification of LDL in the form of acetylation leads to foam cell formation when incubated with macrophages. The uptake of acetylated LDL was shown to take place through a specific receptor, later termed "the acetyl-LDL receptor" (Goldstein et al. 1979). Later, oxidatively modified LDL was also found to be internalized through the same receptor (Parthasarathy et al. 1986). The current theory of oxidative modification hypothesis states that LDL becomes oxidized in the arterial wall where it then lends itself to cellular uptake and foam cell formation. Of critical importance to this hypothesis is the mechanism of LDL oxidation.

The infection-inflammation hypothesis. The facts that inflammation is always present in plaques and atherosclerotic lesions are reminiscent of scar tissue, led to speculation that an inflammatory-reparative reaction caused atherosclerotic remodeling. This idea was further supported by the finding that atherogenesis cannot be totally explained by conventional risk factors (Cook and Lip 1996) and viral and bacterial agents were found to be involved in atherosclerosis (Nicholls and Thomas 1977; Mendall et al. 1994).

1.3 Initiation of atherosclerosis

1.3.1 Structure of a normal vascular wall

Atherosclerosis is an insidious process that may exist for many years before clinical manifestations become evident. Understanding the pathogenesis of atherosclerosis first requires knowledge of the structure and biology of the normal artery and its

indigenous cell types. A normal muscular artery consists of three distinct layers; the intima, media and adventitia. These three layers are separated by concentric layers of elastin known as the internal elastic lamina (between intima and media), and the external elastic lamina (between media and adventitia).

The intima. The intima is adjacent to the vessel lumen which includes the endothelial monolayer that rests upon a basement membrane containing nonfibrillar collagen types and other extracellular matrix molecules (Wight 1995). Endothelial cells are attached to one another by a series of interconnections known as junctional complexes. Endothelial cells regulate a diverse array of functions in the arterial wall, e.g., thrombosis, vascular tone and leukocyte trafficking into the arterial wall (Bachetti and Morbidelli 2000).

The tunica media. The tunica media also consists of a single cell type, the SMCs that are held together by an extracellular matrix comprised largely of elastic fibers and collagen, or cells may be attached also by junctional complexes. The SMCs form well-developed concentric layers that enable the storage of the kinetic energy during circulation, which is crucial in the control of blood pressure. The media varies in size depending on the size of the artery. In small arteries, the media may be only one cell layer thick, whereas in large arteries, e.g., in aortas, media is usually many cell layers thick and consists of a large amount of elastin (Keaney 2000). The external elastic lamina bounds the tunica media abluminally, forming the border with the adventitial layer.

The adventitia. The adventitia of arteries has typically received little attention, although recently interest has increased in its role in arterial homeostasis and pathology. The adventitia consists of a loose matrix of elastin, SMCs, fibroblasts, mast cells and collagen as well as nerves and vaso vasorum (Keaney 2000).

1.3.2 Lipoprotein retention and endothelial activation

A primary initiating event in atherosclerosis is the accumulation of cholesterol-rich very low-density lipoprotein (VLDL) and LDL in the subendothelial matrix. When plasma levels of LDL rise, the transport and retention of lipoproteins are increased in the preferred sites for lesion formation. Lipoprotein accumulation preferentially occurs at sites of arterial branching or curvature, where flow is turbulent, in contrast

to areas of laminar flow, which are less affected. There is also a positive correlation between low shear stress and LDL accumulation (Zand et al. 1999). LDL diffuses passively through endothelial cell junctions and its retention in the vessel wall seems to involve interactions between the apolipoprotein B (apoB) and matrix proteoglycans (Boren et al. 1998). In addition to LDL, other apoB containing lipoproteins (lipoprotein(a), remnants) may also accumulate in the intima (Grainger et al. 1994). Increased LDL retention in the subendothelial space finally exceeds the elimination capacity through lymphatic vessels, which, in turn, increases the amount of retained lipoproteins in the subendothelial matrix. This makes LDL and other lipoproteins vulnerable to enzymatic and non-enzymatic modifications, e.g., proteolysis, aggregation, lipolysis and most importantly, oxidation.

Minimally modified LDL. Oxidation is considered the most significant modification of LDL for the development of an early lesion formation. LDL is first "minimally modified" (minimally oxidized) by e.g., hydroperoxyeicosatetra-enoicacid (HPETE), a reactive oxygen species (ROS) produced by 12/15 lipoxygenase (12-LO) (Kuhn et al. 1994; Folcik et al. 1995), but is still recognized by the normal LDL receptors. The oxidation process is inhibited by a high-density lipoprotein (HDL) associated enzyme, paraoxonase 1 (PON1), antioxidants and cellular enzymatic antioxidants (Watson et al. 1995; Aviram 1996). Minimally modified LDL (mm-LDL) elicits though multiple pro-inflammatory functions crucial for atherosclerotic plaque initiation, e.g., modifications lead to the release of bioactive phospholipids that can activate endothelial cells (Kume et al. 1992). In addition, mm-LDL stimulates the endothelial cells to express adhesion molecules and chemokines such as monocyte chemotactic protein 1 (MCP1), macrophage colony stimulating factor 1 (CSF1), vascular cell adhesion molecule 1 (VCAM1) and other pro-inflammatory molecules and growth factors that promote inflammatory cell migration into the subendothelium (Cushing et al. 1990; Rajavashisth et al. 1990; Yla-Herttuala et al. 1991; Kume et al. 1992; Khan et al. 1995; Lusis 2000). In addition, circulating blood cells are activated by a variety of pro-inflammatory cytokines, including interleukins and tumor necrosis factor alpha produced by intimal cells in response to infiltrating lipoproteins (Takahashi et al. 2002; Sheikine and Hansson 2004; Daugherty et al. 2005).

Oxidized LDL and foam cell formation. Activated inflammatory cells, mainly monocytes and T cells, roll on the surface of activated luminal endothelial cells and

adhere to them in response to signals originating from the intima. The sequential and overlapping actions of chemoattractants, cytokines and adhesion molecules result in the firm arrest of circulating monocytes on sites of activated endothelium (Sheikine and Hansson 2004; Boyle 2005; Daugherty et al. 2005). Once trapped in the arterial wall, monocytes differentiate mainly to macrophages, but also into dendritic cells, depending on micro-enviromental conditions such as the content and composition of cytokines (colony stimulating factor 1 and 2) (Ross 1993; Randolph et al. 1998). In the arterial wall, macrophages react to the vessel microenvironment by internalizing and metabolizing a variety of subendothelial components.

As ROS and several enzymes, e.g., myeloperoxidase (Podrez et al. 2000), sphingomyelinase (Xu and Tabas 1991) and secretory phospholipase (Neuzil et al. 1998), further oxidize LDL, it finally becomes “fully oxidized” (ox-LDL). Ox-LDL is no longer recognized by normal LDL receptors but instead by scavenger receptors on macrophages that rapidly uptake ox-LDL particles and eventually turn into foam cells (Goldstein et al. 1979; Kruth 2001). Macrophages express several scavenger receptors (Matsumoto et al. 1990) but the role of scavenger receptor A (SR-A) and CD (cluster of differentiation) molecule 36 in the foam cell formation has been widely studied (de Villiers and Smart 1999; Linton and Fazio 2001). It has also been suggested that macrophages internalize modified lipoproteins by an alternative uptake mechanism that may contribute to foam cell formation (Moore et al. 2005).

After uptake, lipoproteins are transported within vesicles towards lysosomes (Kruth 2001), and actually, in the early stages of transformation of macrophages into foam cells, lipid inclusions are present within large swollen lysosomes (Jerome and Yancey 2003). The major storage form of cholesterol in macrophages is free cholesterol and cholesteryl fatty acid esters, which are sequestered into membrane-bound cytoplasmic lipid droplets, a process where Acyl-CoA cholesterol acyltransferase (ACAT) is found to play a major role (Kellner-Weibel et al. 1999; Tabas 2000). Cholesterol esters within lipid droplets can be hydrolyzed by hormone-sensitive lipase, generating free cholesterol for incorporation into membranes and transport out of the cell. Membrane incorporation of excess cholesterol, in turn, inhibits the proteolytic activation of the sterol-regulated element binding (SREB) transcription factors required for cholesterol biosynthesis and LDL receptor expression (Brown and Goldstein 1999). While this prevents further

accumulation of cholesterol via these pathways, it does not alter cholesterol uptake via scavenger receptors.

Macrophages can dispose excess cholesterol mainly via ABCA1 (ATP-binding cassette, subfamily A) to HDL or enzymatic modifications (Lawn et al. 1999; Kozarsky et al. 2000). As the amount of cholesterol in macrophages increases, foam cells develop that eventually form an early atherosclerotic lesion, ”the fatty streak”, that are prevalent in young individuals, never cause symptoms, and may either progress into atheromas or disappear with time. The initiating events in atherosclerotic plaque formation are described in Figure 1.

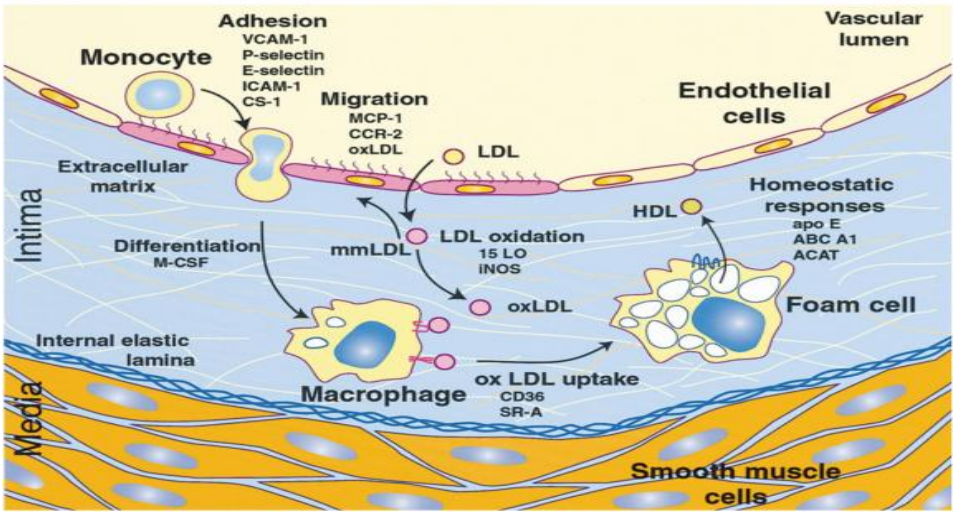

Figure 1. Initiating events in the development of the fatty streak. LDL is vulnerable to oxidative modifications in the subendothelial space, progressing from minimally modified LDL (mmLDL) to extensively oxidized LDL (oxLDL). Monocytes attach to endothelial cells that have been induced to express cell adhesion molecules by mmLDL and inflammatory cytokines. Adherent monocytes migrate into the subendothelial space and differentiate into macrophages. Uptake of oxLDL via scavenger receptors leads to foam cell formation. OxLDL cholesterol taken up by scavenger receptors is subject to esterification and storage in lipid droplets, is converted to more soluble forms, or is exported to extracellular HDL acceptors via cholesterol transporters, such as ABCA1. Abbreviations: VCAM1; vascular cell adhesion molecule 1, ICAM1; inter cellular adhesion molecule 1, CS1connecting segment 1, MCP1; monocyte chemotactic protein 1, CCR2; chemokine receptor 2, ox-LDL; oxidized LDL, HDL; high-density lipoprotein, ABCA1; ATP-binding cassette, member1, ACAT; acetyl-coenzyme A acyltransferase, CD36: CD molecule 36, SR-A; scavenger receptor A, M-CSF; macrophage colony-stimulating factor 1, 15LO; 15 lipoxy-oxygenase, INOS; nitric oxide synthase, inducible. From (Glass and Witztum 2001), with permission.

In addition to promoting foam cell formation, ox-LDL has several other pro-atherogenic effects. For example, ox-LDL is chemotactic for monocytes (Quinn et al. 1987) and T cells (McMurray et al. 1993), reduces the the macrophage mobility (Quinn et al. 1987), reduces the bioactivity of endothelium-derived nitric oxide (Kugiyama et al. 1990) and induces the expression of macrophage scavenger receptors thereby enhancing foam cell formation and LDL uptake (Mietus-Snyder et al. 1997) (Table 1).

Table 1. Potential pro-atherogenic and thrombotic effects of oxidized low-density lipoprotein (ox-LDL). Modified from (Keaney 2000; Stocker and Keaney 2004).

Potential pro-atherogenic and thrombotic activities of oxidized LDL (ox-LDL)
• Supports macrophage foam cell formation
• Ox-LDL derived products are chemotactic for monocytes, T cells and macrophages
• Ox-LDL derived products are cytotoxic and can induce apoptosis
• Mitogenic for smooth muscle cells and macrophages
• Alters inflammatory gene expression, e.g., macrophage scavenger receptors
• Induces the expression and activation of PPARγ (peroxisome proliferator-activated receptor gamma) influencing function of many genes
• Immunogenic and elicits autoantibody formation and activated T cells
• Oxidation renders LDL more susceptible to aggregation which leads to enhanced uptake
• Substrate for sphingomyelinase, which aggregates LDL
• Enhances procoagulant pathways by induction of tissue factor and platelet aggregation
• Products of ox-LDL impair ·NO (nitric oxide) bioactivity
• Binds C-reactive protein activating the complement pathway

1.4 Progression of atherosclerosis

1.4.1 Involvement of T cells in the plaque progression

The presence of T cells in human atherosclerotic plaques was first described in 1985 (Jonasson et al. 1985) and today, the involvement of T cells in atherosclerosis is of particular interest since T cells secrete mediators that influence plaque development and act on most cells in the plaque. Human atheroseclerotic plaques contain numerous T cells. In a plaque, ca 40% of the cells express macrophage markers, ca 10% cells are $CD3^+$ T cells and most of the remainder have characteristics of SMCs (Jonasson et al. 1986). Among T cells, there are also small populations of mast cells (Kovanen 1995), B cells and dendritic cells present in the plaque (Hansson and Libby 2006).

Foam cells present together with other antigen presenting cells, e.g., dendritic cells digested ox-LDL to T cells which initiates an adaptive immune reaction (Ross 1999; Hansson 2001; Jawien 2008). Other antigens promoting atherosclerosis are suggested to be heat shock protein 60 (HSP60)(George et al. 1999), β2-glycoprotein I (George et al. 2000) or fragments of bacterial antigens (Zhu et al. 2001). The exact location for the initial antigen presentation to T cells in atherosclerosis is not known but is thought to occur in regional lymph nodes (Bobryshev 2005).

Activation of naive T cells requires two signals: 1) ligation of the antigen/major histocopatibility complex (MHC) and b) ligation of the costimulatory molecule CD28 on T cells by CD80 or CD86 on the antigen presenting cell (Hansson et al. 2006). In addition, the interaction between the immunological cells requires the presence of CD40 receptor on the antigen presenting cells and CD40L on T cells that results in priming and expansion of antigen-specific $CD4^+$ T cells (Xu and Song 2004). Depending on the antigen, naive T cells differentiate into Th1, Th2, Th17 or regulatory T cells (Tregs). Interestingly, macrophages, platelets, endothelial cells and SMCs in lesions also express CD40 and CD40L, which creates many possible interactions of CD40-CD40L ligation contributing to expression of chemokines, adhesion molecules and leukocyte recruitment (Karmann et al. 1995; Schonbeck and Libby 2001). It is currently believed that the immunological response of Th1 type and its mediators (IFNγ, tumor necrosis factor alpha; TNFα, interleukin 1; IL1, IL12, IL18) accelerate atherosclerosis, whereas the Th2 response (IL4, IL5, IL10,

IL13) inhibits the development of atherosclerosis (Laurat et al. 2001; Daugherty and Rateri 2002; Pinderski et al. 2002). The role of Th17 response in atherosclerosis has been suggested to be insignificant (Song and Schindler 2004), or speculated to be less atherogenic than Th1 (Taleb et al. 2008). Tregs home in peripheral tissues to maintain self-tolerance and to prevent autoimmunity by inhibiting pathogenic lymphocytes. Treg function in atherosclerosis is not fully understood but it has been suggested to play a protective role in the disease (Ait-Oufella et al. 2006; Taleb et al. 2008). The interactions between foam cells, Th1 and Th2 cells are represented in Figure 2.

In human plaques, $CD8^+$ T cells are also present but their role in plaque progression needs to be clarified in the future (Jonasson et al. 1986; Gewaltig et al. 2008).

1.4.2 Fibrous plaques

The transition from fatty streak to a more complex lesion is characterized by the immigration of SMCs from the medial layers of the artery wall past the internal elastic lamina into intima in a process mediated by matrix metalloproteases (Mason et al. 1999). In the intima, SMCs proliferate under the influence of various factors produced by macrophages and T cells, especially to transforming growth factor β and platelet-derived growth factors (Sugiyama et al. 2001; Packard and Libby 2008). IL18 produced by Th1 cells evokes crucial events that facilitate the progression of atherosclerosis, e.g., inducement of VCAM1, chemokines, cytokines and matrix metalloproteases. IL18 signaling also induces IFNγ expression of SMCs activating another proinflammatory pathway (Gerdes et al. 2002) (Figure 2).

In the centre of the mature plaques, atheromas, reside foam cells and extracellular lipid droplets that are surrounded by a cap of SMCs and collagen-rich matrix (Jonasson et al. 1986). T cells and macophages are particularly abundant in the shoulder regions of the plaque (Jonasson et al. 1986). In mature atheromas, denritic cells (Bobryshev and Lord 1995), mast cells (Kovanen et al. 1995), B cells and natural killer T cells have also been found. Neovascularization, promoted by proangiogenic factors, arising from the artery´s vasa vasorum, contributes to lesion progression by providing a route for inflammatory cells to enter the plaque and by

favoring intraplaque hemorrhage (Croce and Libby 2007). With time, the plaque can progress into an even more complex lesion with a considerable amount of cholesterol deposits surrounded by a fibrous cap of varying thickness. The progression of an atherosclerotic lesion also requires the involvement of activated platelets that produce CD40L, platelet-derived growth factor (PDGF) and direct leukocyte adherence into plaques through platelet-mediated leukocyte adhesion, a process that reveals the synergism between inflammation and thrombosis (Croce and Libby 2007).

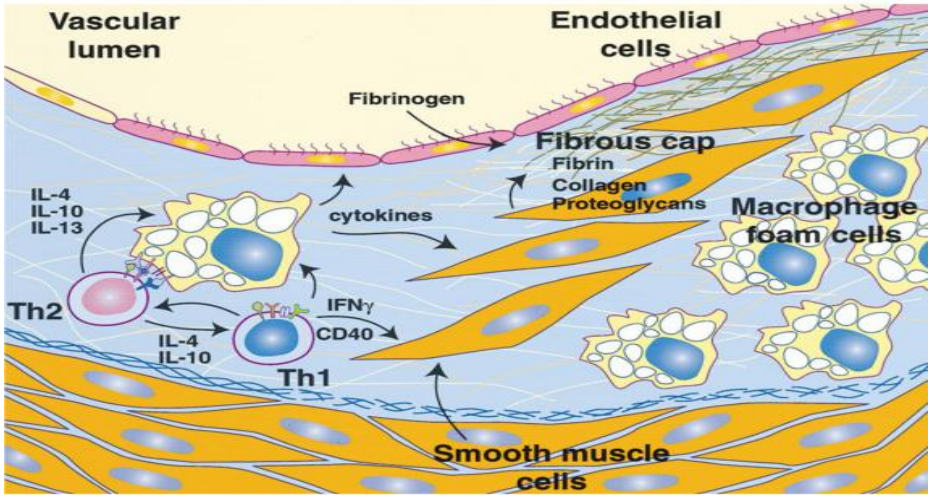

Figure 2. Progression of the lesion. Interactions between foam cells, Th1 and Th2 cells establish a choronic inflammatory process. Cytokines secreted by lymphocytes and macrophages exert both pro- and antiatherogenic effects on each of the cellular elements of the vessel wall. Smooth muscle cells migrate from the medial portion of the arterial wall, proliferate and secrete extracellular matrix proteins that form a fibrous plaque. Abbreviations: IL; interleukin, Th; helper T cell, IFNγ; interferon gamma, CD; cluster of differentiation. From (Glass and Witztum 2001), with permission.

1.4.3 Rupture of the vulnerable plaque and thrombosis

Significant obstruction (~70%) in luminal size can lead to clinical syndromes such as effort angina and intermittent claudication, but even very large unstable plaques may be completely symptomatic (Brown et al. 1993). There is evidence that the development of thrombus-mediated acute coronary events depends principally on the composition and vulnerability of the plaque, rather than the severity of the

stenosis. Patients with acute coronary events have also been found to have elevated levels of circulating cytokines, acute phase reactants and activated T cells (Liuzzo et al. 1994; Caligiuri et al. 2000). Maintenance of the fibrous cap is a balance between matrix production and degradation and inflammatory cells are likely to influence both processes. The stability of advanced plaques is also influenced by calcification and neovascularization (Libby 2001; Croce and Libby 2007).

Advanced complex atheroma exhibits few SMCs at sites of rupture but on the other hand, a number of macrophages, which are key histological characteristics of plaques that have ruptured and caused fatal coronary thrombosis, stroke or critical limb ischemia. Activated macrophages, T cells and mast cells found at sites of plaque rupture, produce several plaque destabilizing molecules, e.g., cytokines, proteases, coagulation factors, radicals and vasoactive molecules (Moreno et al. 1994; van der Wal et al. 1994; Kaartinen et al. 1998; Hansson et al. 2006). The thrombogenity of the lesion core is thought to depend on the presence of tissue factor that initiates the coagulation cascade among other thrombosis mediating molecules (Naghavi et al. 2003a; Naghavi et al. 2003b; Gosk-Bierska et al. 2008).

Acute coronary events most often result from a physical disruption of the fibrous cap that allows the blood to make contact with the thrombogenic material in the lipid core or the subendothelial region of the intima (Libby 2001). This initiates the formation of a thrombus, which can lead to dramatic obstruction of blood flow through the affected artery and cause severe clinical manifestations or even sudden death.

1.5 Classification of atherosclerosis

The atherosclerotic lesions can be classified according to progression of the disease. The American Heart Association (AHA) has published the most recent classification system for atherosclerotic lesions; it recognizes six types of lesions of increasing severity in the atherosclerotic process (Stary et al. 1994; Stary et al. 1995).

Type I. Type I lesions consist of the first microscopically and chemically detectable lipid deposits and small isolated groups of foam cells in the intima and the cell reactions associated with such deposits. The initial histological changes in the intima, however are minimal. These lesions may be present in even a few

months old infants, most likely at sites where later more advanced lesions develop. However, initial type I lesions can also be found in adults, or in locations that are considered lesion resistant (Gerrity 1981; Stary et al. 1994).

Type II. Type II lesions include fatty streaks that are often visible on the intimal surface. Lesions consist primarily of macrophage foam cells stratified in adjacent layers, intimal SMCs containing lipid droplets and T cells (Munro et al. 1987; Katsuda et al. 1992). The number of mast cells is also greater than in the normal intima but their number is still limited (Stary 1990). Most of the lipid in the type II lesions is in cells and the extracellular space contains only small quantities of lipid droplets and vesicular particles. Type II lesions are divided into a "progression-prone" (Type IIa) and "progression resistant" (Type IIb) subgroup. Type IIa lesions localize at typical sites and therefore normally give rise to more advanced lesions, whereas type IIb lesions tend to stabilize (Stary et al. 1994).

Type III. The characteristic microscopical feature in type II lesion is an increase in extracellular lipids with progression from droplets to pools among the layers of SMCs on the intimal thickening. The lipid pools replace intercellular matrix proteoglycans and disrupt their structural uniformity. Type III lesion (preatheroma) represents the bridge to atheroma, the first advanced lesion group.

Type IV. Type IV lesion is also known as "atheroma" and is characterized by the lipid core, a dense accumulation of extracellular lipid that occupies an extensive but well-defined region of the intima. Fibrous tissue increases and the organelles of SMCs may be calcified. Between the lipid core and the endothelial surface, the intima contains macrophages and SMCs, lymphocytes and mast cells. The relative thinness of this tissue explains why atheromas may sometimes be susceptible to rupture, even though atheromas usually fail to narrow the vascular lumen (Stary et al. 1995).

Type V. In type V lesions, a prominent new fibrous connective tissue has formed. Type V lesions may be divided to fibroatheroma (Type Va), calcific (Type Vb) or fibrotic (Type Vc). In fibroatheromas, new fibrous tissue is part of a lesion with a lipid core whereas in a type Vb lesion any part of the lesion is calcified. In type Vc lesion, a lipid core is absent or minimal with a considerable amount of fibrous tissue production possibly due to resorption of the lipid core, organization of thrombi or extensive reparative response of the arterial wall (Stary et al. 1995).

Type VI. Type VI lesions are also called “complicated lesions” and they can be subdivided by the superimposed features. In type VIa, the surface is disrupted, in Type VIb there is hemorrhage or hematoma whereas thrombus formation is typical for Type VIc lesion. There are several factors that promote plaque progress to complicated phenotype, e.g., inflammatory infiltrates in lesions (van der Wal et al. 1994), release of proteolytic enzymes and toxic substances by macrophages (Henney et al. 1991), coronary artery spasm (Nobuyoshi et al. 1991), structural weakness (Richardson et al. 1989).

1.6 Applications of microarrays in atherosclerosis research

Microarrays have been utilized in human atherosclerosis research since the development of the method in the mid 1990s. Microarrays have been used to screen the genes involved in foam cell formation in a cell culture model (Shiffman et al. 2000) as well as the genes expressed in human atherosclerotic arteries. Gene expression profiling has been done on several types on human atherosclerotic plaques, e.g., carotid (Woodside et al. 2003; Dahl et al. 2007), coronary (Randi et al. 2003; King et al. 2005) and peripheral plaques (Fu et al. 2008) and a vast amount of genes involved in cellular turnover, tissue remodeling, lipid metabolism, thrombosis and inflammation are suggested to be involved in the pathologic processes of atherosclerosis. However, the use of whole-mount atherosclerotic lesions or arteries in microarray studies represents many problems since arterial tissue is a very heterogenous collection of cells (Tuomisto et al. 2005). Therefore, the gene expression findings should be localized to certain cell types using *in situ* hybridization or immunohistochemistry. To overcome this problem, microarrays have also been applied to analyse the expression profiles of a single cell type isolated from atherosclerotic plaques (Haley et al. 2000). In addition, microarray methdod has been utilized in the search of suitable biomarkers for cardiovascular diseases (Nakayama et al. 2008).

2. ADAM metalloproteases

The ADAMs (A Disintegrin And Metalloprotease) are a family of transmembrane and secreted proteins of approximately 750 amino acids in length that function in cell adhesion and proteolytic processing of the ectodomains of diverse cell surface receptors and signaling molecules (Wolfsberg et al. 1995; Huovila et al. 2005; Edwards et al. 2008). They belong to the adamalysin subfamily of the metzincin superfamily of Zn-dependent metalloproteinases (Stone et al. 1999) (Figure 3). Of the ADAM family, 38 members have been found in various species. The human genome contains 25 ADAM genes of which four are pseudogenes. Several ADAM genes display alternatively spliced transcripts that produce variant proteins. In humans and other vertebrates, ADAMs are expressed in the testes, hematopoietic cells, nervous system, stem cells and broadly in somatic tissues, see, for example, (Edwards et al. 2008). The typical ADAM gene consists of an N-terminal signal peptide, propeptide, metalloprotease, disintegrin, cysteine-rich and epidermal growth factor-like domains followed by a transmembrane region and a cytoplasmic tail (Figure 4).

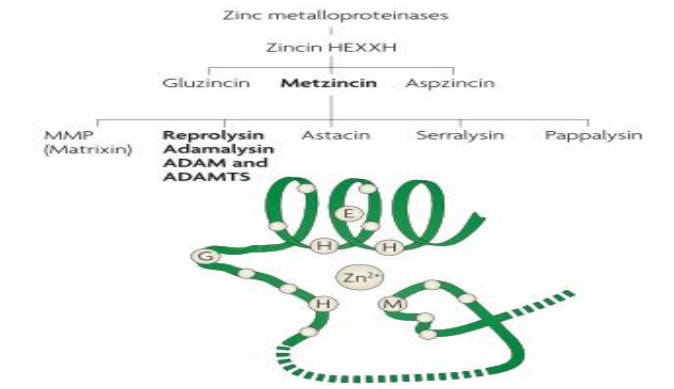

Figure 3. Zinc metalloproteinases. The proteases of the zincin type that have the minimal catalytic zinc-binding motif containing two histidine residues flanking the catalytic glutamate, HEXXH, comprise three superfamilies: the gluzincins, the aspzincins and the metzincins. Within the metzincins the major families are the matrixins or matrix metalloproteinases (MPP), the reprolysins and the astacins. From (Murphy 2008), with permission.

2.1 Prodomain

All ADAM precursors possess signal sequences at their N-terminus, which direct the nascent polypeptides to the secretory pathway. N-terminus is followed by the prodomain which, at least in some ADAMs, acts as an intramolecular chaperone affecting the correct protein folding (Roghani et al. 1999) and functions via the cysteine-switch mechanism to maintain the pro-enzyme latency. According to the cysteine-switch model, a conserved cysteine within the prodomain coordinates the essential active site zinc atom, thus rendering the metalloprotease inactive (Van Wart and Birkedal-Hansen 1990). The prodomain is usually removed during the transit through the secretory pathway. This activation is thought to be mediated by the intracellular pro-protein convertases in a post-Golgi compartment (Lum et al. 1998). Interestingly, there is evidence that the prodomain of ADAM8 and -28 is removed by an autocatalytic mechanism (Howard et al. 2000; Schlomann et al. 2002).

2.2 Metalloprotease domain and catalytic activity of ADAMs

The catalytic metalloproteinase domain shows a remarkable conservation among the various ADAM family members. The active site contains zinc and water atoms that are necessary for the hydrolytic processing of protein substrates, and which are coordinated by three conserved histidine residues and a downstream methionine (Seals and Courtneidge 2003). Of twenty-one human ADAMs, 13 of them possess the reprolysin-type active site (HEXGHXXGXXHD; the one-letter code for amino acids) in the metalloprotease domain followed downstream by the “methione turn” that is the signature of the metzincins (Bode et al. 1993). The presence of the intact active site sequence indicates that the human ADAMs 7, -8, -9, -10, -12, -15, -17, -19, -20, -21, -28, -30, and -33 are catalytically active. Other ADAMs lack one or more essential amino acid residues from the active site, indicating that their metalloproteinase domain is catalytically inactive. Given the high degree of sequence similarity and thus apparent structural conservation, it is tempting to speculate that the inactive metalloprotease domains may participate in other functions such as protein-protein interactions or they may be critical to the overall structure of the ADAM itself.

The most prominent function assigned to ADAM metalloproteases is ectodomain shedding. A variety of cytokines, chemokines, and growth factors are initially produced as transmembrane precrursors. ADAMs constitute the major family of "ectodomain sheddases" proteolytically releasing and activating these mediators, as well as down-regulating their receptors and other membrane proteins, albeit some shedding events are mediated by other membrane-bound proteinases (Blobel 2005; Huovila et al. 2005). Despite the increasing number of the sheddase substrates, their specific recognition by the sheddase ADAMs remains poorly understood. The primary sequence of the cleavage-site does not appear to play a major determinant role. The variability and the tolerance for mutations of the sequences flanking the scissile bond in the substrates of given ADAMs, as well as the fact that several ADAMs can cleave the same peptide mimetics of some subtrates, indicate the importance of more distal interactions (Black et al. 2003; White 2003; Huovila et al. 2005; Arribas and Esselens 2009). Hence, it has been difficult to find specific small-molecule inhibitors for given ADAMs implicated in diverse diseases. On the other hand, ADAM inhibitors based on the tissue inhibitors of metalloproteinases are being pursued at least for some ADAMs, in line with the importance of the distal interactions in ADAM substrate recognition (Lee et al. 2005).

ADAM activities are regulated post-translationally (Doedens and Black 2000; Zheng et al. 2002), by G-protein-coupled receptors (GPCRs), epidermal growth factor receptor (EGFR) activation (Ohtsu et al. 2006) and by ADAM inhibitors e.g., tissue inhibitors of metalloproteinases (TIMPs) and the membrane-associated RECK (reversion-inducing cysteine-rich protein with Kazal motifs) (Baker et al. 2002; Muraguchi et al. 2007).

2.3 Disintegrin domain

The disintegrin domain is named for its presence in the snake venom metalloproteases (SVMPs), being involved in binding of platelet integrin receptors. This prevents the association of platelets with their natural ligands like fibrinogen and blocks platelet aggregation. The disintegrin domain of ADAMs proteins is ~90 amino acids long. Structurally, little is known about the disintegrin domain of ADAMs. "True" snake venom disintegrins have a Arg-Gly-Asp RGD adhesion

sequence (RGD sequence) in the disintegrin loop but with the exeption of ADAM15 other ADAMs lack the RGD sequence (Evans 2001). Despite this, the disintegrin domains of many ADAMs do associate with integrin receptors (Chen et al. 1999; Eto et al. 2002), e.g., through binding to aspartic acid-containing sequences (Zhu and Evans 2002). In many cases these interactions have been shown to influence cell adhesion and cell-cell interactions, see, for example, (Eto et al. 2002; Arribas et al. 2006).

2.4 ADAM cysteine-rich (ACR) domain and EGF-like domain

The cysteine-rich domain is present in all ADAMs and it has been implicated together with the disintegrin domain in the regulation of catalytic activity (Smith et al. 2002), substrate targeting (Janes et al. 2005) and removal of the prodomain from the catalytic domain (Milla et al. 1999). The cysteine-rich domain may also have a role in the binding of cell surface proteoglycans (Iba et al. 1999).

Almost all ADAMs contain an EGF-like domain in their ectodomain. The functional significance of the ADAM EGF-like domain remains poorly understood, but it has been suggested that it may function in cell-cell adhesion and lateral protein interactions.

2.5 Cytosolic domain

The cytosolic tails of the ADAMs vary widely in length and sequence, ranging from 11 to 231 residues (Seals and Courtneidge 2003). The cytosolic tails of most sheddase ADAMs contain putative recognition motifs for signaling proteins and adaptors (Huovila et al. 2005). Several contain one or more PXXP motifs that can act as binding sites for SRC homology 3 domain (SH3) containing proteins and serine, theronine and tyrosine residues that are potential sites for phosphorylation by diverse kinases, suggesting that the cytosolic domains of ADAMs may play important roles in regulating protease function in response to intracellular and outside-in signaling. ADAM9 and ADAM15 bind to endophilin I and SH3-andphox homology (PX) domain-containing protein (SH3PX1). The interactions appear to favor the unprocessed, intracellular forms of these ADAMs (Howard et al. 1999).

As endophilin I and SH3PX1 function in vesicle sorting, is has been speculated that these interactions are involved in the regulation of ADAM maturation and/or subcellular localization (Seals and Courtneidge 2003).

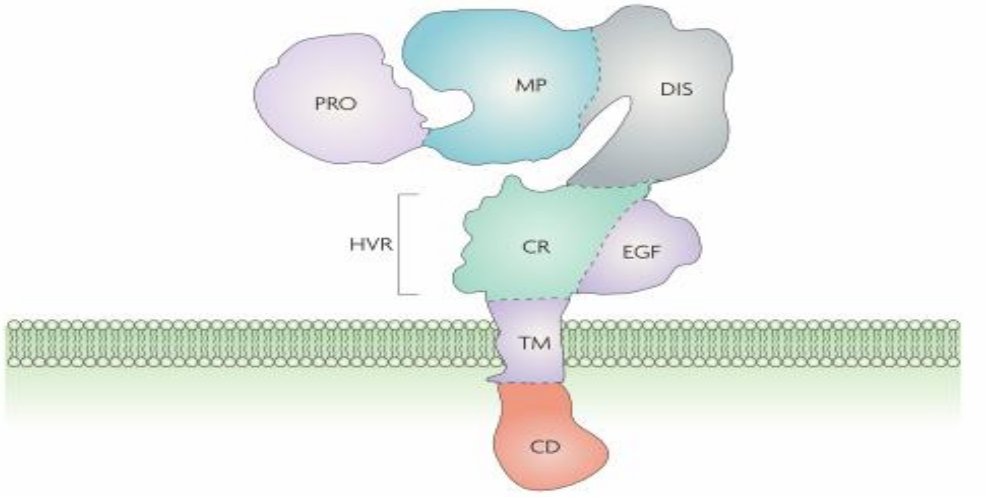

Figure 4. Structure of the ADAM (A Disintegrin And Metalloprotease) family domain. PRO: amino-terminal propeptide, MP: metalloproteinase domain, DIS: disintegrin domain, CR: cysteine-rich region, EGF: epidermal growth factor-like repeat, TM: transmembrane domain, CD: cytoplasmic domain, HVR: hypervariable region. From (Murphy 2008), with permission.

2.6 ADAM8, -9, -15, and -17 genes and expression

ADAM8. ADAM8 (CD156, MS2) is a cell surface glycoprotein originally identified as MS2 cell surface antigen found on mouse macrophages (Yoshida et al. 1990). The human ADAM8 gene includes 23 exons and is located in 10q26.3. Its transcripts encode a 3.1 kb open reading frame which translates to 826 amino acids. ADAM8 protein consists of 16 amino-acid signal peptide, 637 amino acid ectodomain, 25 amino-acid transmembrane region, and 146 amino-acid cytoplasmic region. The deduced ADAM8 protein contains N-glycosylation sites (Yoshiyama et al. 1997) and SH3-binding sequence in the cytosolic region that is generally thought to be involved in the intracellular signaling and regulation of activity (Schlondorff and Blobel 1999; Seals and Courtneidge 2003). ADAM8 is also capable of binding to integrins (Schlomann et al. 2000; Rao et al. 2006) located in disintegrin domain. Zymogen ADAMs are typically activated by furin-catalyzed cut at the pro/metalloprotease domain junction or by other proprotein convertases but

ADAM8 does not contain the consensus cleavage site but, in turn, its prodomain is cleaved off autocatalytically. (Yoshida et al. 1990). ADAM8 is not inhibited by any of the known TIMPs (Amour et al. 2002).

In humans, ADAM8 is expressed in immune cells, particularly in monocytes and granulocytes with the exception of T cells (Yoshiyama et al. 1997). It is also expressed in several tissues, including thymus, cartilage, bone, brain, and, spinal cord and during embryonic development. However, ADAM8 is not essential for the embryogenesis since ADAM8-deficient mice develop normally (Kelly et al. 2005). Although ADAM8 is weakly expressed in normal neurons, its expression is strongly up-regulated in the inflamed murine central nervous system, e.g., in reactive astrocytes, oligodendrocytes, activated microglia and in degenerating neurons (Schlomann et al. 2000). In addition, ADAM8 has also been detected in human osteoclasts (Choi et al. 2001).

ADAM9. ADAM9 (MDC9, meltrin-gamma) was originally cloned from a mouse lung cDNA library (Weskamp et al. 1996). The human ADAM9 gene is located in 8p11.22, comprised of 108 267 bp with 22 exons giving rise to two alternative ADAM9 transcripts through differential splicing, encoding a secreted and a membrane-bound isoform (Hotoda et al. 2002). The mature ADAM9 protein is about 84 kDa protein constituted of 819 amino acids. ADAM9 contains a consensus sequence RXXR for furin through which the pro-metalloprotease domain could be removed (Yamamoto et al. 1999). ADAM9 is also capable of binding integrins through its disintegrin domain and thus suggested to play a role in cell adhesion (Zhou et al. 2001). The cytosolic part of ADAM9 contains two proline-rich (SH3)-binding sequences, suggested to participate in signal transduction, in the C-terminal region of the cytoplasmic tail (Weskamp et al. 1996).

ADAM9 is widely expressed in mouse and human (Weskamp et al. 1996). ADAM9 knockout mice have been reported to be viable and fertile without pathologies in unchallenged conditions (Sahin et al. 2004).

ADAM15. ADAM15 (MDC15, metargidin) was first cloned from adenocarcinoma cells (Kratzschmar et al. 1996) and later from human umbilical vein endothelial cells and cultured human aortic smooth muscle cells (Herren et al. 1997). ADAM15 gene is located in 1q21.3 (Karkkainen et al. 2000) and contains 23 exons of which exons 19 to 21 are used alternatively in human tissues (Kleino et al. 2007). The mass of the mature ADAM15 protein is 85 kDa. The extracellular

moiety contains 5 potential sites of N-linked glycosylation and the zinc-bonding motif suggesting metalloprotease activity. The boundary between prodomain and metalloprotease domain has four consecutive arginine residues providing a potential cleavage site for serine proteases such as the proprotein convertase furin. ADAM15 is the only member the ADAM family that possesses an RGD integrin-binding sequence in the disintegrin loop suggesting a specific role in integrin binding and cell-cell interactions (Kratzschmar et al. 1996). The cytoplasmic tail of ADAM15 presents motifs involved in cell signaling for interaction with SH3-domains of various intracellular proteins (Feng et al. 1994).

ADAM17. ADAM17 (tumor necrosis factor alpha converting enzyme, TACE) was originally identified as the protease responsible for the release of the inflammatory cytokine TNFα from its membrane bound precursor pro-TNF by two groups in 1997 (Black et al. 1997; Moss et al. 1997). The ADAM17, located in 2p25, is 3.0 kb long (Black et al. 1997) and constitutes of several transcripts. The mass of ADAM17 protein is 85 kDa and expression of the protein is largely constitutive. Similarly to ADAM9 and ADAM15, ADAM17 is thought to be activated through cysteine switch mechanism (Black et al. 1997) or by a related enzyme in a late Golgi compartment (Schlondorff et al. 2000). ADAM17 differs from other ADAM family members by having a proline in place of the second cysteine in the consensus EECDCG sequence. There are six potential N-linked glycosylation sites in the extracellular domain (excluding the pro-domain). The cytoplasmic tail ADAM17 is rich in proline and contains a potential tyrosine phosphorylation site, and a Src-homology 3 domain binding site (Moss et al. 1997) that are thought to affect the signaling functions.

2.7 ADAM8, 9-, -15, and -17 in human diseases

2.7.1 ADAM8

The expression of ADAM8 is induced in several diseases indicating a contribution to their pathology. ADAM8 is mainly expressed in the immune systems cells and it

has already been proposed to be involved in several diseases involving immunological processes, e.g., in leukocyte recruitment, rheumatoid arthritis and asthma. In addition, ADAM8 has been associated with cancer.

ADAM8 has been claimed to promote the maturation of bone-destroying osteoclasts and to degrade the hyaline cartilage in rheumatoid arthritis (Koller et al. 2009). Interestingly, a correlation between the degree of joint inflammation and soluble ADAM8 has been established (Gomez-Gaviro et al. 2007). Asthma is a chronic eosinophilic inflammatory disease wherein ADAM8 has recently been found to associate (Matsuno et al. 2006; Foley et al. 2007). In addition, CD23, the low affinity receptor for IgE, has been found to be a substrate of ADAM8 (Moss and Rasmussen 2007), suggesting that ADAM8 may be involved in the CD23 mediated functions, like the regulation of IgE level that initiates the activation cascade leading to hypersensitivity (Gould and Sutton 2008).

ADAM8 mRNA is up-regulated in mice with multiple sclerosis symptoms and in human Parkinson´s disease patients (Koller et al. 2009). ADAM8 has been suggested to exert a neuroprotective effect by preventing neuronal cell death by the release of soluble cell adhesion molecule with homology to L1CAM (CHL1) (Naus et al. 2004) that is an important molecule for neuron survival, neurite outgrowth and cell migration (Schluesener 1998).

Over-expression of ADAM8 has been detected in several cancers. In pancreatic cancer, the over-expression of ADAM8 has been found to be associated with the invasiveness of cancer cells and correlate with reduced patient survival (Valkovskaya et al. 2007). ADAM8 expression has also been found to be associated with severity of prostate cancer (Fritzsche et al. 2006) and tumor invasiveness in brain tumors (Wildeboer et al. 2006). In addition, a diagnostic role has been proposed for ADAM8 as a marker for early stage lung cancer (Ishikawa et al. 2004).
ADAM8 in atherosclerosis. Except for Study III in this thesis, no information exists in the literature about ADAM8 and atherosclerosis.

2.7.2 ADAM9

Most publications concerning the involvement of ADAM9 in human disease have been on cancer. As in the case of ADAM8, the expression of ADAM9 has been

found to be significantly induced in several cancers, including pancreatic cancer and stomach cancer, skin melanoma and hepatocellular carcinoma (Tannapfel et al. 2003; Grutzmann et al. 2004; Carl-McGrath et al. 2005; Zigrino et al. 2005). ADAM9 expression is associated in breast cancer with lymph node metastasis and and correlates positively with neuroblastoma/glioblastoma derived oncogene homolog (HER2), an important prognostic factor for breast cancer (O'Shea et al. 2003).

As a conclusion from the association of ADAM9 with cancer, it has been suggested that ADAM9 may be involved in tumorigenesis, invasion and metastasis through modulation of growth factor activity and integrin function (Mochizuki and Okada 2007).

ADAM9 in atherosclerosis. ADAM9 has previously been reported to be up-regulated in human atherosclerotic carotid artery specimens. Nine-fold up-regulation was seen in the carotid plaques compared to non-atherosclerotic thyroid arteries. In the plaques, ADAM9 was located in smooth muscle cells in the neointima and foam cells of smooth muscle cell origin surrounding lipid cores (Al-Fakhri et al. 2003).

2.7.3 ADAM15

Dysregulation of ADAM15 expression may contribute to a variety of pathological processes. ADAM15 deficient mice have been viable and fertile, but showed reduced tumour neovascularization and accelerated development of osteoarthritis (Horiuchi et al. 2003; Sahin et al. 2004; Bohm et al. 2005). Of human diseases, the association of ADAM15 has mostly been studied with various cancers. ADAM15 up-regulation has been detected e.g., in breast (Lendeckel et al. 2005), prostate (Kuefer et al. 2006), stomach (Carl-McGrath et al. 2005) and lung cancer (Schutz et al. 2005). ADAM15 has been suggested to be involved in tumor invasion and metastasis in lungn cancer and in higher grade disease in prostate cancer (Schutz et al. 2005; Kuefer et al. 2006) Interestingly, ADAM15 knockdown reduced the metastatic spread of prostate cancer cells in vivo and inhibited numerous malignant processes (Najy et al. 2008a). Angiogenesis is an essential process in tumor growth and ADAM15 has actually been detected in areas showing neovascularization in breast cancers (Kuefer et al. 2006).

ADAM15 levels are also up-regulated in osteoarthritis (Bohm et al. 1999) and rheumatoid arthritis (Bohm et al. 2001). Although little is known about the mechanisms of ADAM15-mediated effects on chondrocyte-matrix interactions, it seems clear that ADAM15 plays a role in maintaining homeostasis of cartilage and that disturbation of ADAM15 expression can have pathological consequences (Lucas et al. 2009). ADAM15 has been also linked to Alzheimers´ disease in which it is thought to contribute to the disease via integrin and src protein tyrosin kinase interactions (Bernstein et al. 2003).

ADAM15 in atherosclerosis. There are only few publications on ADAM15 in atherosclerosis, which is quite surprising, since ADAM15 was originally cloned from human umbilical vein endothelial cells (Herren et al. 1997) and endothelial activation is known to play a crucial role in atherosclerosis. In the work of Herren et al., ADAM15 was found to be up-regulated in atherosclerosis of nonhuman primates and localized to the macrophage-rich core. In humans, ADAM15, together with ADAM9, were found to be up-regulated in human atherosclerosis parallel with integrins α5β1 and αvβ3 (Al-Fakhri et al. 2003) that contribute to SMC migration, proliferation and neointima growth (Kim and Yamada 1997; Moiseeva 2001), all crucial elements in the progression of atherosclerosis. ADAM15 was prominently expressed in foam cells of SMC origin and SMCs surrounding lipid cores in the neointima (Al-Fakhri et al. 2003). These data suggest that ADAM15 may contribute to the progression of atherosclerotic lesions.

2.7.4 ADAM17

ADAM17 has been found to play an important role in various physiological and pathophysiological processes, which is not surprising given the wide array of possible ADAM17 substrates (Edwards et al. 2008; Arribas and Esselens 2009; Duffy et al. 2009). ADAM17 knockout mice exhibit perinatal fatality probably due to heart defects, pulmonary hyperplasia and problems with epithelial tissue maturation (Peschon et al. 1998; Zhao et al. 2001; Jackson et al. 2003; Shi et al. 2003).

Similarly to other ADAMs, ADAM17 has been implicated in various cancers, e.g. breast (Lendeckel et al. 2005), ovarian (Tanaka et al. 2005), kidney (Roemer et

al. 2004), colon (Blanchot-Jossic et al. 2005) and prostate cancer (Karan et al. 2003). In breast cancer, the inhibition of ADAM17 has been found to revert the morphology of tumor cells towards nonmalignant phenotype (Kenny and Bissell 2007) whereas the over-expression of ADAM17 has been found to increase the invasion and proliferation of cancer cells (McGowan et al. 2007). Interestingly, treatment of breast cancer cell lines with anti-ADAM17 antibodies decreases cell proliferation (Lendeckel et al. 2005). ADAM17 over-expression in cancers has been suggested to contribute to tumour growth, angiogenesis (Blanchot-Jossic et al. 2005), cancer cell motility (Ali and Knauper 2007) and aggressive behavior of tumors (Tanaka et al. 2005).

ADAM17 has also been found to affect rheumatoid arthritis (Qian et al. 2007) and several inflammatory diseases, e.g., inflammatory bowel disease (Colon et al. 2001), peritonitis (Kermarrec et al. 2005) and polymyositis (Dehmel et al. 2007).

ADAM17 in atherosclerosis. In addition to Study III in this thesis, ADAM17 has previously been detected in human atherosclerosis by Canault et al. (Canault et al. 2006) who showed that human plaques contain catalytically active ADAM17 and suggested the impact of ADAM17 in the inflammatory responses in the lesion (Canault et al. 2007).

AIMS OF THE STUDY

Atherosclerosis is a challenging disease for research of novel therapeutic approaches due to multiple factors affecting its development and progression. Characterizing the typical gene expression changes in the foam cell formation of macrophages and atherosclerotic plaques produces knowledge about the comprehensive mechanisms underlying the disease and discovery of novel candidate genes involved in the disease. The specific aims of the study were:

1) To study and compare the gene expression changes induced by ox-LDL and ox-HDL on healthy cultured human monocyte-macrophages in order to reveal the gene expression changes involved in the foam cell formation of macrophages (I).

2) To investigate the gene expression of all known human genes in advanced human atherosclerotic arteries and non-atherosclerotic arteries using TVS material. More spesifically, the aims were to investigate a) the most up-and down-regulated genes in advanced human atherosclerotic carotid and femoral arteries as well as aortas, b) the site-specific gene expression changes in the arteries studied, c) all pathways and gene sets (available in MSigDB database) that were significantly affected in plaques and d) to verify the expression of all genes in one of the most up-regulated pathways (II).

3) To study the expression of ADAM8 mRNA in advanced atherosclerotic carotid and femoral arteries as well as aortas compared to non-atheroscleortic arteries using TVS material and to study the localization and quantity of ADAM8 protein in the plaques (III).

4) To study the association of ADAM8 2662 T/G allelic variant (rs2995300) with atherosclerosis and myocardial infarction using HSDS material (III).

5) To study the expression and localization and of ADAM9, -15, and -17 mRNA and catalytically active proteins in advanced human atherosclerotic carotid and femoral arteries as well as aortas using TVS material (IV).

1. Cell culture experiments

1.1 Cell culture and lipoprotein treatments

Peripheral mononuclear cells were isolated from leukocyte-rich buffy coats obtained from healthy blood donors from the Finnish Red Cross by Ficoll-Paque (Amersham Pharmacia Biotech UK, Ltd., Buckinghamshire, England) density-gradient centrifugation. Mononuclear cells adhering to dishes (Corning, New York, USA) were cultured in an X-vivo serum-free medium (BioWhittaker, Rockland, California, USA) containing 100 μg / ml penicillin-streptomycin (BioWhittaker). After differentiation into a macrophage-like phenotype, incubations with native and ox-LDL and ox-HDL were done in the serum-free medium for one and three days. Lipoproteins were isolated by sequential ultracentrifugation as previously described (Jaakkola et al. 1989). After isolation, lipoproteins were diluted to 1 mg / ml with phosphate-buffered saline (PBS) and oxidation was done by adding $CuSO_4$ to a final concentration of 20 μmol / l and incubating for 24 h at + 37°C (Lehtimaki et al. 1999). Oxidized lipoproteins were given to the cells in a concentration of 50 μg / ml. Agarose gel electrophoresis (Paragon Lipoprotein (Lipo) Electrophoresis Kit, Beckman Coulter, Fullerton, California, USA) was run and absorbance of conjugated dienes was measured at 234 nm (Perkin Elmer, Boston, Massachusetts, USA) to confirm that lipoproteins were “fully oxidized”. The cells were harvested for total RNA isolation after one and three days of incubation.

1.2 RNA isolation and cDNA expression array

Gene expression profiling was performed using the BD Atlas Human Cardiovascular Array (BD Biosciences Clontech, Palo Alto, California, USA) which included 588 genes potentially involved in cardiovascular diseases. Total RNA was

extracted using the RNeasy® Mini Kit (Qiagen, Valencia, California, USA) following the manufacturer´s instructions. After RNA isolation, 5 µg of total RNA was transcribed, labeled with [α^{33}P] dATP (Amersham Pharmacia Biotech) and hybridized to microarray according to manufacturer´s instructions. Phosphoimaging screens were exposed for ten days and the results scanned with the Storm Phosphoimager (Molecular®Dynamics, Sunnyvale, California, USA).

1.3 Normalization statistical analyses of the microarray data

Raw signal intensities were normalized with ribosomal protein S9 (RPS9) using Atlas Image 2.01 software (BD Biosciences). RPS9 was selected for a housekeeping gene since it showed a constant expression in all microarray experiments. The chi-square test was used to compare the differences in gene expression profiles (number of up-and down-regulated genes) between ox-LDL and ox-HDL experiments (version 8.0; STATA Corporation, Texas, USA). A p-value less than 0.05 was considered statistically significant.

1.4 Quantitative reverse transcription polymerase chain reaction (QRT-PCR)

The expression of macrophage colony stimulating factor 1 (CSF1), which was constantly up-regulated by ox-LDL and down-regulated by ox-HDL after one and three days of incubation in cDNA microarray experiments, was verified by the quantitative RT-PCR (QRT-PCR) using the LightCycler (Roche Diagnostics, Manneheim, Germany). RPS9 showed stable expression between compared samples in QRT-PCR experiments and was thus selected as a housekeeping gene. The primer sequences for CSF1 were from BD Biosciences and the primers were obtained from TIB MolBiol (Berlin, Germany). Total RNA (1 µg was synthesized to cDNA with random primers using the First-Strand cDNA Synthesis Kit for RT-PCR (AMW) (Roche Diagnostics) following the manufacturer´s instructions. PCR analyses were performed in a total volume of 20 µl using the LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics). The PCR reaction mixture included 0.5 µM primers, 4 mM $MgCl_2$ for CSF1 and 3 mM for RPS9, 2 µl LightCycler – Fast Start

Reaction Mix SYBR Green I and 2 µl of 1:10 diluted cDNA. Each sample was analyzed as a duplicate. To verify the amplification specificity, melting curves were run at denaturing + 95°C for 0s, annealing at + 55°C (CSF1) or + 60°C (RPS9) for 30 s followed by denaturing of the samples in + 95°C at a ramping rate of 0.1°C $^{s-1}$. PCR products were further verified by running 1 % agarose gel electrophoresis. QRT-PCR analyses were done using LighCycler Relative Quantification Software with efficiency correction (Roche Diagnostics).

2. Gene expression studies of atherosclerotic plaques

2.1 Vascular samples – Tampere Vascular Study material

The atherosclerotic vascular sample series for genome-wide expression analysis consists of 4 femoral arteries, 9 carotid arteries and 7 abdominal aortas together from 20 patients participating in the ongoing Tampere Vascular Study (TVS). All the patients had a polyvascular disease (i.e. at least two major arterial beds affected by atherosclerotic plaques as evidenced by 1) previous transient ischemic attack and atherosclerotic plaques in the cerebral vasculature or 2) coronary atherosclerosis as evidenced by previous myocardial infarction or 3) angina pectoris and atherosclerotic plaques in coronary angiography or 4) objectively verified peripheral arterial disease by ankle-brachial pressure index < 0.9 or 5) previous arterial surgery due to atherosclerosis or 6) angiographical demonstration of arterial plaques). Six control samples were taken from internal thoracic arteries (ITA) from patients obligated to by-pass surgery due to coronary heart disease. The sample from internal thoracic artery was removed from the distal end of the artery at the beginning of dissection. From these patients, only two had polyvascular disease and all the rest had monovascular disease. The median age for TVS patients was 70 years (45-93) and 75.9% of the subjects were men. The prevalence of risk factors was as follows: dyslipidemia 40.7%, hypertension 77.8%, diabetes 18.5%, history of smoking 86.2%, alcohol consumption more than once a week 42.8%. For the relative gene expression analysis, 24 atherosclerotic tissues samples were used and similarly, the six ITA vessels were used as controls. In a histological study of atherosclerotic vessels, the carotid and femoral artery samples were type V or VI and all aorta

samples were type VI. All control vessels were healthy. The vascular samples were classified according to the American Heart Association classification (AHA) (Stary et al. 1995). The study has been approved by the Ethics Committee of Tampere University Hospital. The samples were taken from patients subjected to open vascular surgical procedures at the Division of Vascular Surgery, Tampere University Hospital. All the patients gave informed consent.

2.2 RNA isolation and genome-wide expression analyses

The fresh tissue samples were soaked in RNALater solution (Ambion Inc., Austin, TX, USA) and isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and the RNAEasy Kit (Qiagen, Valencia, CA, USA). The concentration and quality of the RNA was evaluated spectrophotometrically (BioPhotometer, Eppendorf, Wesseling-Berzdorf, Germany). More than 23,000 known and candidate genes were analyzed using Sentrix Human-8 Expression BeadChips, according to manufacturer´s instructions. (Illumina, San Diego, CA, USA). In brief, a 200 ng aliquot of total RNA from each sample was amplified to cDNA using the Ambion's Illumina RNA Amplification Kit according to the instructions (Ambion, Inc., Austin, TX, USA). Each sample cRNA (1500 ng) was hybridized to Illumina's Sentrix Human-8 Expression BeadChip arrays (Illumina). Hybridized biotinylated cRNA was detected with 1 µg/ml Cyanine3-streptavidine (Amersham Biosciences, Pistacataway, NJ, USA). BeadChips were scanned with the Illumina BeadArray Reader. The method has been described in more detail in our previous work (Oksala et al. 2009).

2.3 Bioinformatics and statistical analyses

Raw intensity data obtained from the IlluminaTM platform were normalized with R language and environment for statistical computing and related Bioconductor module. Bioconductor module was also used to conduct single-probe analysis including fold-change calculations and filtering the probes. Pathway analysis of the expression data was performed using the gene set enrichment analysis (GSEA) implemented in GSEA java desktop application version 2.0 and MsigDB (Molecular Signature Database) version 2.0. Statistical analysis was performed using SPSS

version 14.0. (SPSS Inc., Chicago, IL, USA). The non-parametric Mann-Whitney U-test (SPSS version 14.0, SPSS Inc., Chicago, IL, USA) was used for comparison of mRNA expression between atherosclerotic and control tissues. Data are presented as median and range. A p-value less than 0.05 was considered significant.

2.4 QRT-PCR

Gene expression analyses were performed with TaqMan Low Density arrays (Applied Biosystems, Foster City, CA, USA) using gene specific TaqMan Gene expression assays. Total-RNA (500 ng) was transcribed to cDNA using the High Capacity cDNA Kit (Applied Biosystems) according to manufacturer´s instructions. After the cDNA synthesis, the LDA cards were loaded with 8 µl undiluted cDNA, 42 µl H_20, and 50 µl PCR Universal Master Mix (Applied Biosystems) and run according to the manufacturer`s instructions. Samples were analyzed as duplicates, and both cDNA synthesis and PCR reactions were validated for inhibition. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. The results were analyzed using SDS 2.2 Software (Applied Biosystems) using $\Delta\Delta C_T$method (Livak and Schmittgen 2001).

3. Immunohistochemical stainings

Immunohistochemistry was performed using the ABC-method (Vestastain Elite kit, Vector Laboratories, Burlingame, CA, USA) and paraffin-embedded vascular samples. ADAM-proteins in the vascular wall was detected with a goat anti-human ADAM8 antibody (AF1031, R&D Systems, Minneapolis, MN, USA), rabbit anti-human ADAM9 (CL3ADAM9, Cedarlane Laboratories Ltd., Burlington, NC, USA), mouse anti-human ADAM15 (MAB935), and chicken anti-human ADAM17 (AF930) ectodomain antibodies (R&D Systems). The following primary antibodies were used to detect vascular cell markers in adjacent sections. Muscle actin (mouse anti-human muscle actin, clone HHF35 [DakoCytomation, Glostrup, Denmark]) was used to detect smooth muscle cells. CD68 (mouse anti-human CD68, clone PG-M1 [DakoCytomation]) was used as marker of monocytes and macrophages. For the

detection of endothelial cells, CD31 antibody (mouse anti-human CD31, clone JC70A [DakoCytomation]) was used. The sections were subjected to microwave antigen retrieval treatment as described earlier (Shi et al. 1991). Endogenous peroxidase activity was extinguished by treating the sections with 0.3 % H_2O_2 for 30 minutes. Subsequently, the sections were incubated overnight with the primary antibodies, followed by biotinylated horse anti-goat, goat anti-chicken (1:500, Vector Laboratories) or sheep anti-mouse (1:300, Amersham Int., Buckinhamshire, UK) and ABC-complex for 30 min. Diaminobenzidine was used as a chromogen. All antibodies were diluted in PBS containing 1 % bovine serum albumin and 0.3 % of Triton X-100. Controls included omitting the primary antibody or replacing it with non-immune sera. No staining was seen in the controls.

The co-localization of CD68 and ADAM8, -9 and, -17 in carotid arteries was studied with double staining immunofluorescence. The samples were fixed with 4% paraformaldehyde (in 0.1M PBS, pH 7.3) for 6 h at +4ºC and cryoprotected with 20% sucrose in PBS. Frozen sections (6µm) were cut with Micron HM560 cryostat and thaw-mounted onto Polysine glass slides (Menzel, Braunschweig, Germany). The sections were incubated overnight with mouse monoclonal anti-CD68 (dil. 1:10) and goat anti-ADAM8 (1:10), rabbit anti-ADAM9 (1:100), or with chicken anti-ADAM17 (1:10) followed by a mixture of biotinylated horse anti-mouse antibody (dil. 1:300, Vector Labs) and rhodamine conjugated donkey anti-goat (ADAM8) (dil. 1:50, Jackson Immunoresearch, West Grove, PA, USA) or biotinylated sheep anti-mouse antibody (1:200), Amersham) and rhodamine-conjugated goat anti-rabbit antibody (1:50, Boehringer-Mannheim) (ADAM9), or with a mixture of fluorescein-labeled sheep anti-mouse antibody (1:10, Amersham) and biotinylated goat anti-chicken (ADAM17)(Vector Laboratories, Peterborough, England) for 30 min at +37ºC. Subsequently, the sections were incubated with rhodamine-conjucated avidin (dil. 1:100, Vector Labs) for 30 min. Sections were mounted in a mixture of glycerol and PBS (3:1) containing 0.1% paraphenylenediamine and examined in Nikon Microphot FXA microscope equipped with a proper fluorescence filters. Photographs were obtained with PCO Sensicam digital camera (PCO, Kelheim, Germany). Alternatively the co-distribution of ADAM8, -9, -15, and -17 with CD68 was studied in 5 µm paraffin sections (mirror-image sections). Sections were stained with ABC-method as described above.

4. Western blotting analysis

Four randomly selected samples of atherosclerotic plaques from carotid (two patients) and femoral artery, and aorta were homogenized (100 mg in 350 µl) in ice-cold lysis buffer (10 mM Tris-HCl pH 7.4, 1 % w/v Triton X-100, 0.1 % w/v sodium dodecyl sulphate (SDS), 0.1 % w/v sodium deoxycholate, 150 mM NaCl) (Huovila et al. 1992) containing Roche Complete protease inhibitors (Roche Diagnostics) using a Potter-Elvehjem homogenizer with a Teflon pestle. The amount of protein was not measured but the samples (each 100 mg wet weight) were prepared in equal volumes of homogenization and sample buffers. Insoluble debris was removed with centrifugation (16.2 k x g, 10 min) in a refrigerated microcentrifuge at + 4C. Laemmli SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (5x) with dithiotreitol (DTT) was added to 1x concentration (0.1 M DTT final). The samples were heated at + 99°C for 5 minutes, cooled to room temperature and centrifuged 16.2 x g for 10 minutes prior to loading 100 µl per well onto a 10 % DSD-polyacrylamide gel. Molecular weight markers (Chemichrome Ultimate, C2117, Sigma, St. Louis, MI, USA) were run along the samples. Following the SDS-PAGE, the proteins were electrotransferred onto a polyvinylidene fluoride membrane (BioRad, Hercules, CA, USA) in a semi-dry blotter. The membrane was then treated with 0.1 % w/v Ponceau S in 5 % acetic acid to verify a successful transfer, to ensure an even loading, and to fix the proteins onto the membrane.

The primary antibodies against human ADAM protein ectodomains were from R&D Systems (Abingdon, UK). Goat-anti-ADAM9 (AF939), goat-anti-ADAM15 (AF935), and goat-anti-ADAM17 (AF2129) were diluted 1/1000 in tris-buffered saline with Tween-20 (TBST) containing 5 % non-fat milk (TBS with 0.1 % w/v Tween-20). The peroxidase-coupled secondary antibody, anti-goat/sheep (A9452), was diluted 1/5000 in TBST/milk.

For the immunodetection, the blots were blocked with 5 % w/v non-fat milk in TBST, followed with 1 h incubation at room temperature (RT) with the primary antibodies. After several TBST washes and the secondary antibody incubation (1 h, RT), the blots were washed with TBST, rinsed in TBS, and the bound secondary antibody was visualized with enhanced chemiluminescence (ECL) and a digital imager. Just prior to the ECL reagent incubation, the blots were rinsed in water. The

ECL was done with SuperSignal West Dura reagents according to the manufacturer´s protocol (Pierce, Rockford, Illinois, USA). The ECL was visualized in a ChemoDoc XRS digital imager using Quantity One 4.5.2 software (BioRad, Hercules, California, USA).

5. Genotyping studies

5.1 The Helsinki Sudden Death Study Material

The HSDS material comprises 700 Finnish men subjected to a medicolegal autopsy in the Helsinki region due to the out-of-hospital death of a previously healthy man, accidental death, suspected intoxication, or suicide. The majority of the men studied had died due to cardiac causes (41 %, n=288). Other causes of death were unnatural deaths as the result of accidents or suicides (39 %, n=272) and other diseases (20 %, n=140). Data on coronary artery disease risk factors were collected by interviewing the spouse, relative, or a close friend of the deceased. An informant providing interview data was available for 500 (71 %) of the cases. The interview consisted of more than 50 detailed questions on smoking and alcohol consumption habits, hypertension, diabetes, or other previous illnesses (Karhunen and Penttila 1990). The study was approved by the Ethics Committee of the Department of Forensic Medicine, University of Helsinki and all the medicolegal autopsies were performed at the same department (Ilveskoski et al. 1999).

5.1.1 DNA extraction and genotyping

The samples for genotyping were collected in two series during 1981–1983 (A series, n=400) and 1991–1992 (B series, n=300). In the A series, DNA was extracted from paraffin-embedded samples of cardiac muscle by the method of Isola et al. (Isola et al. 1994). In the B series, DNA was isolated from frozen (-70 °C) cardiac samples by the standard phenol-chloroform method (Ilveskoski et al. 1999). The samples were genotyped using the ABI Prism 7900HT Sequence Detection

System (Applied Biosystems, Foster City, CA, USA), which is based on the 5'-nuclease assay for allelic discrimination. The nucleotide sequences of the primers and the fluorogenic allele-specific oligonucleotide probes used in the PCR reaction were deduced from published sequences in the GenBank database and were chosen and synthesized in conjunction with Applied Biosystems. PCR reaction was performed according to standard protocol for TaqMan MBG probes in a total volume of 5 µl. After cycling, end-point fluorescence was measured and the allelic discrimination analysis module carried out the genotype calling, which was also checked manually. Genotyping was successful in 539 cases out of the 700 samples (298 cases in the A series and 241 in the B series). As a quality control method, we genotyped random blind duplicates from the A and B series.

5.1.2 Measurements of atherosclerotic plaque area

The relative areas of different types of atherosclerotic plaques in the left anterior descending coronary artery (LAD), right coronary artery (RCA), and left circumflex coronary artery (LCX) were measured with the standard computer-assisted planimetric technique, which has been described in detail by Ilveskoski et al. (Ilveskoski et al. 1999). Silicon rubber models of arteries were used to measure the stenosis of the three main epicardial coronary arteries (LAD, RCA and LCX) (Weman et al. 1999). Maximal stenosis was used to define the extent of coronary narrowing for each coronary artery. This data is available for 670 men. To confirm acute myocardial infarction (AMI), the myocardium was examined. The presence of neutrophil granulocytes was used as a definitive marker of AMI, and scar tissue in the myocardium was a marker of previous myocardial infarction (MI). Furthermore, the presence of thrombosis in coronary arteries was studied during the autopsy.

5.1.3 Statistical analyses

Statistical analysis was performed using SPSS version 14.0. (SPSS Inc., Chicago, IL, USA). The non-parametric Mann-Whitney U-test was used for comparison of mRNA expression between atherosclerotic and control tissues. For statistical analyses, the genotypes were merged into two groups: TT homozygotes and carriers

of the G allele (i.e., TG heterozygotes and GG homozygotes), because of the small number of GG homozygotes (n=6). Categorical variables were compared with Pearson χ^2 test and continuous variables using analysis of variance (ANOVA). The relative surface areas of different types of atherosclerotic plaques and total atherosclerotic surface area were square-root transformed for the analyses because of their skewed distributions. The Mann-Whitney U test was used to analyze non-normally distributed variables. To determine adjusted odds ratios for the occurrence of MI, sudden cardiac death (SCD), and different subtypes of SCD by ADAM8 genotype groups, logistic regression analysis was applied. The logistic regression analyses were adjusted for age, body mass index and with hypertension because these covariates significantly associate with the risk of MI and fatal AMI in the present study. Smoking, alcohol consumption, and diabetes were not used as covariates because these data were missing for several cases and including them would have caused too many cases to be excluded from the analysis. There were no significant associations between ADAM8 genotype group and the occurrence of smoking or diabetes and with daily alcohol consumption. The combined group of men who had died of other diseases or by unnatural causes, was used as a reference group when analyzing the association between ADAM8 genotype group and fatal AMI. The ADAM8 genotype group was not associated with the occurrence of death either by other diseases or by unnatural causes. A p-value less than 0.05 was considered statistically significant.

1. Effects of lipoprotein-loading of human macrophages (I)

1.1 cDNA expression array analysis on lipoprotein-loaded macrophages

RNA extracted from cells incubated with native time-matched LDL and HDL was used as controls in the microarray analysis. The genes showing at least 2-fold up- or down-regulation according to the ratio of time-matched oxidized lipoprotein to native lipoprotein-treated cells, (e.g. ratio of oxidized to native LDL or HDL for one or three days) were selected for statistical analysis.

Altogether ox-LDL and ox-HDL treatments of monocyte-macrophages for one day caused an up-regulation of 34 vs. 13 genes ($p= 0.0018$) and for three days 52 vs. 99 genes ($p< 0.0001$) respectively. For one-day incubation, the number of down-regulated genes was 1 vs. 57 ($p< 0.0001$) and for three-day incubation 7 vs. 14 genes ($p= 0.12$) respectively. The genes affected by lipoprotein treatments were divided into five groups: 1) lipid metabolism, 2) growth and chemotaxis, 3) inflammation and coagulation, 4) others and 5) total (all genes). The difference between ox-LDL and ox-HDL incubations was statistically significant at both time-points among up- and down-regulated genes in the lipid metabolism (1), others (4) and in total groups (5) whereas in the growth and chemotaxis group the difference was significant only in one-day incubation among down-regulated genes. In the inflammation and coagulation group, the difference was significant only among genes up-regulated by three-day incubations.

The most constantly expressed genes that showed altered expression in at least two of the four different lipoprotein treatments (ox-LDL one and three day, ox-HDL one and three day) were divided into groups according to their biological functions (Table 2). Genes were divided into groups of 1) lipid metabolism, 2) inflammation, growth and hemostasis, 3) matrix metalloproteinases and tissue inhibitors of matrix

metalloproteinases, 4) other enzymes, 5) structural and binding proteins, and 6) annexins. The genes were further classified according to the effects induced by ox-LDL and ox-HDL; opposite effects (A), parallel (B), only ox-LDL (C) or only ox-HDL (D) induced change in gene expression. Some of the genes have previously been linked to atherosclerosis but in addition, several new candidate genes not previously shown to have altered expression in response to lipoprotein loading in macrophages are presented.

1.2 Expression of colony stimulating factor 1 and ribosomal protein S9

Ox-LDL and ox-HDL incubations induced opposite effects on the expression of certain genes. Since we were interested in this phenomenom, the expression of colony stimulating factor 1 (macrophage) (CSF1) was verified which was constantly up-regulated by ox-LDL and down-regulated by ox-HDL in both one and three-day incubations in microarray experiments. Since ribosomal protein S9 (RPS9) was found to have a stable expression in the microarray analysis as well as in QRT-PCR experiments, it was used as a housekeeping gene. An expression pattern similar to that found in microarray experiments was verified for CSF1 with QRT-PCR. Incubation of monocyte-derived macrophages with ox-LDL for one and three days caused more than 2-fold up-regulation of the CSF1 gene in QRT-PCR analysis, while incubations with ox-HDL had a converse effect at both time-points studied.

Table 2. Comparison of the effects of oxidized low and high density lipoproteins on the gene expression of human cultured macrophages measured by BD ATlas™ Human Cardiovascular array. The gene expression is shown as a ratio of time-matched oxidized lipoprotein-treated cells and native lipoprotein-treated cells. Genes are divided into functional groups and organized into four different classes depending on whether the effect of LDL and HDL was; A) opposite, B) parallel, or whether c) oxidized LDL, or D) oxidized HDL alone induced the gene expression change. Minus (-) indicates down-regulation.

Functional group	**Class**	**Oxidized LDL**		**Oxidized HDL**		**Entrez Gene**
		1 day	**3 days**	**1 day**	**3 days**	
Lipid metabolism (n=12)						
Apolipoprotein E (APOE)	A	2.5	-	- 3.1	-	348
Fatty acid-binding protein 3 (FABP3)	A	2.9	2.1	- 4.3	- 2.3	2170
Lipase A (LIPA)	A	3.9	6.0	- 4.4	-	3988
LDL receptor-related protein-associated protein 1 (LRPAP1)	A	-	- 6.3	-	44.1	4043
CD68 molecule (CD68)	A	2.7	2.0	- 2.4	-	968
Cholesterol acyltransferase (ACAT)	A	-	5.7	- 3.2	-	38
Low density lipoprotein receptor-related protein 1 (LRP1)	B	-	4.3	-	224.0	4035
High density lipoprotein-binding protein (HDLBP)	B	6.2	4.0	-	9.6	3069
Apolipoprotein A precursor (APOA)	B	-	3.0	-	46.8	335
Macrophage scavenger receptor 1 (MSR1)	B	- 4.5	3.4	-	3.0	4481
Acyl-coenzyme A dehydrogenase (VLCAD)	D	-	-	- 14.3	- 5.2	37
Phospholipase C, beta 2 (PLCB2)	D	-	-	- 8.6	- 4.0	5330
Inflammation, growth and chemotaxix (n=23)						
Macrophage colony stimulating factor (CSF1)	A	10.4	9.0	- 5.2	- 19.0	1435
Chemokine ligand 2 (CCL2)	A	-	4.0	- 5.6	- 5.1	6347
Retinoid acid receptor alpha (RARA)	A	-	2.3	- 3.0	-	5914
Major histocompatibility complex, class I, C (HLAC)	A	2.0	- 2.6	-	2.8	3107
CD9 molecule (CD9)	A	2.7	3.4	- 2.3	-	928
Intercellular adhesion molecule 1 (ICAM1)	A	-	2.3	- 4.4	-	3383
Profilin 1 (PFN1)	A	3.2	3.1	- 2.9	-	5216
Calcium and integrin binding protein 1 (CIB1)	A	-	2.9	- 3.1	-	10519
Thrombospondin 2 (THBS2)	B	30.3	13.9	244.6	449.6	7058
Fibrinogen beta chain (FGB)	B	115.3	66.9	2.2	9.4	2244
Platelet-activating factor acetylhydrolase (PAF-AHB1)	B	33.0	11.0	5.5	51.8	5048

Functional group	**Class**	**Oxidized LDL**		**Oxidized HDL**		**Entrez Gene**
		1 day	**3 days**	**1 day**	**3 days**	
Plasminogen activator, tissue (PLAT)	B	19.7	-	10.1	15.4	5327
Neuropilin 2 (NRP2)	B	-	2.6	-	35.8	8828
Chemokine ligand 5 (CCL5)	B	-	2.8	2.8	-	6352
Selectin L (SELL)	B	18.4	-	-	3.4	6402
Integrin, alpha L (ITGAL)	B	8.1	4.8	4.9	-	3683
CD40 molecule (CD40)	B	-	6.9	-	2.1	958
Selectin P ligand (SELPLG)	B	-	2.7	-	13.8	6404
Tissue factor pathway inhibitor (TFPI)	B	-	3.1	-	31.1	7035
Coagulation factor 2 (F2)	B	-	3.3	-	6.1	2147
Integrin, beta 2 (ITGB2)	C	3.4	2.9	-	-	3689
Platelet-derived growth factor A (PDGFA)	D	-	-	- 15.4	3.1	5154
Chemokine ligand 1 (CCL1)	D	-	-	- 3.8	- 8.9	6346
Matrix metalloproteinases and inhibitors (n=5)						
Matrix metalloproteinase 9 (MMP9)	A	4.3	4.8	- 3.6	-	4318
Matrix metalloproteinase 12 (MMP12)	D	-	-	- 16.3	- 5.2	4321
Tissue inhibitor of matrix metalloproteinase 1 (TIMP1)	A	3.2	2.0	- 2.2	-	7076
Tissue inhibitor of matrix metalloproteinase 2 (TIMP2)	A	-	2.6	-3.3	-	7077
Tissue inhibitor of matrix metalloproteinase 3 (TIMP3)	B	20.5	10.0	12.6	77.0	7078
Other enzymes (n=16)						
Superoxide dismutase 2 (SOD2)	A	-	3.5	- 2.3	-	6648
Natriuretic peptide precursor B (NPPB)	A	3.2	2.1	- 5.3	- 2.2	4879
Glucosamine-6 sulfatase (GNS)	A	2.5	3.5	- 3.0	-	2799
Angiotensin I converting enzyme (ACE)	A	4.0	2.6	- 15.7	-	1636
Prolyl 4-hydroxylase, beta (P4HB)	A	3.1	2.9	- 2.9	-	5034
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	A	2.1	-	- 8.0	-	2597
P450 oxidoreductase reductase (POR)	A	-	3.2	- 2.2	-	5447
Peptidyl isomerase B (PPIB)	A	-	2.9	- 2.5	-	5479
Cytochrome B5 reductase 1 (CYB5R1)	A	-	2.6	- 2.5	-	51706
Cytochrome P450 IA1 (CYPA1)	B	24.5	-	23.0	311.0	1543
Endothelin-converting enzyme 1 (ECE1)	D	-	-	- 9.6	- 2.0	1889
Cytochrome P450, family 7 (CYP7A1)	D	-	-	52.0	182.0	1581
Protein kinase, AMP-activated (PRKAA1)	D	-	-	- 10.4	-2.6	5562

Functional group	**Class**	**Oxidized LDL**		**Oxidized HDL**		**Entrez Gene**
		1 day	**3 days**	**1 day**	**3 days**	
Farnesyl diphosphate synthase (FDPS)	D	-	-	4.6	12.4	2224
Acetyl-coenzyme A acyltransferase 1 (ACAA1)	D	-	-	2.7	6.2	30
Structural and binding proteins (n=8)						
Filamin A (FLNA)	A	6.3	5.4	- 4.8	- 2.4	2316
Actin, beta (ACTB)	A	2.8	2.9	- 2.5	-	60
Caveolin 3 (CAV3)	B	3.7	4.0	2.0	9.9	859
Amyloid beta precursor-like protein 2 (APLP2)	C	5.5	3.5	-	-	334
Vimentin (VIM)	C	2.7	2.0	-	-	7431
Ezrin (EZR)	D	-	-	12.8	324.0	7430
Junction plakoglobin (JUP)	D	-	-	4.0	20.5	3728
Lectin, galactosidase binding 1 (LGALS1)	D	-	-	3.6	2.6	3956
Annexins (n=5)						
Annexin 1 (ANXA1)	A	3.2	2.9	- 4.0	-	301
Annexin 3 (ANXA3)	B	80.2	-	11.8	116.0	306
Annexin 4 (ANXA4)	A	2.1	-	- 3.0	-	307
Annexin 5 (ANXA5)	A	2.2	3.4	- 3.8	-	308
Annexin 6 (ANXA6)	D	-	-	- 8.2	- 3.2	309

2. Whole-genome expression analysis of human advanced atherosclerotic plaques (II)

2.1 Significantly altered gene expression in atherosclerotic plaques

Several genes were found to have significantly altered expression in advanced atherosclerotic carotid and femoral arteries as well as in aortas studied with GWEA. According to the used selection criteria (>3.0 fold change and p-value < 0.05), 235 genes were up-regulated and 68 genes down-regulated in type V-VI carotid arteries. For type V-VI femoral arteries, 242 genes were up-regulated and 116 genes down-regulated. In type VI aortas, 256 genes were up-regulated and 49 genes down-regulated. In order to identify globally affected genes, we combined all gene expression results and calculated average fold changes. From these, the 27 most up-regulated and 16 down-regulated genes were verified with QRT-PCR (Table 3 and 4). GWEA and QRT-PCR methods were found to be highly comparable, the congruity was ca 90 %.

Among the most up-regulated genes verified with QRT-PCR in atherosclerotic plaque (Table 3), we found genes already previously connected to atherosclerosis, like matrix metalloproteinases (Raffetto and Khalil 2008), apolipoproteins (Greenow et al. 2005) and osteopontin (Singh et al. 2007), but we also found genes that have not been reported at all in the context of atherosclerosis. Without any prior evidence according to a PubMed search, new potential candidate genes involved in atherosclerosis pathogenesis generally are e.g., acid phosphatase 5 (ACP5), interleukin 4 induced 1 (IL4I1), interferon, gamma-inducible protein (IFI30), SLAM family member 8 (SLAMF8), lysozyme (LYZ) and immunoglobulin J polypeptide (IGJ). These genes are presented in boldface in Table 3.

The most generally down-regulated genes (n=16) in advanced atherosclerotic plaques analyzed QRT-PCR are shown in Table 4. For most of the genes on this list, there are only few studies in the literature and no information about their connection to atherosclerosis.

2.1.1 Site-specific gene expression changes in different arterial regions

As the susceptibility to develop atherosclerosis differs notably between different sites in human vasculature and the severity of atherosclerosis varies from stable calcified and fibrotic plaques to unstable ulcerated plaques, we sought genes that were specifically induced in each atherosclerotic artery type. According to GWEA data, we found eight genes that were induced only in aortic plaques and three genes that were specifically induced in femoral plaques. The genes that were induced in carotid arteries, were also induced in aortic and femoral plaques, thus no specific gene for carotid plaques was found. The genes that were induced only in aortic or femoral plaques, are shown in Table 5.

Table 3. The most generally up-regulated genes in atherosclerotic plaques analyzed with TaqMan Low Density array. The results are shown as an average fold change (FC) compared to control arteries analyzed with $\Delta\Delta C_T$ method (Livak and Schmittgen 2001). New potential candidate genes involved in atherosclerosis pathogenesis are marked in boldface.

Gene abbreviation	***Gene ID***	***Average FC***
MMP12 (matrix metallopeptidase 12)	4321	473.7 (p= 0.000)
MMP7 (matrix metallopeptidase 7)	4316	686.1 (p= 0.000)
SPP1 (secreted phosphoprotein 1)	6696	173.0 (p= 0.000)
APOC1 (apolipoprotein C-I)	341	154.8 (p= 0.000)
MMP9 (matrix metallopeptidase 9)	4318	125.3 (p= 0.000)
CCL18 (CC chemokine ligand 18)	6362	105.7 (p= 0.000)
ACP5 (acid phosphatase 5)	54	57.7 (p= 0.000)
APOE (apolipoprotein E)	348	52.9 (p= 0.000)
IL4I1 (interleukin 4 induced 1)	259307	35.2 (p= 0.000)
RGS1 (regulator of G-protein signaling 1)	5996	19.9 (p= 0.000)
HMOX1 (heme oxygenase (decycling) 1)	3162	20.5 (p= 0.000)
IFI30 (interferon, gamma-inducible protein 30)	10437	23.2 (p= 0.000)
SLAMF8 (SLAM family member 8)	56833	22.0 (p= 0.000)
MGC29506 (hypothetical protein)	51237	19.2 (p= 0.001)
THBS1 (thrombospondin 1)	7057	13.8 (p= 0.000)
LYZ (lysozyme)	4069	10.8 (p= 0.000)
IGJ (immunoglobulin J polypeptide)	3512	14.1 (p= 0.001)
TYMP (thymidine phosphorylase)	1890	14.0 (p= 0.000)
IL8 (interleukin 8)	3576	12.0 (p= 0.000)
COL1A1 (collagen, type I, alpha 1)	1277	5.7 (p= 0.000)
CAPG (capping protein)	822	7.4 (p= 0.000)
ADFB (adipose differentiation-related protein)	123	6.4 (p= 0.000)
LGALS3 (lectin, galactoside-binding, soluble, 3)	3958	1.8 (p= 0.001)
CYBA (cytochrome b-245, alpha polypeptide)	1535	5.4 (p= 0.000)
CFL1 (cofilin 1)	1072	1.3 (p= 0.158)
ALDOA (aldolase A, fructose-bisphosphate)	226	1.2 (p= 0.000)
HLA-DRP3 (major histocompatibility complex, class II, DR beta 3)	3125	- 8.8 (p= 0.561)

Table 4. The most generally down-regulated genes in atherosclerotic plaques analyzed with TaqMan Low Density array. The results are shown as an average fold change (FC) compared to control arteries analyzed with $\Delta\Delta C_T$ method (Livak and Schmittgen 2001).

Gene abbreviation	***Gene ID***	***Average FC***
ITLN1 (intelectin 1)	55600	- 568.2 (p= 0.000)
APOD (apolipoprotein D)	347	- 103.9 (p= 0.000)
DUSP26 (dual specificity phosphatase 26)	78986	- 21.3 (p= 0.000)
CASQ2 (calsequestrin 2)	845	- 23.6 (p= 0.000)
RGS5 (regulator of G-protein signaling 5)	8490	- 13.0 (p= 0.000)
TCEAL2 (transcription elongation factor A (SII)-like 2)	140597	- 18.6 (p= 0.000)
DES (desmin)	1674	- 15.8 (p= 0.025)
CALD1 (caldesmon 1)	800	- 10.4 (p= 0.000)
PPP1R3C (protein phosphatase 1) regulatory (inhibitor) subunit 3C)	5507	- 9.5 (p= 0.000)
ADRA2C (adrenergic, alpha-2C-, receptor)	152	- 12.9 (p= 0.000)
CSRP2 (cysteine and glycine-rich protein 2)	1466	- 8.6 (p= 0.000)
SPEG (SPEG complex locus)	10290	- 4.2 (p= 0.000)
CNN1 (calponin 1)	1264	- 4.3 (p= 0.000)
C6orf117 (chromosome 6 open reading frame 117)	112609	- 8.7 (p= 0.000)
LMOD1 (leiomodin 1)	25802	- 6.5 (p= 0.000)
RAMP1 (receptor (G protein-coupled) activity modifying protein 1)	10267	- 5.4 (p= 0.000)

Table 5. Genes induced only in human advanced aortic or femoral plaques analyzed with genome-wide expression array (GWEA) and represented as fold changes (FC). For all genes, the p-value was less than 0.05.

Gene	***Gene ID***	***FC***	***Function***
Aortic plaques			
CHGA (chromogranin A)	1113	5.8	Secretory granule formation, immunity against microbes, cancer, hypertension
CSF3 (colony stimulating factor 3)	1440	4.6	Survival / proliferation of neutrophils and macrophages
GAGE12I (G antigen 12I)	26748	4.5	Antigen, anti-apoptotic factor
C4orf7 (chromosome 4 open reading frame 7)	260436	4.3	B cell immunity
GAGE6 (G antigen 6)	2578	4.0	Antigen, anti-apoptotic
LTF (lactotransferrin)	4057	3.7	Immune modulator
PRPH (peripherin)	5630	3.6	Intermediate filament
MS4A1 (membrane-spanning 4-domains)	931	3.5	B cell immunity
Femoral plaques			
C1QTNF3 (Ciq and tumor necrosis factor related protein 3)	114899	3.4	Adipose tissue secreted protein
CHAD (chondroadherin)	1101	3.9	Extracellular matrix modification
PTN (pleiotrophin)	5764	4.7	Heparin-binding growth factor, cancer, angiogenesis

2.2 Altered pathways and gene sets in advanced atherosclerotic plaques

In order to identify globally affected pathways in advanced atherosclerosis, we performed GSEA to illuminate dysregulated pathways. In the pathway analyses, 20 pathways appeared to be significantly up-regulated and 8 pathways down-regulated in advanced atherosclerotic arteries compared to non-atherosclerotic arteries according to the criteria recommended by Subramanian et al. (FDR<0.25) (Subramanian et al. 2005). Significantly up-regulated pathways involved apoptotic and pro-inflammatory pathways as well as pathways involved in thrombosis or B cell activation and cell movement. The significantly altered down-regulated pathways included fatty acid metabolism and amino acid metabolism pathways (glutamate, leucine, isoleucine), benzoate degradation pathway and pathway including genes of hormonal functions. A pathway including homeobox genes related to hematopoiesis was also significantly down-regulated.

2.2.1 T cell differentiation pathway

One of the significantly upregulated pathways was a set of genes involved in T cell differentiation (nktPathway). Since T cells are important effectors of plaque inflammatory processes, we quantitated all genes in this pathway with QRT-PCR (Table 6). Many of the genes involved in T cell function have been widely studied with regard to atherosclerosis but now, for the first time, the expression of all the genes involved in T cell differentiation is characterized at the same time from all major atherosclerotic vascular beds. The pathway included a total of 29 genes of which 25 were significantly up-regulated in advanced atherosclerotic lesions compared to non-atherosclerotic internal thoracic arteries (for exact fold changes, see Table 6). In general, the highest fold changes were seen for all genes in aortas. The most up-regulated genes are a set of chemokines and colony stimulating factor 2, interleukin 12B, CD4 molecule and interleukin 12 receptor. Even though the pathway was in general up-regulated, there were also four genes that were generally down-regulated in the arteries studied. The most down-regulated gene was chemokine (C-C motif) receptor 3 (CCR3) (Table 6). By quantitating the whole T cell differentiation pathway we were able also to reveal genes whose role in the

research has commanded less interest. Genes that were significantly up-regulated in this pathway but have not been thoroughly studied with regard to atherosclerosis are chemokine receptors 3, 4, and 7 (CCR3, CCR4, CCR7), interferon gamma receptor 1-2 (IFNGR1, IFNGR2), interleukin 12 receptor beta 1-2 (IL12RB1, IL12RB2), interleukin 18 receptor 1 (IL18R1), interleukin 4 receptor (IL4R), transforming growth factor beta 2 (TGFB2).

Table 6. Expression of nkTPathway genes in atherosclerotic plaques from carotid arteries, aortas and femoral arteries analyzed with TaqMan Low Density array. Fold changes are calculated by comparing the median expression of genes in atherosclerotic arteries vs. controls. Genes marked in boldface represent a specific expression pattern dependable upon arterial bed.

Gene abbreviation	***Gene ID***	***Average FC***
CSF2 (colony stimulating factor 2 (granulocyte-macrophage)	1437	↑↑↑*
IL12B (interleukin 12B)	3593	↑↑*
CCR5 (chemokine (C-C motif) receptor 5)	1234	14.9 (p=0.000)
CXCR4 (chemokine (C-X-C motif) receptor 4)	7852	12.4 (p= 0.001)
CCR1 (chemokine (C-C motif) receptor 1)	1230	10.7 (p= 0.000)
CD4 (CD4 molecule)	920	10.2 (p= 0.000)
CCL4 (chemokine (C-C motif) ligand 4)	6351	8.4 (p= 0.001)
CCR7 (chemokine (C-C motif) receptor 7)	1236	8.1 (p= 0.001)
CCL3 (chemokine (C-C motif) ligand 3)	6348	6.6 (p= 0.000)
IL12RB1 (interleukin 12 receptor, beta 1)	3594	6.0 (p= 0.000)
CD28 (CD28 molecule)	940	5.8 (p= 0.001)
CXCR3 (chemokine (C-X-C motif) receptor 3)	2833	5.6 (p= 0.001)
CCR4 (chemokine (C-C motif) receptor 4)	1233	3.5 (p= 0.033)
CD40LG (CD40 ligand)	959	3.4 (p= 0.008)
IFNG (interferon, gamma)	3458	3.1 (p= 0.022)
IL12RB2 (interleukin 12 receptor, beta 2)	3595	3.0 (p= 0.233)
IFNGR2 (interferon gamma receptor 2)	3460	2.9 (p=0.000)
IL12A (interleukin 12A)	3592	2.9 (p= 0.009)
TGFB1 (transforming growth factor, beta 1)	7040	2.0 (p= 0.000)
IL18R1 (interleukin 18 receptor 1)	8809	2.0 (p= 0.007)
CCR2 (chemokine (C-C motif) receptor 2)	1231	1.9 (p= 0.213)
IFNGR1 (interferon gamma receptor 1)	3459	1.7 (p= 0.005)
IL4R (interleukin 4 receptor)	3566	1.4 (p= 0.002)
TGFB2 (transforming growth factor, beta 2)	7042	1.4 (p= 0.069)
IL5 (interleukin 5)	3567	0.2 (p= 0.500)
IL4 (interleukin 4)	3565	- 0.5 (p= 0.468)
IL2 (interleukin 2)	3558	- 0.1 (p= 0.836)
TGFB3 (transforming growth factor, beta 3)	7043	- 0.1 (p= 0.350)
CCR3 (chemokine (C-C motif) receptor 3)	1232	- 1.7 (p=0.108)

Note. *Highly expressed in atherosclerotic arteries.

3. Comparison of the gene expression profiles of cultured lipoprotein-loaded macrophages and human advanced atherosclerotic plaques (I, II)

Since many of the candidate genes for foam cell formation found in Study I of the thesis, are new with regard to atherosclerosis, we also investigated their expression in human advanced plaques using the GWEA data obtained in Study II. While many of the genes suggested to be crucial for foam cell formation in Study I, several of them were not found to be differentially expressed in advanced atherosclerotic plaques. However, fatty-acid binding protein 3 (FABP3), thrombospondin 2 (THBS2) and filamin A (FLNA) were found to be significantly dysregulated in lipoprotein-loaded macrophages as well as in human advanced plaques suggesting that these genes may have a constant effect in the development of atherosclerosis. These genes are marked in boldface in Table 7 (unpublished data).

Table 7. Comparison the gene expression profiles of selected candidate genes between human lipoprotein-loaded macrophages and advanced atherosclerotic plaques. The gene expression changes in lipoprotein treatments are shown as a ratio of time-matched oxidized lipoprotein-treated cells and native lipoprotein-treated cells. The gene expression changes in atherosclerotic arteries are shown as fold changes (atherosclerotic artery vs. control artery). Genes dysregulated in both lipoprotein-loaded macrophages and atherosclerotic plaques are marked in boldface.

Functional group	**Oxidized LDL 1 day / 3 day**	**Oxidized HDL 1 day / 3 day**	**Carotid arteries**	**Aorta**	**Femoral arteries**
Fatty acid-binding protein 3 (FABP3)	**2.9 / 2.1**	**- 4.3 - 2.3**	**- 1.9**	**- 2.7**	**- 2.4**
LDL receptor-related protein-associated protein 1 (LRPAP1)	- / - 6.3	- / 44.1	1.2	-	1.1
Thrombospondin 2 (THBS2)	**30.3 / 13.9**	**244.6 / 449.6**	**2.2**	**1.7**	**1.9**
Fibrinogen beta chain (FGB)	115.3 / 66.9	2.2 / 9.4	1.1	-	-
Chemokine ligand 1 (CCL1)	- / -	- 3.8 / - 8.9	-	1.1	- 1.1
Natriuretic peptide precursor B (NPPB)	3.2 / 2.1	- 5.3 / - 2.2	1.1	- 1.1	1.1
Acyl-coenzyme A dehydrogenase (VLCAD)	- / -	- 14.3 / - 5.2	1.1	1.3	1.3
Filamin A (FLNA)	**6.3 / 5.4**	**- 4.8 / - 2.4**	**- 1.7**	**- 1.8**	**- 2.0**

4. Expression of ADAM family genes and proteins in atherosclerotic plaques (III, IV)

4.1 ADAM mRNA expression in plaques

ADAM gene expression was examined with GWEA and QRT-PCR between atherosclerotic arteries (types V-VI) and non-atherosclerotic internal thoracic arteries, as well as ADAM expression between different vessel types. All ADAM family genes studied (ADAM8, ADAM9, ADAM15, and ADAM17) were found to be up-regulated generally in atherosclerosis as well as in the various vascular regions studied. The strongest up-regulation was seen for ADAM8 and weakest for ADAM17 (Table 8, unpublished). The expression of ADAM8 was higher in type VI vessels than in type V plaques, whereas no such difference was seen with other ADAM genes.

Table 8. Expression of ADAM genes in advanced human atherosclerotic plaques measured by GWEA and QRT-PCR compared to control arteries. Results are displayed as fold changes and p-values.

ADAM Gene	**Carotid artery**	**Aorta**	**Femoral artery**	**Atherosclerotic vs. control artery**
ADAM8				
GWEA	2.6 (p=0.000)	3.0 (p=0.005)	2.7 (p=0.038)	p < 0.000
QRT-PCR	8.2 (p=0.000)	10.0 (p=0.002)	6.3 (p=0.009)	p < 0.000
ADAM9				
GWEA	1.2 (p=0.012)	1.3 (p=0.001)	1.5 (p=0.010)	p =0.001
QRT-PCR	1.8 (p<0.001)	1.7 (p< 0.001)	1.4 (p=0.065)	
ADAM15				
GWEA	2.2 (p<0.001)	2.7 (p<0.001)	3.0 (p=0.010)	p < 0.001
QRT-PCR	1.4 (p=0.607)	2.1 (p=0.012)	2.2 (p=0.065)	
ADAM17				
GWEA	1.2 (p<0.001)	1.2 (p=0.001)	1.3 (p=0.01)	p <0.001
QRT-PCR	1.1 (p<0.001)	1.4 (p<0.001)	1.3 (p=0.132)	

4.2 ADAM proteins in plaques

Immunohistochemistry of the atherosclerotic lesions revealed that ADAM8 protein is present in both healthy arteries and advanced atherosclerotic lesions but there is a distinct increase in the ADAM8 protein in atherosclerotic plaques. ADAM8 protein

was observed in macrophages and smooth muscle cells (Figure 5). However, endothelial cells displayed no immunoreactivity to the ADAM8. The ADAM8 protein was most prominent in macrophages. The ADAM8 protein presence in macrophages was further verified with double-immunofluorescence and staining adjacent mirror-image section that clearly demonstrated the localization of ADAM8 in macrophages in carotid arteries. The ADAM8 protein was seen mainly in the cell membranes of macrophages (Figure 5).

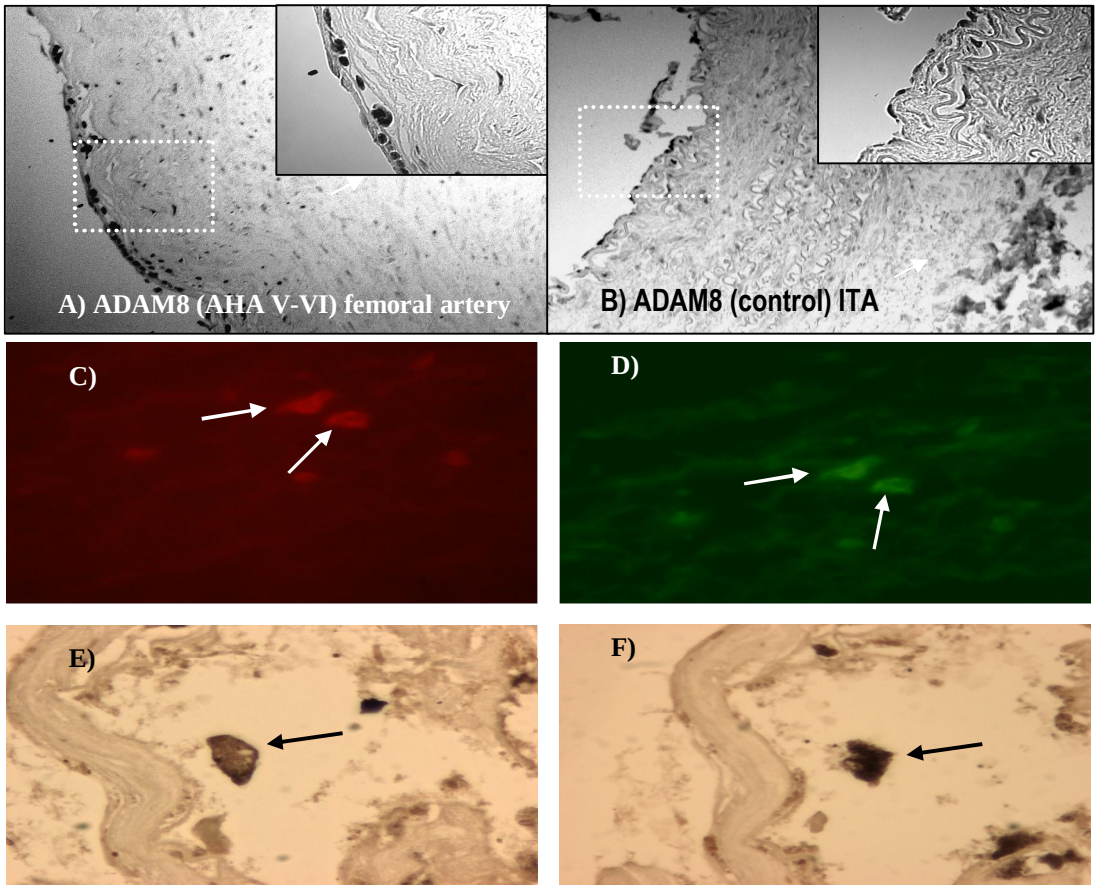

Figure 5. Immunohistochemical stainings of ADAM8 protein in A) AHA type V-VI femoral artery, B) non-atherosclerotic internal thoracic artery, double immunofluorescence images demonstrating co-distribution of C) ADAM8 and D) CD68. Arrows indicate the double labeled cells. Adjacent mirror-image sections showing a macrophage (arrows) heavily stained for E) ADAM8 and F) CD68. ADAM8 immunoreactivity is strongest in the cell membrane.

ADAM9, ADAM15, and ADAM17 proteins were present in the atheromatous core and to some extent in all the vessel wall layers in advanced plaques while only sparse cells were positive in ITA vessels. In aortas, ADAM9, -15, and -17 positive cells co-distributed mainly with CD68-positive macrophages in the plaque but did

not co-distribute with CD31-positive endothelial cells. In the femoral arteries, however, ADAM9, -15, and -17 proteins co-distributed with endothelial cells in addition to macrophages. In the carotid arteries, ADAM9, -15, and -17 proteins co-distributed with macrophages and HHF35-positive smooth muscle cells (Figure 6). Immunofluorescence double-staining microscopy of a representative carotid plaque confirmed that ADAM9 and ADAM17 positive cells co-localized with macrophages, especially in the plasma membrane. ADAM15 co-localized with macrophages in the mirror sections.

The majority of ADAM9, -15, and -17 protein was found to be in the catalytically active form in the atherosclerotic plaques of carotid and femoral arteries and in aortas (Figure 7).

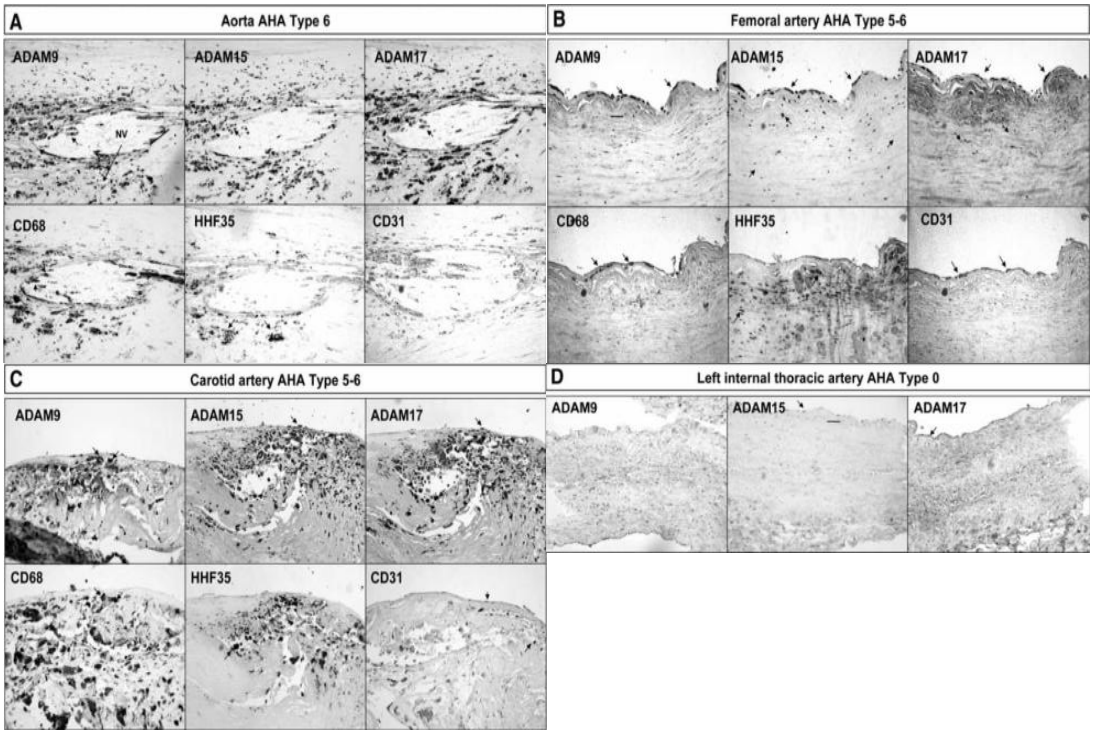

Figure 6. Expression of ADAM9, ADAM15, and ADAM17 in human atherosclerotic plaques. Stainings of monocyte marker CD68, smooth muscle cell marker HHF35 and endothelial cell marker CD31 in human aortic (A), femoral (B), and carotid (C) plaques. Samples from internal thoracic arteries (ITA) were used as controls (D). A straight line indicates the transition between the intima and media. Arrows indicate typical positively stained cells. Arteries were classified according to the American Heart Association (AHA). 100 X magnification.

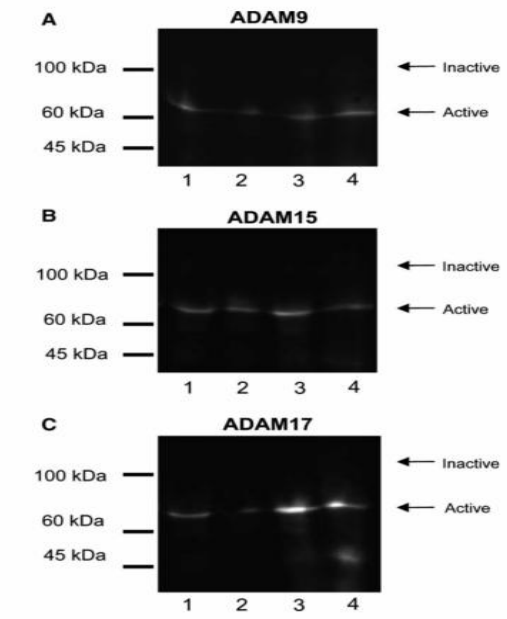

Figure 7. Western detection of A) ADAM9, B), ADAM15, and C) ADAM17 in atherosclerotic carotid (1, 3), femoral (2), and aortic (4) plaques, visualized with enhanced chemiluminescence. The positions of the molecular weight markers and active/inactive forms are indicated.

5. Association of ADAM8 polymorphism (rs2995300) with atherosclerosis (III)

5.1 The ADAM8 rs2995300 polymorphism and the area of different types of atherosclerotic plaques in coronary arteries

According to the coronary measurements of our study, the carriers of the ADAM8 2662G allele had significantly more atherosclerosis (measured as the relative surface area covered by atherosclerotic lesions) in their coronary arteries than the TT homozygotes (p= 0.020 in ANOVA adjusted with age and BMI). When the areas of specific plaque types were compared separately, only the areas of fatty streaks were not significantly associated with this polymorphism (p= 0.229 in ANOVA adjusted with age and BMI).

In line with the results concerning total plaque areas, the G allele carriers had significantly larger relative areas of fibrotic plaques in their coronary arteries (p=0.027 in ANOVA adjusted with age and BMI). A large proportion (23.4%) of

the autopsied men were not found to have calcified plaques in their coronary arteries, and, therefore, we first studied whether ADAM8 polymorphism would affect the occurrence of such plaques. According to logistic regression analysis adjusted with age and BMI, there was no difference in the occurrence of calcified plaques. However, in the group of men who were found to have calcified plaques in their coronary arteries, the relative areas of such plaques were significantly larger among the G allele carriers than among the TT homozygotes (p=0.011 by ANOVA adjusted with age and BMI). The proportion of men without complicated plaques was also very high (53.7%), and the same protocol was therefore followed as with analyzing the occurrence and surface areas of calcified plaques. The G allele carriers a had higher occurrence rate for complicated plaques (age and BMI adjusted OR 1.75 with 95% CI 1.04 to 2.96, p=0.037), and among the men who had complicated plaques, the G allele carriers had significantly larger areas covered by such plaques than the TT homozygotes (p=0.011 by ANOVA adjusted with BMI and age) (Table 2).

5.2 The ADAM8 rs2995300 polymorphism and the occurrence of autopsy-verified myocardial infarctions and fatal prehospital coronary events

In the whole Helsinki Sudden Death study population, the G allele carriers had a higher occurrence rate for autopsy-verified MI (old or acute) compared to T allele carriers, 23.9% (n=101) of the TT homozygotes and 35.0 % (n=41) of the G allele carriers had suffered an MI (adjusted OR 2.03 with 95% CI 1.25 to 3.31, p=0.004). Correspondingly, the G allele carriers had a significantly higher risk of SCD by acute MI (AMI): 12.3% (n=52) of all TT homozygotes and 23.9% (n=28) of all G allele carriers had suffered a fatal prehospital AMI (adjusted OR 2.41 with 95% CI 1.34 to 4.32, p=0.003). When the same analysis was limited to men whose death was caused by autopsy-verified coronary thrombus, the association became more significant: 6.9% (n=29) vs. 17.9% (n=20) (adjusted OR 3.18 with 95% CI 1.59 to 6.34, p= 0.001).

In our material the relative areas of complicated plaques significantly associated with the occurrence of fatal AMI. We found that the ADAM8 genotype group associated with both the surface areas of complicated plaques and the

occurrence of fatal AMI. Therefore, we tested whether the higher susceptibility of the G allele carriers to fatal AMI was due to the larger surface areas of complicated plaques in their coronary arteries. When the surface areas of complicated plaques were included as a covariate into the regression analyses, the effect of the G allele on the occurrence of fatal AMI was markedly weakened markedly and was no longer statistically significant (adjusted OR 1.56 with 95% CI 0.69 to 3.5, p= 0.284).

DISCUSSION

1. Effect of lipoprotein loading of human macrophages (I)

Foam cell and fatty streak formation are the first hallmarks of atherosclerosis (Goldstein et al. 1979; Kruth 2001). To reveal the gene expression changes that prevail in the early phases of atherosclerosis, we studied in the Study I the effects of native and oxidised lipoproteins on human monocyte-macrophages. Even though ox-LDL is thought to be a major factor in foam cell formation, HDL is also susceptible to oxidative modifications (Nishigaki et al. 1981) which could affect foam cell formation (Nagano et al. 1991; Hurtado et al. 1996). The overall gene expression profiles were significantly affected by both ox-LDL and oxHDL treatments. Both oxidized lipoproteins induced similar as well as different, or even contrasting, effects on the gene expression of human monocyte-macrophages. Ox-LDL and ox-HDL induced mainly opposite gene expression but a significant proportion of the genes behaved similarly to ox-LDL and ox-HDL treatments. In Study I, genes that were new with regard atherosclerosis, were discussed in more detail.

Lipid metabolism. Fatty acid-binding proteins are thought to be markers of tissue injury, and actually fatty acid-bonding protein 3 (FABP3) has been found to predict long-term mortality after acute coronary syndrome (Kilcullen et al. 2007) and thought to be a useful biomarker in acute coronary syndromes (Valle et al. 2008). The role of FAPB3 in the development of atherosclerosis is not known, but it is known to modulate intracellular lipid metabolism (Coe and Bernlohr 1998). In Study I, ox-LDL induced over 2-fold increase in FABP3 expression as compared to non-treated cells. We have later done genome-wide gene expression studies of human atherosclerotic plaques where the expression of all the genes in the disease can be evaluated. Interestingly, FABP3 was also ~2-fold up-regulated in atherosclerotic arteries, suggesting that in addition to being involved in the early phases of atherosclerosis, FABP3 is also associated with later stages of the disease.

Another protein involved in lipid metabolism that induced conversely to ox-LDL and ox-HDL treatments was LDL receptor-associated protein 1 (LRPAP1), which is required for normal processing of LDL receptor (Willnow et al. 1995). The biological meaning of LRPAP1 dysregulation in atherosclerosis is not known, but, interestingly, LRPAP1 has already been associated to MI (Gonzalez et al. 2002) and metabolic syndrome (McCarthy et al. 2003). The dysregulation of very-long-chain acyl-CoA dehydrogenase (VLCAD) was also found in Study I. Until now, the role of VLCAD in atherosclerosis is not known. According to our GWEA data, VLCAD is slightly up-regulated in human plaques, further suggesting the involvement of VLCAD in atherosclerosis.

Inflammation, growth and hemostasis. Natriuretic peptide precursor B (NPPB) has been found to inhibit monocyte migration, change their shape and inhibit their adhesion to human umbilical vein endothelial cells (Kim et al. 2004). Interestingly, ox-LDL enhanced the expression of NPPB, which could result in atheroprotective effects, whereas ox-HDL down-regulated NPPB, suggesting an atherosclerosis promoting effect for ox-HDL. In the atherosclerotic plaque however, there were no differences in NPPB levels compared to healthy arteries, suggesting the role for NPPB in the early phases of atherosclerosis.

An ox-LDL inducible up-regulation of filamin A (FLNA) could, in turn, be considered to promote foam cell formation and atheroclerosis since FLNA mediates the attachment of platelets and leukocytes. Ox-HDL seemed to down-regulate FLNA, suggesting an atheroprotective role for HDL that is not influenced by oxidation. In the advanced plaques, FLNA was slightly down-regulated.

Chemokine ligand 1 (CCL1) is another suggested candidate gene for foam cell formation presented in Study I based on its strong down-regulation in response to lipoprotein treatments. Apolipoprotein (Apo) (a) treatment of human vascular endothelial cells has been found to induce CCL1 as the principal monocyte chemoattractant protein (Haque et al. 2000) strengthening the involvement of CCL1 in the early phases of atherosclerosis. However, in the advanced human plaques the expression for CCL1 was not changed. Similarly, the expression of neuropilin 2 (NRP2) was increased in response to ox-LDL and ox-HDL but, according to our GWEA data, the expression of NRP2 is not changed in advanced plaques. The expression of fibrinogen B polypeptide (FGB) was also induced in response to ox-LDL and ox-HDL but in the advanced plaques, no difference was seen.

Thrombosponding 2 (THBS2), which showed a strong up-regulation in response to ox-LDL treatments, was also ~ 2-fold up-regulated in advanced plaques suggesting that THBS2 may be involved in the early atherogenic processes as well as later stages of the disease. THBS2 is suggested to modulate collagen fibrillogenesis and angiogenesis (Bornstein et al. 2000) and its single nucleotide polymorphisms have been found to be associated with coronary heart disease (McCarthy et al. 2004) and plaque erosion (Burke et al. 2009) but still, more research is required to reveal its biological function in atherosclerosis.

2. Whole-genome expression analysis of advanced atherosclerosis (II)

2.1 Significant gene expression alterations in human advanced atherosclerotic arteries

Microarray method has previously been used to reveal the gene expression changes prevailing in the atherosclerotic plaque (Hiltunen et al. 2002; Tyson et al. 2002; Tuomisto et al. 2003; Seo et al. 2004) but in Study II, for the first time, the expression profiling was done out of three major arterial beds affected by atherosclerosis. A considerable number of gene expression changes were found in advanced human atherosclerotic arteries compared to healthy non-atherosclerotic arteries. Many of the genes have already previously been connected to atherosclerosis, whereas several new candidate genes are also presented. In the following, a gathering of some of the interesting new candidate genes, are presented.

Up-regulated genes. Among the 27 generally up-regulated genes was chemokine ligand 18 (CCL18) with ~100 –fold up-regulation. CCL18 has also previously been found to be expressed in human atherosclerotic plaques, predominantly in macrophages and suggested to play a role in T lymphocyte attraction (Reape et al. 1999; Hagg et al. 2008). In addition, the plasma level of CCL18 has been found to increase in patients with unstable angina pectoris (Kraaijeveld et al. 2007) suggesting the suitability of CCL18 for use as a biomarker in cardiovascular diseases. A substantial up-regulation was also observed for the regulator of G-protein signalling 1 (RGS1) which could be an interesting new candidate in the

atherosclerosis pathogenesis as RGS proteins are implicated in blocking the signal transduction of chemokine receptors and thus are likely to have an important suppressive impact on lymphocyte migration and function (Moratz et al. 2004). Thrombospondin 1 (THBS1) was found to be strongly up-regulated in all atherosclerotic plaques studied, average fold change being 10.9. THBS1 has previously been detected in atherosclerotic plaques of mice and has been suggested to enhance endothelial dysfunction, apoptosis and smooth muscle cell proliferation. However, once the lesion has been formed, the expression of THBS1 may in turn be protective as it may reduce inflammation as well as maturation and plaque rupture (Moura et al. 2008; Stenina and Plow 2008). Galectin-3 gene (LGALS3) and capping protein (CAPG) were also among the most up-regulated genes. LGALS3 has previously been found to be expressed in plaques (Nachtigal et al. 1998) and the deficiency of LGALS3 was found to be associated with less severe atherosclerotic lesions and decreased adventitial inflammation in mice (Nachtigal et al. 2008). CAPG in turn, has been suggested to modulate the protective effects of unidirectional shear stress (Pellieux et al. 2003).

Down-regulated genes. Most of the significantly down-regulated genes found in Study II, are new with regard to atherosclerosis. The most markedly down-regulated gene was intelectin 1 (ITLN1), which was almost absent in atherosclerotic plaques compared to non-atherosclerotic internal thoracic arteries. ITLN1 is a cell surface phagocytotic receptor that recognizes specific bacterial cell wall components (Tsuji et al. 2001) and the absence of ITLN1 has been suggested to alter immune responses to infection and facilitate inflammation (Schaffler et al. 2005). Another significantly down-regulated gene was the regulator of G-protein signaling 5 (RGS5). Recently, the blockage of RGS5 has been suggested to provide an alternative approach to treat hypertension but the biological impact of the reduced expression of RGS5 in the plaques is not known. Traditionally, in gene expression profiling studies, research has focused on genes found to be up-regulated in various diseases. The evaluation of down-regulated genes may, however, be equally important in the ascertaining the pathogenic processes underlying different diseases.

2.1.1 Site-specific gene expression changes in the vascular regions studied

The susceptibility to develop atherosclerosis differs noticeably between different sites in human vasculature and the type of atherosclerosis ranges from stable calcified plaques and fibrotic plaques all the way to unstable ulcerated plaques and the prevalence of these lesions varies according to vascular bed region. To better understand the molecular basis of this phenomenon, we determined genes that were specifically induced in only one arterial bed. Eight genes were found to characterize specially the aortic plaques and three genes the femoral plaques. An apparent observation was that the genes characterizing the aortic plaques were mainly involved in immunity, e.g., B cell regulation and antigen presenting, whereas the genes characterizing the femoral plaques were involved in extra-cellular environment modifications, adipose tissue metabolism and angiogenesis. This suggests that in aortic plaques the immune mechanisms are pronounced compared to femoral and carotid plaques. In addition, the three genes observed to be specifically induced in femoral plaques may contribute to the commonly observed stable phenotype of lower limb atherosclerosis.

2.2 Dysregulated pathways in atherosclerosis

Apoptotic activity is a well-known feature of atherosclerotic plaques (Isner et al. 1995; Hegyi et al. 1996) thus it is not surprising that among the significantly up-regulated pathways were several central pro-apoptotic pathways including tumor necrosis factor alpha and beta signaling genes as well as caspases. In addition, a careful examination of the genes belonging to these pathways may reveal new genes involved in the apoptotic activity of plaques. Several pro-inflammatory pathways involved in T cell differentiation, bacterial infection, interleukin signaling and B cell activation were found to be induced in advanced plaques, especially the B cell-related pathways seemed to be significantly induced. The involvement of T cells in atherosclerosis has been widely studied but little is known about the role of B cells in the disease. Two pathways involved in thrombus formation and blood coagulation were found to be up-regulated. These pathways may have emerged in this study since the carotid artery plaques analyzed in this study were mainly from patients

with symptomatic carotid artery disease where plaque rupture and thrombosis are known to be activated. One of the thrombogenic pathways, Par1Pathway, includes protease-activated receptors 1 and 4 that have been found to cause platelet aggregation (Kim et al. 2002) and a G protein-coupled receptor kinase-5 that regulates thrombin-activated signaling in endothelial cells (Tiruppathi et al. 2000). Another pathway involved in blood coagulation was a pathway involving genes in complement activation. Although complement activation has been recognized in atherosclerotic lesions, the functional role of the complement system in the atherogenesis is not known. The complement has been considered to be a part of the pro-inflammatory system, while there is also evidence that that it may protect tissues from the accumulation of debris through opsonization of apoptotic cells (Haskard et al. 2008).

A totally different phenomenon was seen among the most down-regulated pathways compared to the up-regulated pathways. The significantly down-regulated pathways were involved in the basic metabolism of fatty and amino acids, benzoate degradation and hormonal functions. In addition, a pathway related to hematopoiesis was significantly down-regulated in the atherosclerotic plaques. This suggests that the basic metabolic functions are significantly lower in advanced atherosclerotic arteries compared to healthy non-atherosclerotic vessels. The biological impact of this phenomenon remains to be elucidated in the future.

2.2.1 T cell differentiation pathway in atherosclerotic plaque

T cells have crucial effects in the regulation of the inflammatory response in the atherosclerotic plaques, which makes them interesting targets in the prevention and treatment of atherosclerosis (Hansson et al. 2006). Among the most up-regulated pathways, we chose a pathway involved in T cell differentiation for further inspection. Several T cell markers, chemokines and chemokine receptors were found to be highly up-regulated in the plaques. Instead of focusing on well-known genes involved in T cell activation, which are widely discussed with regard atherosclerosis, we focused on genes whose role in T cell mediated functions in the disease is not known.

Chemokine receptor 7 (CCR7), which was highly up-regulated in the atherosclerotic plaques contributes to macrophage and dendritic cell emigration and homing (Llodra et al. 2004) and is involved in immunity and peripheral immunotolerance (Forster et al. 2008). CCR7 has been demonstrated to have a major functional role in the regression of atherosclerotic plaques (Trogan et al. 2006) making it an interesting candidate for pharmacological studies. Another two chemokine receptors, CCR4 and CCR3, showed a different expression profile between the arteries studied compared to other chemokine receptors included in the pathway. CCR4 was significantly up-regulated only in the aortas (fold change 5.5, p= 0.008) whereas the up-regulation in the carotid and femoral arteries was not significant. This suggests a vascular bed specific function for CCR4. CCR4 is expressed in SMCs as well as in Th2 cells. Pronounced up-regulation may be due to a greater number of SMCs in the aorta than in the carotid and femoral arteries but the consequence of up-regulation of CCR4 specifically in the aortas remains to be ascertained in the future. CCR3, a marker for Th2 cells, showed significant down-regulation (fold change – 4.5, p = 0.012) only in the carotid arteries, suggesting that the Th2 response in carotid artery disease is significantly lower than in femoral arteries and in aortas.

Interferon gamma has been suggested to accelerate atherosclerosis e.g. by activating macrophages and increasing their production of nitric oxide, pro-inflammatory cytokines and, pro-thrombotic and vasoactive mediators (Hansson and Libby 2006). In our study, the expression of interferon gamma receptors 1 and 2 (IFNGR1, IFNGR2) was significantly up-regulated in all atherosclerotic arteries studied. To the best of our knowledge, this is the first study to report the up-regulation of IFNGR1 and IFNGR2 in human atherosclerotic plaques. It remains to be seen whether INFG-mediated actions in atherosclerotic plaques could be modulated through manipulation of its receptors.

Transforming growth factors beta 1-3 (TGFB1-3) also showed a different expression pattern in the atherosclerotic arteries studied. TGFB1, which has previously been shown to be atheroprotective (Amento et al. 1991) was ~ 2.0-fold up-regulated in the plaques, whereas TGFB2 was ~ 1.5 fold down-regulated in all arteries studied. Interestingly, the expression of TGFB3 was up-regulated only in the aortas and femoral arteries but significantly down-regulated in the carotid arteries

suggesting different roles for TGFB molecules in arterial atherosclerosis that may, in addition, be dependent upon vascular bed.

3. ADAMs in atherosclerosis (III, IV)

3.1 ADAM8

Apart from the present thesis (Study III), no information was found in the literature about the role of ADAM8 in atherosclerosis. The study results in the third article clearly represent the up-regulation of ADAM8 in atherosclerotic plaques and reveal an association between its single nucleotide polymorphism and complicated plaque area as well as fatal myocardial infarction, clearly showing that ADAM8 is somehow involved in plaque progression. An obvious way for ADAM8 to affect the plaque progression arises from its capability to cleave adhesion molecules on the cell surfaces.

ADAM8 in leukocyte recruitment and inflammation. The recruitment of leukocytes into the inflammatory sites is a crucial event in the inflammatory response that plays a fundamental role in atherosclerosis. Since ADAM8 is capable of cleaving adhesion molecules L-selectin and VCAM1, it is possible that ADAM8 could be involved in the regulation of inflammatory cell recruitment to the atherosclerotic plaque.

Neutrophils are the first immune cells to be recruited to the inflammatory foci, where they phagocytose foreign bodies and release several antimicrobial polypeptides (Borregaard and Cowland 1997) and generate oxygen-free radicals. In the early stages of adhesion, neutrophils utilize the L-selectin adhesion molecule which aids the neutrophil rolling along the endothelium. The rolling speed of neutrophils was reduced and their accumulation into tissues increased when the shedding of L-selectin was inhibited (Walcheck et al. 1996). Upon neutrophil activation and degranulation, ADAM8 was translocated from the granule membrane to the cell membrane and a soluble form of ADAM8 was released. Both the cell membrane and the soluble form of ADAM8 were found to enhance L-selectin shedding (Gomez-Gaviro et al. 2007). ADAM8 can thus reasonably be considered

as a physiological anti-inflammatory agent regulating the leukocyte invasion through its L-selectin sheddase activity.

Endothelial cells are activated during the early atherogenic process and they express leukocyte adhesion molecules contributing to the progression of atherosclerosis (Cybulsky and Gimbrone 1991). While the first step in the adhesion, the "rolling" of leukocytes along the endothelial surface, is mediated by selectins, vascular cell adhesion molecule (VCAM1) interacts with leukocyte integrins (Springer 1994) to establish a firm adhesion to the endothelium. Matsuno et al. found that ADAM8 is able to shed VCAM1 and suggested that ADAM8 may have a suppressive role in leukocyte trafficking through VCAM1 clearance from endothelial cells (Matsuno et al. 2006).

ADAM8 is also capable of shedding CD23, which has been implicated in several functions ranging from cellular adhesion, antigen presentation, growth and differentiation of B and T cells, rescue from apoptosis, release of cytotoxic mediators and regulation of IgE synthesis (Bonnefoy et al. 1997). The consequences of CD23 shedding in atherosclerotic plaque need to be studied in the future.

It is also interesting to note that peroxisome proliferator-activated receptor gamma (PPARG) activation of macrophages induced the up-regulation of ADAM8 (Hodgkinson and Ye 2003). PPARG is an important regulator of macrophage gene expression inhibiting the production of inflammatory cytokines by macrophages (Jiang et al. 1998) and promoting cholesterol efflux from macrophages (Chinetti et al. 2001). PPARG has also been shown to block the proliferation and to increase apoptosis of SMCs (Bruemmer et al. 2003a; Bruemmer et al. 2003b). It remains to be seen in the future whether ADAM8 is involved in the PPARG mediated anti-inflammatory mechanisms.

ADAM8 in the progression of atherosclerosis. The probability of developing thrombogenic events rises as atherosclerosis proceeds (Libby 2001). It is interesting to note that ADAM8 is capable of interacting with alpha9beta1 integrin (Rao et al. 2006) that is also a ligand for coagulation factor XIII and von Willebrand factor (Takahashi et al. 2000). Since there is evidence suggesting that soluble ADAM8 (sADAM8) may be physiologically active (Schlomann et al. 2002), it is intriguing to speculate whether sADAM8 could prevent the thrombotic events in atherosclerosis by blocking the binding site for these essential factors in blood coagulation and thus prevent the thrombotic complications of atherosclerosis.

Another typical feature, besides thrombosis, of advanced atherosclerosis, is calcification. Although calcification has previously been thought to be a passive precipitation phenomena, there is accumulating evidence that arterial calcification is an organized, regulated process (Abedin et al. 2004). Interestingly, ADAM8 is an important factor in osteoclast formation (OCL) (Choi et al. 2001), which could be an important finding considerating that the monocytotic line of hematopoietic cells have been reported to undergo osteoclastic differentiation and OCL-like cells have been detected in atherosclerotic plaques (Jeziorska et al. 1998). It remains to be seen in the future whether ADAM8 is involved in the calcification process of atherosclerosis.

3.1.1 Association of ADAM8 rs2995300 polymorphism with advanced atherosclerotic lesion area and risk of MI and fatal acute MI

The G allele carriers of ADAM8 2662 T/G allelic variant had significantly more atherosclerosis as well as increased areas of fibrotic, calcified, and complicated plaques in their coronary arteries, than did the TT homozygotes. The observed SNP appears to be related to the extent of the atherosclerotic plaque area suggesting that ADAM8 is somehow involved in the plaque formation.

Patients with the G variant of 2662 T/G (TG and GG genotypes) appeared to have higher rates of MI and fatal acute MI, possibly explained by the association with the increased areas of complicated lesions in the coronary arteries of the G allele carriers. However, when the MI prevalence was adjusted with the areas of complicated lesions, the correlation disappeared. This suggests that the G allele might have an effect on the emergence of MI at the level of complicated lesion areas rather than directly affecting the thrombus formation. Even though the 2662 T/G is located in the 3'-UTR region of the gene, it has been suggested that the 3'-UTR can affect gene expression by influencing mRNA stability and translation (Misquitta et al. 2001). Since the 2662 T/G belongs to bin (SNPs in high linkage disequilibrium) with four other SNPs it is also possible that the 2662 T/G is a neutral marker of some other SNP belonging to same subset.

3.2 ADAM9

ADAM9 in atherosclerotic plaques. In addition to Study II in this thesis, ADAM9 has previously been reported to be up-regulated in human atherosclerotic carotid artery specimens. ADAM9 was found to be ninefold up-regulated in the carotid plaques compared to non-atherosclerotic thyroid arteries and located in smooth muscle cells in the neointima and foam cells of SMC origin surrounding lipid cores (Al-Fakhri et al. 2003). We observed a notably lower up-regulation of ADAM9 in atherosclerotic arteries, which may be due to the fact that our control samples were obtained form subjects with coronary atherosclerosis and the systemic nature of atherosclerosis may affect several arterial beds, even though the control arteries in this study were histologically healthy. We also found that, in addition to being expressed in SMCs, ADAM9 was also found in macrophages in carotid and femoral arteries as well as in aortas and in addition, in endothelial cells in femoral arteries.

ADAM9 in cell adhesion. A complex process of atherosclerosis is initiated by the recruitment of inflammatory cells into the vascular wall. In the process, cell adhesion allowing the firm contact of blood cells with the endothelium, and proteolytic degradation of the extracellular matrix allowing cell migration into the subendothelium, are crucial. The binding of leukocytes to vascular endothelium is mainly mediated by integrins and selectins (Hillis 2003). In the study by Al-Fakhri *et al*., integrins α5β1 and αvβ3, involved in the migration of smooth muscle cells to sites of vascular injury, were parallel up-regulated along with ADAM9 and ADAM15. This process can be regulated, among others, by integrin binding through different ligands. ADAMs have been suggested to function as integrin regulators, e.g., allowing cells to specifically open or block integrin binding to different ligands and regulating cell-cell, cell-matrix interactions, migration and proliferation (Al-Fakhri et al. 2003; Karadag et al. 2006).

ADAM9 could also affect the leukocyte recruitment by shedding laminin, a molecule that monocytes utilize in the attachement to the endothelium (Mazzocca et al. 2005; Konstantinidis et al. 2009). Vascular endothelial growth factor (VEGF), a cytokine crucial for atherosclerosis (Kimura et al. 2007), has been found to induce laminin expression in umbilical arteries (Infanger et al. 2008). Despite being recognized as a substrate of ADAM9, it remains to be seen in the future whether

ADAM9 is a functional sheddase of these substrates in atherosclerosis and thus contributes to plaque progression.

As thrombogenic features are typical in advanced atherosclerosis and predispose to fatal complications of atherosclerosis, it is interesting to note that ADAM9 is able to inhibit the adhesion of activated platelets to collagen type I (Cominetti et al. 2009). Collagen type I is a major component of atherosclerotic lesions (McCullagh et al. 1980) and serves as a ligand for macrophages which, through collagen type I binding, may influence the progression of the disease. Further study is needed to elucidate the possible role of ADAM9 and collagen type I interaction of macrophages in atherosclerosis.

Atherosclerosis-related ADAM9 substrates. ADAM9 has several substrates that could be relevant for atherosclerosis (Edwards et al. 2008; Duffy et al. 2009). ADAM9 has been proposed to activate epidermal growth factor (EGF) and heparin-binding epidermal growth factor (HB-EGF), both associated with atherosclerosis, through ectodomain shedding. Soluble EGF concentrations have been found to be positively associated with protective lipid concentrations, e.g., apolipoprotein A1 and high-density lipoprotein, (Berrahmoune et al. 2009) whereas soluble HB-EGF has been found to stimulate proliferation and migration of SMCs and fibroblasts (Dreux et al. 2006). The HB-EGF signaling pathway was also associated with increased leukocyte density, wall hypertrophy and lumen narrowing (Zhang et al. 2008). However, even though HB-EGF and EGF are potential substrates for ADAM9, the major sheddase for HB-EGF is thought to be ADAM17 (Lee et al. 2003a).

3.3 ADAM15

ADAM15 in atherosclerotic plaque. ADAM15 was originally cloned from human umbilical vein endothelial cells and found to be up-regulated in atherosclerosis of nonhuman primates (Herren et al. 1997). Later ADAM15 up-regulation was found in human atherosclerosis parallel with integrins $\alpha5\beta1$ and $\alpha v\beta3$ (Al-Fakhri et al. 2003) that contribute to SMC migration, proliferation and neointima growth (Kim and Yamada 1997; Moiseeva 2001), all crucial factors in the progression of atherosclerosis. ADAM15 was prominently expressed in foam cells of SMC origin

and SMCs surrounding lipid cores in the neointima (Al-Fakhri et al. 2003). We also localized ADAM15 to macrophages and SMCs and in addition, observed ADAM15 protein in endothelial cells in femoral arteries. Whether the rheological properties of different vascular beds affect the expression profile of ADAMs in specific cell types remains to be elucidated in the future.

ADAM15 and integrins. Besides the observation that ADAM15 was parallel up-regulated with integrins (Al-Fakhri et al. 2003), there are several studies suggesting the role for ADAM15 in integrin regulation (Blobel 1997; Herren et al. 1997; Al-Fakhri et al. 2003; Charrier et al. 2005; Mosnier et al. 2006) which makes ADAM15 an intriguing molecule in the field of atherosclerosis.

A complex process of atherosclerosis is initiated by the recruitment of inflammatory cells into the vascular wall. In the process, cell adhesion allowing the firm contact of blood cells with the endothelium, and proteolytic degradation of the extracellular matrix allowing cell migration into the subendothelium, are crucial (Krieglstein and Granger 2001; Lu et al. 2008). ADAM family members have both of these properties making them interesting targets in the search for putative molecules for future therapies in atherosclerosis. Considering the major role of ADAM15 in integrin functions, and the observed up-regulation of ADAM15 in atherosclerosis, ADAM15 makes an interesting candidate for pharmacological studies in atherosclerosis being involved in inflammatory cell and platelet recruitment, SMC migration and proliferation. In addition, ADAM15 may be involved in thrombogenic events, since platelets were found to adhere to ADAM15 via GPIIb-IIIa integrin (αIIb/β3) and recruit further platelets to form a microthrombus. ADAM15 was also found to induce platelet activation and secretion of CD40 Ligand (Langer et al. 2005), which has been found to contribute to atherosclerosis (Mach et al. 1998; Langer et al. 2005).

Atherosclerosis-related ADAM15 substrates. Since ADAM15 already has several potential substrates (Edwards et al. 2008; Duffy et al. 2009; Lucas et al. 2009) and the number is constantly growing, it is likely that ADAM15 could affect atherosclerosis by several mechanisms. Cadherins are another family of cell surface adhesion molecules suggested to be involved in cardiovascular diseases. For example, the shedding of E-cahderin by ADAM15 induces the ErbB transactivation (Najy et al. 2008b) that has been suggested to play a critical role in cardiovascular diseases (Eguchi et al. 2003).

In addition to ADAM9 and -17, ADAM15 is able to cleave HB-EGF, the signaling of which is associated with increased leukocyte density, vessel wall hypertrophy and lumen narrowing (Zhang et al. 2008). ADAM15 expression has also been found to be elevated after infarction in myocardium and has been suggested to be involved in inflammatory response and cardiac remodeling (Li et al. 2009).

3.4 ADAM17

ADAM17 in atherosclerotic plaque. Previously ADAM17 has been detected in human atherosclerosis by Canault et al. (Canault et al. 2006), who showed that human plaques contain catalytically active ADAM17 and suggested the impact of ADAM17 in the inflammatory responses in the lesion (Canault et al. 2007). In Study III, a slight up-regulation of ADAM17 was observed in the carotid artery plaques and in the aortas where it was localized to macrophages and SMCs. ADAM17 has already been found to be involved in the survival, proliferation and differentiation of mononuclear phagocytes based on its ability to shed the macrophage colony-stimulating factor receptor (M-CSFR) (Clinton et al. 1992; Rovida et al. 2001).

Atherosclerosis-related ADAM17 substrates. Many of the several ADAM17 substrates (Arribas and Esselens 2009; Duffy et al. 2009) have already been suggested to be involved in atherosclerosis as it is able to cleave several molecules crucial to atherosclerosis e.g, vascular cell adhesion molecule (VCAM), intercellular adhesion molecule (ICAM), L-selectin, TNF and its receptors and HB-EGF. As discussed regarding ADAM8 and its L-selectin shedding, it is evident that ADAM17 mediated adhesion molecule shedding may also have a crucial effect in the regulation of inflammatory cell recruitment into the atherosclerotic plaque. ADAM17 is also able to cleave endothelial chemokines that may result in diminished inflammatory cell attachment (Clinton et al. 1992).

An obvious effect of ADAM17 to atherosclerotic plaque biology arises from its capability to shed TNFα and its receptor tumor necrosis factor receptor 1 (TNFR1) (Canault et al. 2006; Canault et al. 2007). TNFα induces the gene expression of various inflammatory cytokines and chemokines that accelerate atherogenesis, thrombosis, vascular remodeling, vascular inflammation, endothelium apoptosis,

vascular oxidative stress and dimished NO bioavailability contributing to the blunted vascular function (Zhang et al. 2009). TNFα level has also been found to be elevated after acute myocardial infarction (Maury and Teppo 1989). Whether ADAM17 mediated TFNα and its receptor shedding is a promoting or a protective phenomenon in atherosclerosis remains to be ascertained in the future. Interestingly, though, the increased expression and activity of ADAM17 has been found to decrease the lesion formation in mice (Holdt et al. 2008) supporting the protective role of ADAM17.

ADAM17 shedding is also known to affect lipoprotein functions since ADAM17 is able to shed low-density lipoprotein receptor-related protein 1 (LRP1) (Liu et al. 2009) and its shedding activity is activated by HDL (Tellier et al. 2008). LRP1 is an endocytic receptor that is thought to regulate the cellular microenvironment in injury and inflammation that is found to act protectively in atherosclerosis (Boucher et al. 2003) and of which soluble form has been suggested to have anti-inflammatory properties (Raghavendra et al. 2002; Gaultier et al. 2008) suggesting another anti-atherogenic role for ADAM17.

4. Limitations of the study

HSDS study material. In the autopsy series, the survival bias is avoided, a phenomenon typical in conventional cross-sectional or retrospective studies. The HSDS subjects may, however, present more severe atherosclerosis that randomly selected subjects and the cardiovascular risk factors may be differentially distributed, e.g., due to the high alcohol consumption in the HSDS subjects. The risk factor data for HSDS subjects is limited as there are no blood samples from this study population. The data obtained by interviewing spouses, relatives, or close friends of the deceased, is however, considered reliable enough to be used in statistical analyses. All HSDS subjects are men thus the results obtained with this study polulation cannot necessarily be generalized to women.

TVS study material. The atherosclerotic vascular samples were collected from the carotid, aorta and femoral arteries, where the local hemodynamic conditions are different from each other. The majority of the samples were though from histologically advanced plaques (types V-VI), however, which allows us to suggest

that they reflect the systemic nature of atherosclerosis. We compared the expression of mRNA and protein levels between atherosclerotic plaques and non atherosclerotic internal thoracic arteries. Even though comparison of diseased and healthy arteries from the same source would be more accurate, unfortunately we were not able to obtain any corresponding healthy arteries from the carotid, aortic and femoral regions due to ethical issues. The fact that each arterial sample was obtained from a different individual with different medication may also have influenced the results. Nevertheless, we believe that comparison of atherosclerotic plaques to ITA vessels provides sufficient information to reveal the biological processes going on in the diseased vessels.

Gene expression studies. In this thesis, the level of mRNA was evaluated with cDNA microarray, oligonucleotide microarray and QRT-PCR. cDNA and oligonucleotide microarrays are fundamentally two different methods for mRNA quantification. In cDNA microarrays, PCR products are spotted on a nitrocellulose membrane (Duggan et al. 1999) whereas oligonucleotide microarrays are fabricated by in situ synthesis and immobilization on a glass matrix (Lipshutz et al. 1999). Despite the advantages and disadvantages of these two methods, both microarrays are thought to be suitable for the identification of specific gene alterations in diseases (Duggan et al. 1999; Lipshutz et al. 1999; Maughan et al. 2001; Lee et al. 2003b). The main concern with microarray studies arises from data mining, which can greatly influence the results (Forster et al. 2003). The QRT-PCR reactions in the thesis were done with TaqMan Gene expression assays based on 5´nuclease chemistry that are highly specific to measure even minor changes in gene expression. The crucial matter in quantitating QRT-PCR runs is normalizing the results with regard to a stable expressed housekeeping gene. Disruption of the expression of the housekeeping gene between samples studied will result in erroneous results (Bustin 2000). In the QRT-PCR experiments done in this thesis, the stable expression of a housekeeping gene was assured and standard recommendations for the validation of a QRT-PCR assay was performed, thus the results obtained by the $\Delta\Delta C_T$ method are considered reliable.

The most critical issue in RNA studies arises from the radip degradation of RNA molecules. The tissues in TVS material were stored immediately after the surgical procedures in an RNA preserving solution, RNALater that efficiently inhibits RNA degradation. The integrity of RNA was also verified spectrofotometrically as well as

electrophoretically. Another issue about gene expression analyses is that the correlation between mRNA and protein expression is not linear, which poses many challenges in genomic analyses. This discrepancy arises from various post-transcriptional and post-translational modifications, functional interactions of proteins, and gene regulation, which could function only at the mRNA level, at the protein level, or at both levels. Analysing gene expression using whole-mount atherosclerotic plaques mainly reflect the genes induced in inflammatory cells recruited into plaques rather than a different gene expression profile of basic arterial cells in different arteries. The data obtained in gene expression profiling studies is purely descriptive which makes it challenging to make any conclusions about the biological function of dysregulated genes in various diseases.

Even though theoretical pathways can be constructed out of microarray or QRT-PCR data (Subramanian et al. 2005), the results will not reveal the function of the genes nor the biological consequences of the gene dysregulation.

Immunohistochemistry. Immunohistochemistry was applied to study the localization of ADAM proteins in this study. Immunohistochemistry is considered reliable and powerful research tool when appropriately used, whereas inappropriate use may easily produce confusing and misleading results (Mighell et al. 1998). In this study, the immunohistochemical procedures were thoroughly validated. Tissues were originally stored in RNALater that should not interfere with immunohistocemistry. However, it cannot be ruled out that RNALater may have compromised some factors involved in immunohistochemical stainings. To ascertain the immunohistochemical stainings, the results were verified with double-labeled fluorescence and mirror image methods from fresh-frozen tissues.

5. Future prospects

The gene expression profiling done in this thesis on human monocyte-macrophages and human advanced plaques revealed several novel candidate genes, e.g., ADAMs that could be involved in atherosclerotic processes. In the future, the role of ADAMs` dysregulation in atherosclerosis will be studied in more detail. Cell culture experiments will be employed to study the effect of ADAM gene silencing to an overall gene expression profile of the cells and the functional ADAM substrates.

Since the regulation of ADAM acitivity is not fully understood, we will characterize the transcription factors specific for ADAM genes. In addition, the usability of soluble ADAMs as biomarkers in human atherosclerosis will be evaluated. In the future, ADAM apoE knockout mice or mice overexpressing a particular ADAM would provide functional information valuable in evaluating the role of ADAMs in atherosclerosis.

SUMMARY AND CONCLUSIONS

The present study was conducted to identify the gene expression changes specific to the foam cell formation of macrophages by treating human cultured human monocyte-macrophages with ox-LDL and ox-HDL particles as well as to later stages of the disease by screening the gene expression changes involved in human advanced atherosclerotic plaques. Several new candidate genes were found to be involved in these processes of which ADAM8, -9, -15, and -17 were studied in more detail according to their multiple functions that may be crucial in the pathogenesis of atherosclerosis.

The major findings of the study were:

1. cDNA microarray method was found to be applicable in analyzing the gene expression changes induced by ox-LDL and ox-HDL particles in human monocyte-macrophages. The overall gene expression profiles were significantly affected by both oxidized lipoproteins. Mainly, the effects of oxidized lipoproteins were found to be opposite, but a substantial number of genes also behaved similarly to lipoprotein treatments. In the study, several new candidate genes involved in the foam cell formation of macrophages were found.

2. Significant gene expression changes were observed in human advanced atherosclerotic plaques compared to non-atherosclerotic arteries and several new candidate genes for atherosclerosis pathogenesis were presented. Site-specific gene expression changes were observed for aortic and femoral plaques. In addition, pathways dysregulated in plaques were characterized with special emphasis on a T cell chemokine pathway.

3. For the first time, the present study implicates ADAM8 in the pathogenesis of human atherosclerosis. ADAM8 mRNA and protein levels are up-regulated in advanced atherosclerotic lesions wherein ADAM8 was co-distributed to monocyte-

macrophages and SMCs. Furthermore, ADAM8 rs2995300 polymorphism was associated with the areas of complicated plaques and risk of MI and fatal acute MI.

4. ADAM9, -15, and -17 mRNA and protein levels were found to be up-regulated in human advanced plaques in their catalytically active forms. In the plaques, ADAMs were located mainly in macrophages but they were also seen in SMCs and endothelial cells depending upon arterial bed, thereby suggesting roles in inflammatory cell recruitment and profileration.

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REFERENCES

Abedin M, Tintut Y, Demer LL (2004) Vascular calcification: mechanisms and clinical ramifications. Arterioscler Thromb Vasc Biol 24:1161-1170.

Ait-Oufella H, Salomon BL, Potteaux S, Robertson AK, Gourdy P, Zoll J, Merval R, Esposito B, Cohen JL, Fisson S, Flavell RA, Hansson GK, Klatzmann D, Tedgui A, Mallat Z (2006) Natural regulatory T cells control the development of atherosclerosis in mice. Nat Med 12:178-180.

Al-Fakhri N, Wilhelm J, Hahn M, Heidt M, Hehrlein FW, Endisch AM, Hupp T, Cherian SM, Bobryshev YV, Lord RS, Katz N (2003) Increased expression of disintegrin-metalloproteinases ADAM-15 and ADAM-9 following upregulation of integrins alpha5beta1 and alphavbeta3 in atherosclerosis. J Cell Biochem 89:808-823.

Ali N, Knauper V (2007) Phorbol ester-induced shedding of the prostate cancer marker transmembrane protein with epidermal growth factor and two follistatin motifs 2 is mediated by the disintegrin and metalloproteinase-17. J Biol Chem 282:37378-37388.

Amento EP, Ehsani N, Palmer H, Libby P (1991) Cytokines and growth factors positively and negatively regulate interstitial collagen gene expression in human vascular smooth muscle cells. Arterioscler Thromb 11:1223-1230.

Amour A, Knight CG, English WR, Webster A, Slocombe PM, Knauper V, Docherty AJ, Becherer JD, Blobel CP, Murphy G (2002) The enzymatic activity of ADAM8 and ADAM9 is not regulated by TIMPs. FEBS Lett 524:154-158.

Arribas J, Bech-Serra JJ, Santiago-Josefat B (2006) ADAMs, cell migration and cancer. Cancer Metastasis Rev 25:57-68.

Arribas J, Esselens C (2009) ADAM17 as a therapeutic target in multiple diseases. Curr Pharm Des 15:2319-2335.

Aviram M (1996) Interaction of oxidized low density lipoprotein with macrophages in atherosclerosis, and the antiatherogenicity of antioxidants. Eur J Clin Chem Clin Biochem 34:599-608.

Bachetti T, Morbidelli L (2000) Endothelial cells in culture: a model for studying vascular functions. Pharmacol Res 42:9-19.

Baker AH, Edwards DR, Murphy G (2002) Metalloproteinase inhibitors: biological actions and therapeutic opportunities. J Cell Sci 115:3719-3727.

Benditt EP, Benditt JM (1973) Evidence for a monoclonal origin of human atherosclerotic plaques. Proc Natl Acad Sci U S A 70:1753-1756.

Bernstein HG, Bukowska A, Krell D, Bogerts B, Ansorge S, Lendeckel U (2003) Comparative localization of ADAMs 10 and 15 in human cerebral cortex normal aging, Alzheimer disease and Down syndrome. J Neurocytol 32:153-160.

Berrahmoune H, Lamont JV, Herbeth B, Lambert D, Masson C, McPhillips M, Fitzgerald PS, Visvikis-Siest S (2009) Association between EGF and lipid concentrations: a benefit role in the atherosclerotic process? Clin Chim Acta 402:196-198.

Black RA, Doedens JR, Mahimkar R, Johnson R, Guo L, Wallace A, Virca D, Eisenman J, Slack J, Castner B, Sunnarborg SW, Lee DC, Cowling R, Jin G, Charrier K, Peschon JJ, Paxton R (2003) Substrate specificity and inducibility of TACE (tumour necrosis factor alpha-converting enzyme) revisited: the Ala-Val preference, and induced intrinsic activity. Biochem Soc Symp:39-52.

Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S, Nelson N, Boiani N, Schooley KA, Gerhart M, Davis R, Fitzner JN, Johnson RS, Paxton RJ, March CJ, Cerretti DP (1997) A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature 385:729-733.

Blanchot-Jossic F, Jarry A, Masson D, Bach-Ngohou K, Paineau J, Denis MG, Laboisse CL, Mosnier JF (2005) Up-regulated expression of ADAM17 in human colon carcinoma: co-expression with EGFR in neoplastic and endothelial cells. J Pathol 207:156-163.

Blobel CP (1997) Metalloprotease-disintegrins: links to cell adhesion and cleavage of TNF alpha and Notch. Cell 90:589-592.

Blobel CP (2005) ADAMs: key components in EGFR signalling and development. Nat Rev Mol Cell Biol 6:32-43.

Bobryshev YV (2005) Dendritic cells in atherosclerosis: current status of the problem and clinical relevance. Eur Heart J 26:1700-1704.

Bobryshev YV, Lord RS (1995) S-100 positive cells in human arterial intima and in atherosclerotic lesions. Cardiovasc Res 29:689-696.

Bode W, Gomis-Ruth FX, Stockler W (1993) Astacins, serralysins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the 'metzincins'. FEBS Lett 331:134-140.

Bohm BB, Aigner T, Blobel CP, Kalden JR, Burkhardt H (2001) Highly enhanced expression of the disintegrin metalloproteinase MDC15 (metargidin) in rheumatoid synovial tissue. Arthritis Rheum 44:2046-2054.

Bohm BB, Aigner T, Gehrsitz A, Blobel CP, Kalden JR, Burkhardt H (1999) Up-regulation of MDC15 (metargidin) messenger RNA in human osteoarthritic cartilage. Arthritis Rheum 42:1946-1950.

Bohm BB, Aigner T, Roy B, Brodie TA, Blobel CP, Burkhardt H (2005) Homeostatic effects of the metalloproteinase disintegrin ADAM15 in degenerative cartilage remodeling. Arthritis Rheum 52:1100-1109.

Bonnefoy JY, Lecoanet-Henchoz S, Gauchat JF, Graber P, Aubry JP, Jeannin P, Plater-Zyberk C (1997) Structure and functions of CD23. Int Rev Immunol 16:113-128.

Boren J, Olin K, Lee I, Chait A, Wight TN, Innerarity TL (1998) Identification of the principal proteoglycan-binding site in LDL. A single-point mutation in apo-B100 severely affects proteoglycan interaction without affecting LDL receptor binding. J Clin Invest 101:2658-2664.

Bornstein P, Kyriakides TR, Yang Z, Armstrong LC, Birk DE (2000) Thrombospondin 2 modulates collagen fibrillogenesis and angiogenesis. J Investig Dermatol Symp Proc 5:61-66.

Borregaard N, Cowland JB (1997) Granules of the human neutrophilic polymorphonuclear leukocyte. Blood 89:3503-3521.

Boucher P, Gotthardt M, Li WP, Anderson RG, Herz J (2003) LRP: role in vascular wall integrity and protection from atherosclerosis. Science 300:329-332.

Boyle JJ (2005) Macrophage activation in atherosclerosis: pathogenesis and pharmacology of plaque rupture. Curr Vasc Pharmacol 3:63-68.

Brown BG, Zhao XQ, Sacco DE, Albers JJ (1993) Lipid lowering and plaque regression. New insights into prevention of plaque disruption and clinical events in coronary disease. Circulation 87:1781-1791.

Brown MS, Goldstein JL (1999) A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. Proc Natl Acad Sci U S A 96:11041-11048.

Bruemmer D, Yin F, Liu J, Berger JP, Kiyono T, Chen J, Fleck E, Van Herle AJ, Forman BM, Law RE (2003a) Peroxisome proliferator-activated receptor gamma inhibits expression of minichromosome maintenance proteins in vascular smooth muscle cells. Mol Endocrinol 17:1005-1018.

Bruemmer D, Yin F, Liu J, Berger JP, Sakai T, Blaschke F, Fleck E, Van Herle AJ, Forman BM, Law RE (2003b) Regulation of the growth arrest and DNA damage-inducible gene 45 (GADD45) by peroxisome proliferator-activated receptor gamma in vascular smooth muscle cells. Circ Res 93:e38-47.

Burke A, Creighton W, Tavora F, Li L, Fowler D (2009) Decreased frequency of the 3'UTR T>G single nucleotide polymorphism of thrombospondin-2 gene in sudden death due to plaque erosion. Cardiovasc Pathol

Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 25:169-193.

Caligiuri G, Paulsson G, Nicoletti A, Maseri A, Hansson GK (2000) Evidence for antigen-driven T-cell response in unstable angina. Circulation 102:1114-1119.

Canault M, Leroyer AS, Peiretti F, Leseche G, Tedgui A, Bonardo B, Alessi MC, Boulanger CM, Nalbone G (2007) Microparticles of human atherosclerotic plaques enhance the shedding of the tumor necrosis factor-alpha converting enzyme/ADAM17 substrates, tumor necrosis factor and tumor necrosis factor receptor-1. Am J Pathol 171:1713-1723.

Canault M, Peiretti F, Kopp F, Bonardo B, Bonzi MF, Coudeyre JC, Alessi MC, Juhan-Vague I, Nalbone G (2006) The TNF alpha converting enzyme (TACE/ADAM17) is expressed in the atherosclerotic lesions of apolipoprotein E-deficient mice: possible contribution to elevated plasma levels of soluble TNF alpha receptors. Atherosclerosis 187:82-91.

Carl-McGrath S, Lendeckel U, Ebert M, Roessner A, Rocken C (2005) The disintegrin-metalloproteinases ADAM9, ADAM12, and ADAM15 are upregulated in gastric cancer. Int J Oncol 26:17-24.

Charrier L, Yan Y, Driss A, Laboisse CL, Sitaraman SV, Merlin D (2005) ADAM-15 inhibits wound healing in human intestinal epithelial cell monolayers. Am J Physiol Gastrointest Liver Physiol 288:G346-353.

Chen MS, Almeida EA, Huovila AP, Takahashi Y, Shaw LM, Mercurio AM, White JM (1999) Evidence that distinct states of the integrin alpha6beta1 interact with laminin and an ADAM. J Cell Biol 144:549-561.

Chinetti G, Lestavel S, Bocher V, Remaley AT, Neve B, Torra IP, Teissier E, Minnich A, Jaye M, Duverger N, Brewer HB, Fruchart JC, Clavey V, Staels B (2001) PPAR-alpha and PPAR-gamma activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. Nat Med 7:53-58.

Choi SJ, Han JH, Roodman GD (2001) ADAM8: a novel osteoclast stimulating factor. J Bone Miner Res 16:814-822.
Cifkova R, Mancia G, Kjeldsen SE, Laurent S (2008) European guidelines on cardiovascular disease prevention in clinical practice: past, present, and future: a need for joint forces. J Hypertens 26:157-160.
Clinton SK, Underwood R, Hayes L, Sherman ML, Kufe DW, Libby P (1992) Macrophage colony-stimulating factor gene expression in vascular cells and in experimental and human atherosclerosis. Am J Pathol 140:301-316.
Coe NR, Bernlohr DA (1998) Physiological properties and functions of intracellular fatty acid-binding proteins. Biochim Biophys Acta 1391:287-306.
Colon AL, Menchen LA, Hurtado O, De Cristobal J, Lizasoain I, Leza JC, Lorenzo P, Moro MA (2001) Implication of TNF-alpha convertase (TACE/ADAM17) in inducible nitric oxide synthase expression and inflammation in an experimental model of colitis. Cytokine 16:220-226.
Cominetti MR, Martin AC, Ribeiro JU, Djaafri I, Fauvel-Lafeve F, Crepin M, Selistre-de-Araujo HS (2009) Inhibition of platelets and tumor cell adhesion by the disintegrin domain of human ADAM9 to collagen I under dynamic flow conditions. Biochimie 91:1045-1052.
Cook PJ, Lip GY (1996) Infectious agents and atherosclerotic vascular disease. Qjm 89:727-735.
Croce K, Libby P (2007) Intertwining of thrombosis and inflammation in atherosclerosis. Curr Opin Hematol 14:55-61.
Cushing SD, Berliner JA, Valente AJ, Territo MC, Navab M, Parhami F, Gerrity R, Schwartz CJ, Fogelman AM (1990) Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. Proc Natl Acad Sci U S A 87:5134-5138.
Cybulsky MI, Gimbrone MA, Jr. (1991) Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. Science 251:788-791.
Dahl TB, Yndestad A, Skjelland M, Oie E, Dahl A, Michelsen A, Damas JK, Tunheim SH, Ueland T, Smith C, Bendz B, Tonstad S, Gullestad L, Froland SS, Krohg-Sorensen K, Russell D, Aukrust P, Halvorsen B (2007) Increased expression of visfatin in macrophages of human unstable carotid and coronary atherosclerosis: possible role in inflammation and plaque destabilization. Circulation 115:972-980.
Daugherty A, Rateri DL (2002) T lymphocytes in atherosclerosis: the yin-yang of Th1 and Th2 influence on lesion formation. Circ Res 90:1039-1040.
Daugherty A, Webb NR, Rateri DL, King VL (2005) Thematic review series: The immune system and atherogenesis. Cytokine regulation of macrophage functions in atherogenesis. J Lipid Res 46:1812-1822.
de Villiers WJ, Smart EJ (1999) Macrophage scavenger receptors and foam cell formation. J Leukoc Biol 66:740-746.
Dehmel T, Janke A, Hartung HP, Goebel HH, Wiendl H, Kieseier BC (2007) The cell-specific expression of metalloproteinase-disintegrins (ADAMs) in inflammatory myopathies. Neurobiol Dis 25:665-674.
Doedens JR, Black RA (2000) Stimulation-induced down-regulation of tumor necrosis factor-alpha converting enzyme. J Biol Chem 275:14598-14607.
Dreux AC, Lamb DJ, Modjtahedi H, Ferns GA (2006) The epidermal growth factor receptors and their family of ligands: their putative role in atherogenesis. Atherosclerosis 186:38-53.

Duffy MJ, McKiernan E, O'Donovan N, McGowan PM (2009) The role of ADAMs in disease pathophysiology. Clin Chim Acta 403:31-36.

Duggan DJ, Bittner M, Chen Y, Meltzer P, Trent JM (1999) Expression profiling using cDNA microarrays. Nat Genet 21:10-14.

Edwards DR, Handsley MM, Pennington CJ (2008) The ADAM metalloproteinases. Mol Aspects Med 29:258-289.

Eguchi S, Frank GD, Mifune M, Inagami T (2003) Metalloprotease-dependent ErbB ligand shedding in mediating EGFR transactivation and vascular remodelling. Biochem Soc Trans 31:1198-1202.

Eto K, Huet C, Tarui T, Kupriyanov S, Liu HZ, Puzon-McLaughlin W, Zhang XP, Sheppard D, Engvall E, Takada Y (2002) Functional classification of ADAMs based on a conserved motif for binding to integrin alpha 9beta 1: implications for sperm-egg binding and other cell interactions. J Biol Chem 277:17804-17810.

Evans JP (2001) Fertilin beta and other ADAMs as integrin ligands: insights into cell adhesion and fertilization. Bioessays 23:628-639.

Feng S, Chen JK, Yu H, Simon JA, Schreiber SL (1994) Two binding orientations for peptides to the Src SH3 domain: development of a general model for SH3-ligand interactions. Science 266:1241-1247.

Folcik VA, Nivar-Aristy RA, Krajewski LP, Cathcart MK (1995) Lipoxygenase contributes to the oxidation of lipids in human atherosclerotic plaques. J Clin Invest 96:504-510.

Foley SC, Mogas AK, Olivenstein R, Fiset PO, Chakir J, Bourbeau J, Ernst P, Lemiere C, Martin JG, Hamid Q (2007) Increased expression of ADAM33 and ADAM8 with disease progression in asthma. J Allergy Clin Immunol 119:863-871.

Forster R, Davalos-Misslitz AC, Rot A (2008) CCR7 and its ligands: balancing immunity and tolerance. Nat Rev Immunol 8:362-371.

Forster T, Roy D, Ghazal P (2003) Experiments using microarray technology: limitations and standard operating procedures. J Endocrinol 178:195-204.

Fritzsche FR, Jung M, Xu C, Rabien A, Schicktanz H, Stephan C, Dietel M, Jung K, Kristiansen G (2006) ADAM8 expression in prostate cancer is associated with parameters of unfavorable prognosis. Virchows Arch 449:628-636.

Fu S, Zhao H, Shi J, Abzhanov A, Crawford K, Ohno-Machado L, Zhou J, Du Y, Kuo WP, Zhang J, Jiang M, Jin JG (2008) Peripheral arterial occlusive disease: global gene expression analyses suggest a major role for immune and inflammatory responses. BMC Genomics 9:369.

Gaultier A, Arandjelovic S, Li X, Janes J, Dragojlovic N, Zhou GP, Dolkas J, Myers RR, Gonias SL, Campana WM (2008) A shed form of LDL receptor-related protein-1 regulates peripheral nerve injury and neuropathic pain in rodents. J Clin Invest 118:161-172.

George J, Harats D, Gilburd B, Afek A, Shaish A, Kopolovic J, Shoenfeld Y (2000) Adoptive transfer of beta(2)-glycoprotein I-reactive lymphocytes enhances early atherosclerosis in LDL receptor-deficient mice. Circulation 102:1822-1827.

George J, Shoenfeld Y, Afek A, Gilburd B, Keren P, Shaish A, Kopolovic J, Wick G, Harats D (1999) Enhanced fatty streak formation in C57BL/6J mice by immunization with heat shock protein-65. Arterioscler Thromb Vasc Biol 19:505-510.

Gerdes N, Sukhova GK, Libby P, Reynolds RS, Young JL, Schonbeck U (2002) Expression of interleukin (IL)-18 and functional IL-18 receptor on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for atherogenesis. J Exp Med 195:245-257.

Gerrity RG (1981) The role of the monocyte in atherogenesis: I. Transition of blood-borne monocytes into foam cells in fatty lesions. Am J Pathol 103:181-190.

Gewaltig J, Kummer M, Koella C, Cathomas G, Biedermann BC (2008) Requirements for CD8 T-cell migration into the human arterial wall. Hum Pathol 39:1756-1762.

Glass CK, Witztum JL (2001) Atherosclerosis. the road ahead. Cell 104:503-516.

Goldstein JL, Ho YK, Basu SK, Brown MS (1979) Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. Proc Natl Acad Sci U S A 76:333-337.

Gomez-Gaviro M, Dominguez-Luis M, Canchado J, Calafat J, Janssen H, Lara-Pezzi E, Fourie A, Tugores A, Valenzuela-Fernandez A, Mollinedo F, Sanchez-Madrid F, Diaz-Gonzalez F (2007) Expression and regulation of the metalloproteinase ADAM-8 during human neutrophil pathophysiological activation and its catalytic activity on L-selectin shedding. J Immunol 178:8053-8063.

Gonzalez P, Alvarez R, Reguero JR, Batalla A, Alvarez V, Cortina A, Cubero GI, Garcia-Castro M, Coto E (2002) Variation in the lipoprotein receptor-related protein, alpha2-macroglobulin and lipoprotein receptor-associated protein genes in relation to plasma lipid levels and risk of early myocardial infarction. Coron Artery Dis 13:251-254.

Gosk-Bierska I, Wysokin Ski W, Karnicki K, Adamiec R (2008) Tissue factor, tissue pathway factor inhibitor and risk factors of atherosclerosis in patients with chronic limbs ischemia: preliminary study. Int Angiol 27:296-301.

Gould HJ, Sutton BJ (2008) IgE in allergy and asthma today. Nat Rev Immunol 8:205-217.

Grainger DJ, Kemp PR, Liu AC, Lawn RM, Metcalfe JC (1994) Activation of transforming growth factor-beta is inhibited in transgenic apolipoprotein(a) mice. Nature 370:460-462.

Greenow K, Pearce NJ, Ramji DP (2005) The key role of apolipoprotein E in atherosclerosis. J Mol Med 83:329-342.

Grutzmann R, Luttges J, Sipos B, Ammerpohl O, Dobrowolski F, Alldinger I, Kersting S, Ockert D, Koch R, Kalthoff H, Schackert HK, Saeger HD, Kloppel G, Pilarsky C (2004) ADAM9 expression in pancreatic cancer is associated with tumour type and is a prognostic factor in ductal adenocarcinoma. Br J Cancer 90:1053-1058.

Hagg DA, Olson FJ, Kjelldahl J, Jernas M, Thelle DS, Carlsson LM, Fagerberg B, Svensson PA (2008) Expression of chemokine (C-C motif) ligand 18 in human macrophages and atherosclerotic plaques. Atherosclerosis

Haley KJ, Lilly CM, Yang JH, Feng Y, Kennedy SP, Turi TG, Thompson JF, Sukhova GH, Libby P, Lee RT (2000) Overexpression of eotaxin and the CCR3 receptor in human atherosclerosis: using genomic technology to identify a potential novel pathway of vascular inflammation. Circulation 102:2185-2189.

Hansson GK (2001) Immune mechanisms in atherosclerosis. Arterioscler Thromb Vasc Biol 21:1876-1890.
Hansson GK, Libby P (2006) The immune response in atherosclerosis: a double-edged sword. Nat Rev Immunol 6:508-519.
Hansson GK, Robertson AK, Soderberg-Naucler C (2006) Inflammation and atherosclerosis. Annu Rev Pathol 1:297-329.
Haque NS, Zhang X, French DL, Li J, Poon M, Fallon JT, Gabel BR, Taubman MB, Koschinsky M, Harpel PC (2000) CC chemokine I-309 is the principal monocyte chemoattractant induced by apolipoprotein(a) in human vascular endothelial cells. Circulation 102:786-792.
Haskard DO, Boyle JJ, Mason JC (2008) The role of complement in atherosclerosis. Curr Opin Lipidol 19:478-482.
Hegyi L, Skepper JN, Cary NR, Mitchinson MJ (1996) Foam cell apoptosis and the development of the lipid core of human atherosclerosis. J Pathol 180:423-429.
Henney AM, Wakeley PR, Davies MJ, Foster K, Hembry R, Murphy G, Humphries S (1991) Localization of stromelysin gene expression in atherosclerotic plaques by in situ hybridization. Proc Natl Acad Sci U S A 88:8154-8158.
Herren B (2002) ADAM-mediated shedding and adhesion: a vascular perspective. News Physiol Sci 17:73-76.
Herren B, Raines EW, Ross R (1997) Expression of a disintegrin-like protein in cultured human vascular cells and in vivo. Faseb J 11:173-180.
Hillis GS (2003) Soluble integrin adhesion receptors and atherosclerosis: much heat and a little light? J Hum Hypertens 17:449-453.
Hiltunen MO, Tuomisto TT, Niemi M, Brasen JH, Rissanen TT, Toronen P, Vajanto I, Yla-Herttuala S (2002) Changes in gene expression in atherosclerotic plaques analyzed using DNA array. Atherosclerosis 165:23-32.
Hodgkinson CP, Ye S (2003) Microarray analysis of peroxisome proliferator-activated receptor-gamma induced changes in gene expression in macrophages. Biochem Biophys Res Commun 308:505-510.
Holdt LM, Thiery J, Breslow JL, Teupser D (2008) Increased ADAM17 mRNA expression and activity is associated with atherosclerosis resistance in LDL-receptor deficient mice. Arterioscler Thromb Vasc Biol 28:1097-1103.
Horiuchi K, Weskamp G, Lum L, Hammes HP, Cai H, Brodie TA, Ludwig T, Chiusaroli R, Baron R, Preissner KT, Manova K, Blobel CP (2003) Potential role for ADAM15 in pathological neovascularization in mice. Mol Cell Biol 23:5614-5624.
Hotoda N, Koike H, Sasagawa N, Ishiura S (2002) A secreted form of human ADAM9 has an alpha-secretase activity for APP. Biochem Biophys Res Commun 293:800-805.
Howard L, Maciewicz RA, Blobel CP (2000) Cloning and characterization of ADAM28: evidence for autocatalytic pro-domain removal and for cell surface localization of mature ADAM28. Biochem J 348 Pt 1:21-27.
Howard L, Nelson KK, Maciewicz RA, Blobel CP (1999) Interaction of the metalloprotease disintegrins MDC9 and MDC15 with two SH3 domain-containing proteins, endophilin I and SH3PX1. J Biol Chem 274:31693-31699.
Huovila AP, Eder AM, Fuller SD (1992) Hepatitis B surface antigen assembles in a post-ER, pre-Golgi compartment. J Cell Biol 118:1305-1320.

Huovila AP, Turner AJ, Pelto-Huikko M, Karkkainen I, Ortiz RM (2005) Shedding light on ADAM metalloproteinases. Trends Biochem Sci 30:413-422.

Hurtado I, Fiol C, Gracia V, Caldu P (1996) In vitro oxidised HDL exerts a cytotoxic effect on macrophages. Atherosclerosis 125:39-46.

Iba K, Albrechtsen R, Gilpin BJ, Loechel F, Wewer UM (1999) Cysteine-rich domain of human ADAM 12 (meltrin alpha) supports tumor cell adhesion. Am J Pathol 154:1489-1501.

Ilveskoski E, Perola M, Lehtimaki T, Laippala P, Savolainen V, Pajarinen J, Penttila A, Lalu KH, Mannikko A, Liesto KK, Koivula T, Karhunen PJ (1999) Age-dependent association of apolipoprotein E genotype with coronary and aortic atherosclerosis in middle-aged men: an autopsy study. Circulation 100:608-613.

Infanger M, Grosse J, Westphal K, Leder A, Ulbrich C, Paul M, Grimm D (2008) Vascular endothelial growth factor induces extracellular matrix proteins and osteopontin in the umbilical artery. Ann Vasc Surg 22:273-284.

Ishikawa N, Daigo Y, Yasui W, Inai K, Nishimura H, Tsuchiya E, Kohno N, Nakamura Y (2004) ADAM8 as a novel serological and histochemical marker for lung cancer. Clin Cancer Res 10:8363-8370.

Isner JM, Kearney M, Bortman S, Passeri J (1995) Apoptosis in human atherosclerosis and restenosis. Circulation 91:2703-2711.

Isola J, DeVries S, Chu L, Ghazvini S, Waldman F (1994) Analysis of changes in DNA sequence copy number by comparative genomic hybridization in archival paraffin-embedded tumor samples. Am J Pathol 145:1301-1308.

Jaakkola O, Yla-Herttuala S, Sarkioja T, Nikkari T (1989) Macrophage foam cells from human aortic fatty streaks take up beta-VLDL and acetylated LDL in primary culture. Atherosclerosis 79:173-182.

Jackson LF, Qiu TH, Sunnarborg SW, Chang A, Zhang C, Patterson C, Lee DC (2003) Defective valvulogenesis in HB-EGF and TACE-null mice is associated with aberrant BMP signaling. Embo J 22:2704-2716.

Janes PW, Saha N, Barton WA, Kolev MV, Wimmer-Kleikamp SH, Nievergall E, Blobel CP, Himanen JP, Lackmann M, Nikolov DB (2005) Adam meets Eph: an ADAM substrate recognition module acts as a molecular switch for ephrin cleavage in trans. Cell 123:291-304.

Jawien J (2008) New insights into immunological aspects of atherosclerosis. Pol Arch Med Wewn 118:127-131.

Jerome WG, Yancey PG (2003) The role of microscopy in understanding atherosclerotic lysosomal lipid metabolism. Microsc Microanal 9:54-67.

Jeziorska M, McCollum C, Wooley DE (1998) Observations on bone formation and remodelling in advanced atherosclerotic lesions of human carotid arteries. Virchows Arch 433:559-565.

Jiang C, Ting AT, Seed B (1998) PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. Nature 391:82-86.

Jonasson L, Holm J, Skalli O, Bondjers G, Hansson GK (1986) Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. Arteriosclerosis 6:131-138.

Jonasson L, Holm J, Skalli O, Gabbiani G, Hansson GK (1985) Expression of class II transplantation antigen on vascular smooth muscle cells in human atherosclerosis. J Clin Invest 76:125-131.

Kaartinen M, van der Wal AC, van der Loos CM, Piek JJ, Koch KT, Becker AE, Kovanen PT (1998) Mast cell infiltration in acute coronary syndromes: implications for plaque rupture. J Am Coll Cardiol 32:606-612.

Karadag A, Zhou M, Croucher PI (2006) ADAM-9 (MDC-9/meltrin-gamma), a member of the a disintegrin and metalloproteinase family, regulates myeloma-cell-induced interleukin-6 production in osteoblasts by direct interaction with the alpha(v)beta5 integrin. Blood 107:3271-3278.

Karan D, Lin FC, Bryan M, Ringel J, Moniaux N, Lin MF, Batra SK (2003) Expression of ADAMs (a disintegrin and metalloproteases) and TIMP-3 (tissue inhibitor of metalloproteinase-3) in human prostatic adenocarcinomas. Int J Oncol 23:1365-1371.

Karhunen PJ, Penttila A (1990) Validity of post-mortem alcohol reports. Alcohol Alcohol 25:25-32.

Karkkainen I, Karhu R, Huovila AP (2000) Assignment of the ADAM15 gene to human chromosome band 1q21.3 by in situ hybridization. Cytogenet Cell Genet 88:206-207.

Karmann K, Hughes CC, Schechner J, Fanslow WC, Pober JS (1995) CD40 on human endothelial cells: inducibility by cytokines and functional regulation of adhesion molecule expression. Proc Natl Acad Sci U S A 92:4342-4346.

Katsuda S, Boyd HC, Fligner C, Ross R, Gown AM (1992) Human atherosclerosis. III. Immunocytochemical analysis of the cell composition of lesions of young adults. Am J Pathol 140:907-914.

Keaney JF, Jr. (2000) Atherosclerosis: from lesion formation to plaque activation and endothelial dysfunction. Mol Aspects Med 21:99-166.

Kellner-Weibel G, Yancey PG, Jerome WG, Walser T, Mason RP, Phillips MC, Rothblat GH (1999) Crystallization of free cholesterol in model macrophage foam cells. Arterioscler Thromb Vasc Biol 19:1891-1898.

Kelly K, Hutchinson G, Nebenius-Oosthuizen D, Smith AJ, Bartsch JW, Horiuchi K, Rittger A, Manova K, Docherty AJ, Blobel CP (2005) Metalloprotease-disintegrin ADAM8: expression analysis and targeted deletion in mice. Dev Dyn 232:221-231.

Kenny PA, Bissell MJ (2007) Targeting TACE-dependent EGFR ligand shedding in breast cancer. J Clin Invest 117:337-345.

Kermarrec N, Selloum S, Plantefeve G, Chosidow D, Paoletti X, Lopez A, Mantz J, Desmonts JM, Gougerot-Pocidalo MA, Chollet-Martin S (2005) Regulation of peritoneal and systemic neutrophil-derived tumor necrosis factor-alpha release in patients with severe peritonitis: role of tumor necrosis factor-alpha converting enzyme cleavage. Crit Care Med 33:1359-1364.

Khan BV, Parthasarathy SS, Alexander RW, Medford RM (1995) Modified low density lipoprotein and its constituents augment cytokine-activated vascular cell adhesion molecule-1 gene expression in human vascular endothelial cells. J Clin Invest 95:1262-1270.

Kilcullen N, Viswanathan K, Das R, Morrell C, Farrin A, Barth JH, Hall AS (2007) Heart-type fatty acid-binding protein predicts long-term mortality after acute coronary syndrome and identifies high-risk patients across the range of troponin values. J Am Coll Cardiol 50:2061-2067.

Kim LT, Yamada KM (1997) The regulation of expression of integrin receptors. Proc Soc Exp Biol Med 214:123-131.

Kim MJ, Cheng G, Agrawal DK (2004) Cl- channels are expressed in human normal monocytes: a functional role in migration, adhesion and volume change. Clin Exp Immunol 138:453-459.

Kim S, Foster C, Lecchi A, Quinton TM, Prosser DM, Jin J, Cattaneo M, Kunapuli SP (2002) Protease-activated receptors 1 and 4 do not stimulate G(i) signaling pathways in the absence of secreted ADP and cause human platelet aggregation independently of G(i) signaling. Blood 99:3629-3636.

Kimura K, Hashiguchi T, Deguchi T, Horinouchi S, Uto T, Oku H, Setoyama S, Maruyama I, Osame M, Arimura K (2007) Serum VEGF--as a prognostic factor of atherosclerosis. Atherosclerosis 194:182-188.

King JY, Ferrara R, Tabibiazar R, Spin JM, Chen MM, Kuchinsky A, Vailaya A, Kincaid R, Tsalenko A, Deng DX, Connolly A, Zhang P, Yang E, Watt C, Yakhini Z, Ben-Dor A, Adler A, Bruhn L, Tsao P, Quertermous T, Ashley EA (2005) Pathway analysis of coronary atherosclerosis. Physiol Genomics 23:103-118.

Kleino I, Ortiz RM, Huovila AP (2007) ADAM15 gene structure and differential alternative exon use in human tissues. BMC Mol Biol 8:90.

Koller G, Schlomann U, Golfi P, Ferdous T, Naus S, Bartsch JW (2009) ADAM8/MS2/CD156, an emerging drug target in the treatment of inflammatory and invasive pathologies. Curr Pharm Des 15:2272-2281.

Konstantinidis D, Paletas K, Koliakos G, Kaloyianni M (2009) Signaling components involved in leptin-induced amplification of the atherosclerosis-related properties of human monocytes. J Vasc Res 46:199-208.

Kovanen PT (1995) Role of mast cells in atherosclerosis. Chem Immunol 62:132-170.

Kovanen PT, Kaartinen M, Paavonen T (1995) Infiltrates of activated mast cells at the site of coronary atheromatous erosion or rupture in myocardial infarction. Circulation 92:1084-1088.

Kozarsky KF, Donahee MH, Glick JM, Krieger M, Rader DJ (2000) Gene transfer and hepatic overexpression of the HDL receptor SR-BI reduces atherosclerosis in the cholesterol-fed LDL receptor-deficient mouse. Arterioscler Thromb Vasc Biol 20:721-727.

Kraaijeveld AO, de Jager SC, de Jager WJ, Prakken BJ, McColl SR, Haspels I, Putter H, van Berkel TJ, Nagelkerken L, Jukema JW, Biessen EA (2007) CC chemokine ligand-5 (CCL5/RANTES) and CC chemokine ligand-18 (CCL18/PARC) are specific markers of refractory unstable angina pectoris and are transiently raised during severe ischemic symptoms. Circulation 116:1931-1941.

Kratzschmar J, Lum L, Blobel CP (1996) Metargidin, a membrane-anchored metalloprotease-disintegrin protein with an RGD integrin binding sequence. J Biol Chem 271:4593-4596.

Krieglstein CF, Granger DN (2001) Adhesion molecules and their role in vascular disease. Am J Hypertens 14:44S-54S.

Kruth HS (2001) Macrophage foam cells and atherosclerosis. Front Biosci 6:D429-455.

Kuefer R, Day KC, Kleer CG, Sabel MS, Hofer MD, Varambally S, Zorn CS, Chinnaiyan AM, Rubin MA, Day ML (2006) ADAM15 disintegrin is associated with aggressive prostate and breast cancer disease. Neoplasia 8:319-329.

Kugiyama K, Kerns SA, Morrisett JD, Roberts R, Henry PD (1990) Impairment of endothelium-dependent arterial relaxation by lysolecithin in modified low-density lipoproteins. Nature 344:160-162.

Kuhn H, Belkner J, Zaiss S, Fahrenklemper T, Wohlfeil S (1994) Involvement of 15-lipoxygenase in early stages of atherogenesis. J Exp Med 179:1903-1911.

Kume N, Cybulsky MI, Gimbrone MA, Jr. (1992) Lysophosphatidylcholine, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured human and rabbit arterial endothelial cells. J Clin Invest 90:1138-1144.

Langer H, May AE, Bultmann A, Gawaz M (2005) ADAM 15 is an adhesion receptor for platelet GPIIb-IIIa and induces platelet activation. Thromb Haemost 94:555-561.

Laurat E, Poirier B, Tupin E, Caligiuri G, Hansson GK, Bariety J, Nicoletti A (2001) In vivo downregulation of T helper cell 1 immune responses reduces atherogenesis in apolipoprotein E-knockout mice. Circulation 104:197-202.

Lawn RM, Wade DP, Garvin MR, Wang X, Schwartz K, Porter JG, Seilhamer JJ, Vaughan AM, Oram JF (1999) The Tangier disease gene product ABC1 controls the cellular apolipoprotein-mediated lipid removal pathway. J Clin Invest 104:R25-31.

Lee DC, Sunnarborg SW, Hinkle CL, Myers TJ, Stevenson MY, Russell WE, Castner BJ, Gerhart MJ, Paxton RJ, Black RA, Chang A, Jackson LF (2003a) TACE/ADAM17 processing of EGFR ligands indicates a role as a physiological convertase. Ann N Y Acad Sci 995:22-38.

Lee JK, Bussey KJ, Gwadry FG, Reinhold W, Riddick G, Pelletier SL, Nishizuka S, Szakacs G, Annereau JP, Shankavaram U, Lababidi S, Smith LH, Gottesman MM, Weinstein JN (2003b) Comparing cDNA and oligonucleotide array data: concordance of gene expression across platforms for the NCI-60 cancer cells. Genome Biol 4:R82.

Lee MH, Rapti M, Murphy G (2005) Total conversion of tissue inhibitor of metalloproteinase (TIMP) for specific metalloproteinase targeting: fine-tuning TIMP-4 for optimal inhibition of tumor necrosis factor-{alpha}-converting enzyme. J Biol Chem 280:15967-15975.

Lehtimaki T, Lehtinen S, Solakivi T, Nikkila M, Jaakkola O, Jokela H, Yla-Herttuala S, Luoma JS, Koivula T, Nikkari T (1999) Autoantibodies against oxidized low density lipoprotein in patients with angiographically verified coronary artery disease. Arterioscler Thromb Vasc Biol 19:23-27.

Lendeckel U, Kohl J, Arndt M, Carl-McGrath S, Donat H, Rocken C (2005) Increased expression of ADAM family members in human breast cancer and breast cancer cell lines. J Cancer Res Clin Oncol 131:41-48.

Li JK, Du WJ, Jiang SL, Tian H (2009) Expression of ADAM-15 in rat myocardial infarction. Int J Exp Pathol 90:347-354.

Liapis CD, Avgerinos ED, Kadoglou NP, Kakisis JD (2009) What a vascular surgeon should know and do about atherosclerotic risk factors. J Vasc Surg 49:1348-1354.

Libby P (2001) Current concepts of the pathogenesis of the acute coronary syndromes. Circulation 104:365-372.

Linton MF, Fazio S (2001) Class A scavenger receptors, macrophages, and atherosclerosis. Curr Opin Lipidol 12:489-495.

Lipshutz RJ, Fodor SP, Gingeras TR, Lockhart DJ (1999) High density synthetic oligonucleotide arrays. Nat Genet 21:20-24.

Liu Q, Zhang J, Tran H, Verbeek MM, Reiss K, Estus S, Bu G (2009) LRP1 shedding in human brain: roles of ADAM10 and ADAM17. Mol Neurodegener 4:17.

Liuzzo G, Biasucci LM, Gallimore JR, Grillo RL, Rebuzzi AG, Pepys MB, Maseri A (1994) The prognostic value of C-reactive protein and serum amyloid a protein in severe unstable angina. N Engl J Med 331:417-424.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

Llodra J, Angeli V, Liu J, Trogan E, Fisher EA, Randolph GJ (2004) Emigration of monocyte-derived cells from atherosclerotic lesions characterizes regressive, but not progressive, plaques. Proc Natl Acad Sci U S A 101:11779-11784.

Lockhart DJ, Winzeler EA (2000) Genomics, gene expression and DNA arrays. Nature 405:827-836.

Lu X, Lu D, Scully MF, Kakkar VV (2008) The role of integrin-mediated cell adhesion in atherosclerosis: pathophysiology and clinical opportunities. Curr Pharm Des 14:2140-2158.

Lucas N, Najy AJ, Day ML (2009) The therapeutic potential of ADAM15. Curr Pharm Des 15:2311-2318.

Lum L, Reid MS, Blobel CP (1998) Intracellular maturation of the mouse metalloprotease disintegrin MDC15. J Biol Chem 273:26236-26247.

Lusis AJ (2000) Atherosclerosis. Nature 407:233-241.

Mach F, Schonbeck U, Sukhova GK, Atkinson E, Libby P (1998) Reduction of atherosclerosis in mice by inhibition of CD40 signalling. Nature 394:200-203.

Mason DP, Kenagy RD, Hasenstab D, Bowen-Pope DF, Seifert RA, Coats S, Hawkins SM, Clowes AW (1999) Matrix metalloproteinase-9 overexpression enhances vascular smooth muscle cell migration and alters remodeling in the injured rat carotid artery. Circ Res 85:1179-1185.

Matsumoto A, Naito M, Itakura H, Ikemoto S, Asaoka H, Hayakawa I, Kanamori H, Aburatani H, Takaku F, Suzuki H, et al. (1990) Human macrophage scavenger receptors: primary structure, expression, and localization in atherosclerotic lesions. Proc Natl Acad Sci U S A 87:9133-9137.

Matsuno O, Miyazaki E, Nureki S, Ueno T, Kumamoto T, Higuchi Y (2006) Role of ADAM8 in experimental asthma. Immunol Lett 102:67-73.

Maughan NJ, Lewis FA, Smith V (2001) An introduction to arrays. J Pathol 195:3-6.

Maury CP, Teppo AM (1989) Circulating tumour necrosis factor-alpha (cachectin) in myocardial infarction. J Intern Med 225:333-336.

Mazzocca A, Coppari R, De Franco R, Cho JY, Libermann TA, Pinzani M, Toker A (2005) A secreted form of ADAM9 promotes carcinoma invasion through tumor-stromal interactions. Cancer Res 65:4728-4738.

McCarthy JJ, Meyer J, Moliterno DJ, Newby LK, Rogers WJ, Topol EJ (2003) Evidence for substantial effect modification by gender in a large-scale genetic association study of the metabolic syndrome among coronary heart disease patients. Hum Genet 114:87-98.

McCarthy JJ, Parker A, Salem R, Moliterno DJ, Wang Q, Plow EF, Rao S, Shen G, Rogers WJ, Newby LK, Cannata R, Glatt K, Topol EJ (2004) Large scale association analysis for identification of genes underlying premature

coronary heart disease: cumulative perspective from analysis of 111 candidate genes. J Med Genet 41:334-341.
McCullagh KG, Duance VC, Bishop KA (1980) The distribution of collagen types I, III and V (AB) in normal and atherosclerotic human aorta. J Pathol 130:45-55.
McGowan PM, Ryan BM, Hill AD, McDermott E, O'Higgins N, Duffy MJ (2007) ADAM-17 expression in breast cancer correlates with variables of tumor progression. Clin Cancer Res 13:2335-2343.
McMurray HF, Parthasarathy S, Steinberg D (1993) Oxidatively modified low density lipoprotein is a chemoattractant for human T lymphocytes. J Clin Invest 92:1004-1008.
Mendall MA, Goggin PM, Molineaux N, Levy J, Toosy T, Strachan D, Camm AJ, Northfield TC (1994) Relation of Helicobacter pylori infection and coronary heart disease. Br Heart J 71:437-439.
Mietus-Snyder M, Friera A, Glass CK, Pitas RE (1997) Regulation of scavenger receptor expression in smooth muscle cells by protein kinase C: a role for oxidative stress. Arterioscler Thromb Vasc Biol 17:969-978.
Mighell AJ, Hume WJ, Robinson PA (1998) An overview of the complexities and subtleties of immunohistochemistry. Oral Dis 4:217-223.
Milla ME, Leesnitzer MA, Moss ML, Clay WC, Carter HL, Miller AB, Su JL, Lambert MH, Willard DH, Sheeley DM, Kost TA, Burkhart W, Moyer M, Blackburn RK, Pahel GL, Mitchell JL, Hoffman CR, Becherer JD (1999) Specific sequence elements are required for the expression of functional tumor necrosis factor-alpha-converting enzyme (TACE). J Biol Chem 274:30563-30570.
Misquitta CM, Iyer VR, Werstiuk ES, Grover AK (2001) The role of 3'-untranslated region (3'-UTR) mediated mRNA stability in cardiovascular pathophysiology. Mol Cell Biochem 224:53-67.
Mochizuki S, Okada Y (2007) ADAMs in cancer cell proliferation and progression. Cancer Sci 98:621-628.
Moiseeva EP (2001) Adhesion receptors of vascular smooth muscle cells and their functions. Cardiovasc Res 52:372-386.
Moore KJ, Kunjathoor VV, Koehn SL, Manning JJ, Tseng AA, Silver JM, McKee M, Freeman MW (2005) Loss of receptor-mediated lipid uptake via scavenger receptor A or CD36 pathways does not ameliorate atherosclerosis in hyperlipidemic mice. J Clin Invest 115:2192-2201.
Moratz C, Harrison K, Kehrl JH (2004) Regulation of chemokine-induced lymphocyte migration by RGS proteins. Methods Enzymol 389:15-32.
Moreno PR, Falk E, Palacios IF, Newell JB, Fuster V, Fallon JT (1994) Macrophage infiltration in acute coronary syndromes. Implications for plaque rupture. Circulation 90:775-778.
Mosnier JF, Jarry A, Bou-Hanna C, Denis MG, Merlin D, Laboisse CL (2006) ADAM15 upregulation and interaction with multiple binding partners in inflammatory bowel disease. Lab Invest 86:1064-1073.
Moss ML, Jin SL, Milla ME, Bickett DM, Burkhart W, Carter HL, Chen WJ, et al. (1997) Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha. Nature 385:733-736.
Moss ML, Rasmussen FH (2007) Fluorescent substrates for the proteinases ADAM17, ADAM10, ADAM8, and ADAM12 useful for high-throughput inhibitor screening. Anal Biochem 366:144-148.

Moura R, Tjwa M, Vandervoort P, Van Kerckhoven S, Holvoet P, Hoylaerts MF (2008) Thrombospondin-1 deficiency accelerates atherosclerotic plaque maturation in ApoE-/- mice. Circ Res 103:1181-1189.

Munro JM, van der Walt JD, Munro CS, Chalmers JA, Cox EL (1987) An immunohistochemical analysis of human aortic fatty streaks. Hum Pathol 18:375-380.

Muraguchi T, Takegami Y, Ohtsuka T, Kitajima S, Chandana EP, Omura A, Miki T, Takahashi R, Matsumoto N, Ludwig A, Noda M, Takahashi C (2007) RECK modulates Notch signaling during cortical neurogenesis by regulating ADAM10 activity. Nat Neurosci 10:838-845.

Murphy G (2008) The ADAMs: signalling scissors in the tumour microenvironment. Nat Rev Cancer 8:929-941.

Murray CJ, Lopez AD (1997) Alternative projections of mortality and disability by cause 1990-2020: Global Burden of Disease Study. Lancet 349:1498-1504.

Nachtigal M, Al-Assaad Z, Mayer EP, Kim K, Monsigny M (1998) Galectin-3 expression in human atherosclerotic lesions. Am J Pathol 152:1199-1208.

Nachtigal M, Ghaffar A, Mayer EP (2008) Galectin-3 gene inactivation reduces atherosclerotic lesions and adventitial inflammation in ApoE-deficient mice. Am J Pathol 172:247-255.

Nagano Y, Arai H, Kita T (1991) High density lipoprotein loses its effect to stimulate efflux of cholesterol from foam cells after oxidative modification. Proc Natl Acad Sci U S A 88:6457-6461.

Naghavi M, Libby P, Falk E, Casscells SW, Litovsky S, Rumberger J, Badimon JJ, et al. (2003a) From vulnerable plaque to vulnerable patient: a call for new definitions and risk assessment strategies: Part II. Circulation 108:1772-1778.

Naghavi M, Libby P, Falk E, Casscells SW, Litovsky S, Rumberger J, Badimon JJ, et al. (2003b) From vulnerable plaque to vulnerable patient: a call for new definitions and risk assessment strategies: Part I. Circulation 108:1664-1672.

Najy AJ, Day KC, Day ML (2008a) ADAM15 supports prostate cancer metastasis by modulating tumor cell-endothelial cell interaction. Cancer Res 68:1092-1099.

Najy AJ, Day KC, Day ML (2008b) The ectodomain shedding of E-cadherin by ADAM15 supports ErbB receptor activation. J Biol Chem 283:18393-18401.

Nakayama M, Kudoh T, Kaikita K, Yoshimura M, Oshima S, Miyamoto Y, Takeya M, Ogawa H (2008) Class A macrophage scavenger receptor gene expression levels in peripheral blood mononuclear cells specifically increase in patients with acute coronary syndrome. Atherosclerosis 198:426-433.

Naus S, Richter M, Wildeboer D, Moss M, Schachner M, Bartsch JW (2004) Ectodomain shedding of the neural recognition molecule CHL1 by the metalloprotease-disintegrin ADAM8 promotes neurite outgrowth and suppresses neuronal cell death. J Biol Chem 279:16083-16090.

Neuzil J, Upston JM, Witting PK, Scott KF, Stocker R (1998) Secretory phospholipase A2 and lipoprotein lipase enhance 15-lipoxygenase-induced enzymic and nonenzymic lipid peroxidation in low-density lipoproteins. Biochemistry 37:9203-9210.

Nicholls AC, Thomas M (1977) Coxsackie virus infection in acute myocardial infarction. Lancet 1:883-884.

Nishigaki I, Hagihara M, Tsunekawa H, Maseki M, Yagi K (1981) Lipid peroxide levels of serum lipoprotein fractions of diabetic patients. Biochem Med 25:373-378.

Nobuyoshi M, Tanaka M, Nosaka H, Kimura T, Yokoi H, Hamasaki N, Kim K, Shindo T, Kimura K (1991) Progression of coronary atherosclerosis: is coronary spasm related to progression? J Am Coll Cardiol 18:904-910.

O'Shea C, McKie N, Buggy Y, Duggan C, Hill AD, McDermott E, O'Higgins N, Duffy MJ (2003) Expression of ADAM-9 mRNA and protein in human breast cancer. Int J Cancer 105:754-761.

Ohtsu H, Dempsey PJ, Eguchi S (2006) ADAMs as mediators of EGF receptor transactivation by G protein-coupled receptors. Am J Physiol Cell Physiol 291:C1-10.

Oksala N, Levula M, Airla N, Pelto-Huikko M, Ortiz RM, Jarvinen O, Salenius JP, Ozsait B, Komurcu-Bayrak E, Erginel-Unaltuna N, Huovila AP, Kytomaki L, Soini JT, Kahonen M, Karhunen PJ, Laaksonen R, Lehtimaki T (2009) ADAM-9, ADAM-15, and ADAM-17 are upregulated in macrophages in advanced human atherosclerotic plaques in aorta and carotid and femoral arteries--Tampere vascular study. Ann Med 41:279-290.

Packard RR, Libby P (2008) Inflammation in atherosclerosis: from vascular biology to biomarker discovery and risk prediction. Clin Chem 54:24-38.

Parthasarathy S, Printz DJ, Boyd D, Joy L, Steinberg D (1986) Macrophage oxidation of low density lipoprotein generates a modified form recognized by the scavenger receptor. Arteriosclerosis 6:505-510.

Pellieux C, Desgeorges A, Pigeon CH, Chambaz C, Yin H, Hayoz D, Silacci P (2003) Cap G, a gelsolin family protein modulating protective effects of unidirectional shear stress. J Biol Chem 278:29136-29144.

Peschon JJ, Slack JL, Reddy P, Stocking KL, Sunnarborg SW, Lee DC, Russell WE, Castner BJ, Johnson RS, Fitzner JN, Boyce RW, Nelson N, Kozlosky CJ, Wolfson MF, Rauch CT, Cerretti DP, Paxton RJ, March CJ, Black RA (1998) An essential role for ectodomain shedding in mammalian development. Science 282:1281-1284.

Pinderski LJ, Fischbein MP, Subbanagounder G, Fishbein MC, Kubo N, Cheroutre H, Curtiss LK, Berliner JA, Boisvert WA (2002) Overexpression of interleukin-10 by activated T lymphocytes inhibits atherosclerosis in LDL receptor-deficient Mice by altering lymphocyte and macrophage phenotypes. Circ Res 90:1064-1071.

Podrez EA, Abu-Soud HM, Hazen SL (2000) Myeloperoxidase-generated oxidants and atherosclerosis. Free Radic Biol Med 28:1717-1725.

Qian M, Bai SA, Brogdon B, Wu JT, Liu RQ, Covington MB, Vaddi K, Newton RC, Fossler MJ, Garner CE, Deng Y, Maduskuie T, Trzaskos J, Duan JJ, Decicco CP, Christ DD (2007) Pharmacokinetics and pharmacodynamics of DPC 333 ((2R)-2-((3R)-3-amino-3{4-[2-methyl-4-quinolinyl) methoxy] phenyl}-2-oxopyrrolidinyl)-N-hydroxy-4-methylpentanamide)), a potent and selective inhibitor of tumor necrosis factor alpha-converting enzyme in rodents, dogs, chimpanzees, and humans. Drug Metab Dispos 35:1916-1925.

Quinn MT, Parthasarathy S, Fong LG, Steinberg D (1987) Oxidatively modified low density lipoproteins: a potential role in recruitment and retention of monocyte/macrophages during atherogenesis. Proc Natl Acad Sci U S A 84:2995-2998.

Raffetto JD, Khalil RA (2008) Matrix metalloproteinases and their inhibitors in vascular remodeling and vascular disease. Biochem Pharmacol 75:346-359.

Raghavendra V, Rutkowski MD, DeLeo JA (2002) The role of spinal neuroimmune activation in morphine tolerance/hyperalgesia in neuropathic and sham-operated rats. J Neurosci 22:9980-9989.

Rajavashisth TB, Andalibi A, Territo MC, Berliner JA, Navab M, Fogelman AM, Lusis AJ (1990) Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low-density lipoproteins. Nature 344:254-257.

Randi AM, Biguzzi E, Falciani F, Merlini P, Blakemore S, Bramucci E, Lucreziotti S, Lennon M, Faioni EM, Ardissino D, Mannucci PM (2003) Identification of differentially expressed genes in coronary atherosclerotic plaques from patients with stable or unstable angina by cDNA array analysis. J Thromb Haemost 1:829-835.

Randolph GJ, Beaulieu S, Lebecque S, Steinman RM, Muller WA (1998) Differentiation of monocytes into dendritic cells in a model of transendothelial trafficking. Science 282:480-483.

Rao H, Lu G, Kajiya H, Garcia-Palacios V, Kurihara N, Anderson J, Patrene K, Sheppard D, Blair HC, Windle JJ, Choi SJ, Roodman GD (2006) Alpha9beta1: a novel osteoclast integrin that regulates osteoclast formation and function. J Bone Miner Res 21:1657-1665.

Reape TJ, Rayner K, Manning CD, Gee AN, Barnette MS, Burnand KG, Groot PH (1999) Expression and cellular localization of the CC chemokines PARC and ELC in human atherosclerotic plaques. Am J Pathol 154:365-374.

Richardson PD, Davies MJ, Born GV (1989) Influence of plaque configuration and stress distribution on fissuring of coronary atherosclerotic plaques. Lancet 2:941-944.

Roemer A, Schwettmann L, Jung M, Roigas J, Kristiansen G, Schnorr D, Loening SA, Jung K, Lichtinghagen R (2004) Increased mRNA expression of ADAMs in renal cell carcinoma and their association with clinical outcome. Oncol Rep 11:529-536.

Roghani M, Becherer JD, Moss ML, Atherton RE, Erdjument-Bromage H, Arribas J, Blackburn RK, Weskamp G, Tempst P, Blobel CP (1999) Metalloprotease-disintegrin MDC9: intracellular maturation and catalytic activity. J Biol Chem 274:3531-3540.

Rokitansky (ed) (1852) A Manual of Pathological Anatomy

Ross R (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 362:801-809.

Ross R (1999) Atherosclerosis--an inflammatory disease. N Engl J Med 340:115-126.

Ross R, Glomset JA (1973) Atherosclerosis and the arterial smooth muscle cell: Proliferation of smooth muscle is a key event in the genesis of the lesions of atherosclerosis. Science 180:1332-1339.

Rovida E, Paccagnini A, Del Rosso M, Peschon J, Dello Sbarba P (2001) TNF-alpha-converting enzyme cleaves the macrophage colony-stimulating factor receptor in macrophages undergoing activation. J Immunol 166:1583-1589.

Sahin U, Weskamp G, Kelly K, Zhou HM, Higashiyama S, Peschon J, Hartmann D, Saftig P, Blobel CP (2004) Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands. J Cell Biol 164:769-779.

Schaffler A, Neumeier M, Herfarth H, Furst A, Scholmerich J, Buchler C (2005) Genomic structure of human omentin, a new adipocytokine expressed in omental adipose tissue. Biochim Biophys Acta 1732:96-102.

Schlomann U, Rathke-Hartlieb S, Yamamoto S, Jockusch H, Bartsch JW (2000) Tumor necrosis factor alpha induces a metalloprotease-disintegrin, ADAM8 (CD 156): implications for neuron-glia interactions during neurodegeneration. J Neurosci 20:7964-7971.

Schlomann U, Wildeboer D, Webster A, Antropova O, Zeuschner D, Knight CG, Docherty AJ, Lambert M, Skelton L, Jockusch H, Bartsch JW (2002) The metalloprotease disintegrin ADAM8. Processing by autocatalysis is required for proteolytic activity and cell adhesion. J Biol Chem 277:48210-48219.

Schlondorff J, Becherer JD, Blobel CP (2000) Intracellular maturation and localization of the tumour necrosis factor alpha convertase (TACE). Biochem J 347 Pt 1:131-138.

Schlondorff J, Blobel CP (1999) Metalloprotease-disintegrins: modular proteins capable of promoting cell-cell interactions and triggering signals by protein-ectodomain shedding. J Cell Sci 112 (Pt 21):3603-3617.

Schluesener HJ (1998) The disintegrin domain of ADAM 8 enhances protection against rat experimental autoimmune encephalomyelitis, neuritis and uveitis by a polyvalent autoantigen vaccine. J Neuroimmunol 87:197-202.

Schonbeck U, Libby P (2001) CD40 signaling and plaque instability. Circ Res 89:1092-1103.

Schutz A, Hartig W, Wobus M, Grosche J, Wittekind C, Aust G (2005) Expression of ADAM15 in lung carcinomas. Virchows Arch 446:421-429.

Seals DF, Courtneidge SA (2003) The ADAMs family of metalloproteases: multidomain proteins with multiple functions. Genes Dev 17:7-30.

Seo D, Wang T, Dressman H, Herderick EE, Iversen ES, Dong C, Vata K, Milano CA, Rigat F, Pittman J, Nevins JR, West M, Goldschmidt-Clermont PJ (2004) Gene expression phenotypes of atherosclerosis. Arterioscler Thromb Vasc Biol 24:1922-1927.

Sheikine Y, Hansson GK (2004) Chemokines and atherosclerosis. Ann Med 36:98-118.

Shi SR, Key ME, Kalra KL (1991) Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem 39:741-748.

Shi W, Chen H, Sun J, Buckley S, Zhao J, Anderson KD, Williams RG, Warburton D (2003) TACE is required for fetal murine cardiac development and modeling. Dev Biol 261:371-380.

Shiffman D, Mikita T, Tai JT, Wade DP, Porter JG, Seilhamer JJ, Somogyi R, Liang S, Lawn RM (2000) Large scale gene expression analysis of cholesterol-loaded macrophages. J Biol Chem 275:37324-37332.

Singh M, Ananthula S, Milhorn DM, Krishnaswamy G, Singh K (2007) Osteopontin: a novel inflammatory mediator of cardiovascular disease. Front Biosci 12:214-221.

Smith KM, Gaultier A, Cousin H, Alfandari D, White JM, DeSimone DW (2002) The cysteine-rich domain regulates ADAM protease function in vivo. J Cell Biol 159:893-902.

Song L, Schindler C (2004) IL-6 and the acute phase response in murine atherosclerosis. Atherosclerosis 177:43-51.

Springer TA (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 76:301-314.

Stary HC (1990) The sequence of cell and matrix changes in atherosclerotic lesions of coronary arteries in the first forty years of life. Eur Heart J 11 Suppl E:3-19.

Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W, Jr., Rosenfeld ME, Schwartz CJ, Wagner WD, Wissler RW (1995) A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Arterioscler Thromb Vasc Biol 15:1512-1531.

Stary HC, Chandler AB, Glagov S, Guyton JR, Insull W, Jr., Rosenfeld ME, Schaffer SA, Schwartz CJ, Wagner WD, Wissler RW (1994) A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation 89:2462-2478.

Stenina OI, Plow EF (2008) Counterbalancing forces: what is thrombospondin-1 doing in atherosclerotic lesions? Circ Res 103:1053-1055.

Stocker R, Keaney JF, Jr. (2004) Role of oxidative modifications in atherosclerosis. Physiol Rev 84:1381-1478.

Stone AL, Kroeger M, Sang QX (1999) Structure-function analysis of the ADAM family of disintegrin-like and metalloproteinase-containing proteins (review). J Protein Chem 18:447-465.

Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 102:15545-15550.

Sugiyama S, Okada Y, Sukhova GK, Virmani R, Heinecke JW, Libby P (2001) Macrophage myeloperoxidase regulation by granulocyte macrophage colony-stimulating factor in human atherosclerosis and implications in acute coronary syndromes. Am J Pathol 158:879-891.

Tabas I (2000) Cholesterol and phospholipid metabolism in macrophages. Biochim Biophys Acta 1529:164-174.

Takahashi H, Isobe T, Horibe S, Takagi J, Yokosaki Y, Sheppard D, Saito Y (2000) Tissue transglutaminase, coagulation factor XIII, and the pro-polypeptide of von Willebrand factor are all ligands for the integrins alpha 9beta 1 and alpha 4beta 1. J Biol Chem 275:23589-23595.

Takahashi K, Takeya M, Sakashita N (2002) Multifunctional roles of macrophages in the development and progression of atherosclerosis in humans and experimental animals. Med Electron Microsc 35:179-203.

Taleb S, Tedgui A, Mallat Z (2008) Regulatory T-cell immunity and its relevance to atherosclerosis. J Intern Med 263:489-499.

Tanaka Y, Miyamoto S, Suzuki SO, Oki E, Yagi H, Sonoda K, Yamazaki A, Mizushima H, Maehara Y, Mekada E, Nakano H (2005) Clinical significance of heparin-binding epidermal growth factor-like growth factor and a disintegrin and metalloprotease 17 expression in human ovarian cancer. Clin Cancer Res 11:4783-4792.

Tannapfel A, Anhalt K, Hausermann P, Sommerer F, Benicke M, Uhlmann D, Witzigmann H, Hauss J, Wittekind C (2003) Identification of novel proteins

associated with hepatocellular carcinomas using protein microarrays. J Pathol 201:238-249.

Tellier E, Canault M, Poggi M, Bonardo B, Nicolay A, Alessi MC, Nalbone G, Peiretti F (2008) HDLs activate ADAM17-dependent shedding. J Cell Physiol 214:687-693.

Tiruppathi C, Yan W, Sandoval R, Naqvi T, Pronin AN, Benovic JL, Malik AB (2000) G protein-coupled receptor kinase-5 regulates thrombin-activated signaling in endothelial cells. Proc Natl Acad Sci U S A 97:7440-7445.

Trogan E, Feig JE, Dogan S, Rothblat GH, Angeli V, Tacke F, Randolph GJ, Fisher EA (2006) Gene expression changes in foam cells and the role of chemokine receptor CCR7 during atherosclerosis regression in ApoE-deficient mice. Proc Natl Acad Sci U S A 103:3781-3786.

Tsuji S, Uehori J, Matsumoto M, Suzuki Y, Matsuhisa A, Toyoshima K, Seya T (2001) Human intelectin is a novel soluble lectin that recognizes galactofuranose in carbohydrate chains of bacterial cell wall. J Biol Chem 276:23456-23463.

Tuomisto TT, Binder BR, Yla-Herttuala S (2005) Genetics, genomics and proteomics in atherosclerosis research. Ann Med 37:323-332.

Tuomisto TT, Korkeela A, Rutanen J, Viita H, Brasen JH, Riekkinen MS, Rissanen TT, Karkola K, Kiraly Z, Kolble K, Yla-Herttuala S (2003) Gene expression in macrophage-rich inflammatory cell infiltrates in human atherosclerotic lesions as studied by laser microdissection and DNA array: overexpression of HMG-CoA reductase, colony stimulating factor receptors, CD11A/CD18 integrins, and interleukin receptors. Arterioscler Thromb Vasc Biol 23:2235-2240.

Tyson KL, Weissberg PL, Shanahan CM (2002) Heterogeneity of gene expression in human atheroma unmasked using cDNA representational difference analysis. Physiol Genomics 9:121-130.

Valkovskaya N, Kayed H, Felix K, Hartmann D, Giese NA, Osinsky SP, Friess H, Kleeff J (2007) ADAM8 expression is associated with increased invasiveness and reduced patient survival in pancreatic cancer. J Cell Mol Med 11:1162-1174.

Valle HA, Riesgo LG, Bel MS, Gonzalo FE, Sanchez MS, Oliva LI (2008) Clinical assessment of heart-type fatty acid binding protein in early diagnosis of acute coronary syndrome. Eur J Emerg Med 15:140-144.

van der Wal AC, Becker AE, van der Loos CM, Das PK (1994) Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. Circulation 89:36-44.

Van Wart HE, Birkedal-Hansen H (1990) The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. Proc Natl Acad Sci U S A 87:5578-5582.

Virchow R (1989) Cellular pathology. As based upon physiological and pathological histology. Lecture XVI--Atheromatous affection of arteries. 1858. Nutr Rev 47:23-25.

Walcheck B, Kahn J, Fisher JM, Wang BB, Fisk RS, Payan DG, Feehan C, Betageri R, Darlak K, Spatola AF, Kishimoto TK (1996) Neutrophil rolling altered by inhibition of L-selectin shedding in vitro. Nature 380:720-723.

Watson AD, Berliner JA, Hama SY, La Du BN, Faull KF, Fogelman AM, Navab M (1995) Protective effect of high density lipoprotein associated paraoxonase. Inhibition of the biological activity of minimally oxidized low density lipoprotein. J Clin Invest 96:2882-2891.

Weman SM, Salminen US, Penttila A, Mannikko A, Karhunen PJ (1999) Post-mortem cast angiography in the diagnostics of graft complications in patients with fatal outcome following coronary artery bypass grafting (CABG). Int J Legal Med 112:107-114.

Weskamp G, Kratzschmar J, Reid MS, Blobel CP (1996) MDC9, a widely expressed cellular disintegrin containing cytoplasmic SH3 ligand domains. J Cell Biol 132:717-726.

White JM (2003) ADAMs: modulators of cell-cell and cell-matrix interactions. Curr Opin Cell Biol 15:598-606.

Wight TN (1995) The extracellular matrix and atherosclerosis. Curr Opin Lipidol 6:326-334.

Wildeboer D, Naus S, Amy Sang QX, Bartsch JW, Pagenstecher A (2006) Metalloproteinase disintegrins ADAM8 and ADAM19 are highly regulated in human primary brain tumors and their expression levels and activities are associated with invasiveness. J Neuropathol Exp Neurol 65:516-527.

Williams KJ, Tabas I (1995) The response-to-retention hypothesis of early atherogenesis. Arterioscler Thromb Vasc Biol 15:551-561.

Williams KJ, Tabas I (1998) The response-to-retention hypothesis of atherogenesis reinforced. Curr Opin Lipidol 9:471-474.

Willnow TE, Armstrong SA, Hammer RE, Herz J (1995) Functional expression of low density lipoprotein receptor-related protein is controlled by receptor-associated protein in vivo. Proc Natl Acad Sci U S A 92:4537-4541.

Wolfsberg TG, Straight PD, Gerena RL, Huovila AP, Primakoff P, Myles DG, White JM (1995) ADAM, a widely distributed and developmentally regulated gene family encoding membrane proteins with a disintegrin and metalloprotease domain. Dev Biol 169:378-383.

Woodside KJ, Hernandez A, Smith FW, Xue XY, Hu M, Daller JA, Hunter GC (2003) Differential gene expression in primary and recurrent carotid stenosis. Biochem Biophys Res Commun 302:509-514.

Xu XX, Tabas I (1991) Sphingomyelinase enhances low density lipoprotein uptake and ability to induce cholesteryl ester accumulation in macrophages. J Biol Chem 266:24849-24858.

Xu Y, Song G (2004) The role of CD40-CD154 interaction in cell immunoregulation. J Biomed Sci 11:426-438.

Yamamoto S, Higuchi Y, Yoshiyama K, Shimizu E, Kataoka M, Hijiya N, Matsuura K (1999) ADAM family proteins in the immune system. Immunol Today 20:278-284.

Yla-Herttuala S, Lipton BA, Rosenfeld ME, Sarkioja T, Yoshimura T, Leonard EJ, Witztum JL, Steinberg D (1991) Expression of monocyte chemoattractant protein 1 in macrophage-rich areas of human and rabbit atherosclerotic lesions. Proc Natl Acad Sci U S A 88:5252-5256.

Yoshida S, Setoguchi M, Higuchi Y, Akizuki S, Yamamoto S (1990) Molecular cloning of cDNA encoding MS2 antigen, a novel cell surface antigen strongly expressed in murine monocytic lineage. Int Immunol 2:585-591.

Yoshiyama K, Higuchi Y, Kataoka M, Matsuura K, Yamamoto S (1997) CD156 (human ADAM8): expression, primary amino acid sequence, and gene location. Genomics 41:56-62.

Zand T, Hoffman AH, Savilonis BJ, Underwood JM, Nunnari JJ, Majno G, Joris I (1999) Lipid deposition in rat aortas with intraluminal hemispherical plug stenosis. A morphological and biophysical study. Am J Pathol 155:85-92.

Zhang H, Park Y, Wu J, Chen X, Lee S, Yang J, Dellsperger KC, Zhang C (2009) Role of TNF-alpha in vascular dysfunction. Clin Sci (Lond) 116:219-230.

Zhang H, Sunnarborg SW, McNaughton KK, Johns TG, Lee DC, Faber JE (2008) Heparin-binding epidermal growth factor-like growth factor signaling in flow-induced arterial remodeling. Circ Res 102:1275-1285.

Zhao J, Chen H, Peschon JJ, Shi W, Zhang Y, Frank SJ, Warburton D (2001) Pulmonary hypoplasia in mice lacking tumor necrosis factor-alpha converting enzyme indicates an indispensable role for cell surface protein shedding during embryonic lung branching morphogenesis. Dev Biol 232:204-218.

Zheng Y, Schlondorff J, Blobel CP (2002) Evidence for regulation of the tumor necrosis factor alpha-convertase (TACE) by protein-tyrosine phosphatase PTPH1. J Biol Chem 277:42463-42470.

Zhou M, Graham R, Russell G, Croucher PI (2001) MDC-9 (ADAM-9/Meltrin gamma) functions as an adhesion molecule by binding the alpha(v)beta(5) integrin. Biochem Biophys Res Commun 280:574-580.

Zhu J, Nieto FJ, Horne BD, Anderson JL, Muhlestein JB, Epstein SE (2001) Prospective study of pathogen burden and risk of myocardial infarction or death. Circulation 103:45-51.

Zhu X, Evans JP (2002) Analysis of the roles of RGD-binding integrins, alpha(4)/alpha(9) integrins, alpha(6) integrins, and CD9 in the interaction of the fertilin beta (ADAM2) disintegrin domain with the mouse egg membrane. Biol Reprod 66:1193-1202.

Zigrino P, Mauch C, Fox JW, Nischt R (2005) Adam-9 expression and regulation in human skin melanoma and melanoma cell lines. Int J Cancer 116:853-859.

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ORIGINAL ARTICLE

Effects of oxidized low- and high-density lipoproteins on gene expression of human macrophages

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Abstract

Objective. Oxidized low-density lipoprotein (ox-LDL) is a major factor in foam cell formation, whereas the role of oxidized high-density lipoprotein (ox-HDL) in this process is not known. The objective of the present study was to examine the effects of ox-LDL and ox-HDL on the gene expression of cultured human macrophages. ***Material and methods.*** Gene expression of human macrophages was studied after incubation for 1 day and 3 days with native and oxidized LDL and HDL using cDNA expression array. Expression of granulocyte-macrophage colony-stimulating factor 1, which was constantly up-regulated by ox-LDL and down-regulated by ox-HDL after 1- and 3 days of incubation in cDNA microarray experiments, was verified by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). ***Results.*** Genes that showed altered expression were divided into six groups; 1) lipid metabolism, 2) inflammation, growth and hemostasis, 3) matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases, 4) enzymes, 5) structural and binding proteins and 6) annexins. ***Conclusions.*** The microarray method was found to be applicable in analyzing changes in gene expression induced by oxidized lipoproteins in cultured human macrophages. Our results reflect different functional roles of ox-LDL and ox-HDL in foam cell formation.

Key Words: *Atherosclerosis, foam cell, microarray, ox-HDL, ox-LDL*

Introduction

Monocyte-derived macrophages play a crucial role in the pathogenesis of atherosclerosis. Macrophages are present in the initial events of atherosclerosis as well as in more advanced

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stages of the disease. The early lesions of atherosclerosis (fatty streaks) consist of cholesterol-engorged macrophages, i.e. foam cells and T lymphocytes [1]. It is suggested that macrophages internalize oxidized low-density lipoprotein (ox-LDL) via scavenger receptors [2] that function in the uptake of various harmful agents, such as oxidized lipids. This kind of process is not controlled by the normal negative feedback regulation of cholesterol metabolism. Therefore, macrophages are thought to accumulate cholesterol until they become full of cholesterol and cholesterol esters and eventually turn into foam cells [3]. Macrophages have also been found to internalize lipoprotein aggregates by a mechanism that resembles phagocytosis [4]. Although cellular and molecular interactions between monocyte-macrophages and low-density lipoprotein (LDL) play a key role in atherogenesis, overall the complexity of the pathogenesis also involves several other additional risk factors that interact with several types of artery wall cell lines such as smooth muscle cells, T-cell lymphocytes, mast cells and endothelial cells [5,6]. The major coronary risk factors, which can also cause endothelial dysfunction, include elevated and modified LDL, free radicals caused by cigarette smoking, hypertension, elevated plasma homocysteine concentrations, several infectious microorganisms, diabetes mellitus, genetic alterations and combinations of these or other factors [5,6].

LDL is retained in the artery wall by the interaction of its apolipoprotein B-100 with the extracellular matrix [7], and becomes vulnerable to oxidative modifications. In the intima, several factors are capable of oxidizing LDL, e.g. tyrosyl radical, hypochlorite, peroxynitrite and lipoxygenase [8]. In addition, the less effective antioxidant capacity in the intima compared to plasma [9] is thought to contribute to the oxidation of LDL.

Modified lipoproteins have been suggested to modulate gene expression of vascular cells. Ox-LDL has been shown to contribute to the progression of atherosclerosis by attracting more monocytes to enter the intima. Ox-LDL induces monocyte migration and differentiation by stimulating the release of monocyte chemotactic protein (MCP1) [10] and granulocyte-macrophage colony stimulating factor 1 (G-MCF1) [11] in vascular cells. Once the macrophages become activated by cholesterol loading, they enhance the progression of atherosclerosis by secreting several cytokines [12] and thus affect the gene expression profile of other cells involved in atherosclerotic plaque formation.

In contrast to LDL, high-density lipoprotein (HDL) has several beneficial protective properties [13]. HDL functions as a reverse cholesterol transporter [14] and has been proposed to inhibit LDL oxidation [15,16]. HDL has also been found to suppress ox-LDL-induced adhesion of monocyte-like cells and may thus inhibit the uptake of modified LDL [17]. On the other hand, HDL has been found to be even more susceptible to oxidation than LDL. Nishigaki et al. first suggested the *in vivo* oxidation of HDL [18] and ox-HDL has been found to have properties resembling those of ox-LDL. Nagano et al. reported that oxidatively modified HDL had a diminished cholesterol efflux capacity from macrophage-derived foam cells *in vitro* compared with native HDL [19]. It has been claimed that ox-HDL is cytotoxic to endothelial cells [20] and causes aggregation of apolipoproteins [21].

Oxidized LDL is a major factor in macrophage-derived foam cell formation but the role of ox-HDL in this process is not fully understood. Therefore, the objective of the present study was to examine and compare the effects of oxidized LDL and HDL on changes in gene expression in human macrophages. Macrophages obtained from healthy blood donors were treated with native and oxidized LDL and HDL for 1 and 3 days and altered gene expression was detected using the cDNA microarray method. Gene expression changes induced by ox-LDL have previously been investigated using THP-1 cells [22,23] but this is the first study to investigate changes in gene expression induced by ox-LDL and ox-HDL in healthy human macrophages.

Material and methods

Cell culture

Peripheral mononuclear cells were isolated from leukocyte-rich buffy coats obtained from healthy blood donors from the Finnish Red Cross by Ficoll-Paque (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, England) density-gradient centrifugation. Mononuclear cells adhering to dishes (Corning, New York, USA) were cultured in an *X-vivo* serum-free medium (BioWhittaker, Rockland, Calif., USA) containing 100 μg/mL penicillin-streptomycin (BioWhittaker). After differentiation into a macrophage-like phenotype, incubations with native and oxidized LDL and HDL were done in the serum-free medium for 1 and 3 days. Lipoproteins were isolated by sequential ultracentrifugation as previously described [24]. After isolation, lipoproteins were diluted to 1 mg/mL with phosphate-buffered saline (PBS) and oxidation was done by adding $CuSO_4$ to a final concentration of 20 μmol/L and by incubating for 24 h at +37°C [25]. Oxidized lipoproteins were given to the cells in a concentration of 50 μg/mL. Agarose gel electrophoresis (Paragon Lipoprotein (Lipo) Electrophoresis Kit, Beckman Coulter, Fullerton, Calif., USA) was run and absorbance of conjugated dienes was measured at 234 nm (Perkin Elmer, Boston, Mass., USA) to confirm that lipoproteins were ''fully oxidized''. The cells were harvested for total RNA isolation after 1 and 3 days of incubation.

RNA isolation and cDNA expression array

Gene expression profiling was performed using the BD Atlas Human Cardiovascular Array (BD Biosciences Clontech, Palo Alto, Calif., USA) which included 588 genes potentially involved in cardiovascular diseases. The list of all the genes on the microarray is shown in the Internet (http://www.bdbiosciences.com/clontech/atlas/genelists/7734-1_HuCardio.pdf). Total RNA was extracted using the RNeasy® Mini Kit (Qiagen, Valencia, Calif., USA) following the manufacturer's instructions. After extraction, 5 μg total RNA was transcribed, labelled with [α^{33}P] dATP (Amersham Pharmacia Biotech) and hybridized to microarray according to the manufacturer's instructions. Phosphoimaging screens were exposed for 10 days and the results scanned with the Storm Phosphoimager (Molecular® Dynamics, Sunnyvale, Calif., USA).

Normalization of the microarray results and statistical analysis

Raw signal intensities were normalized with ribosomal protein S9 (RPS9) using Atlas Image 2.01 software (BD Biosciences). RPS9 was selected as a housekeeping gene since it showed a constant expression in all microarray experiments. The chi-square test was used to compare the differences in gene expression profiles (number of up- and down-regulated genes) between ox-LDL and ox-HDL experiments (version 8.0; STATA Corporation, Tex., USA). A p-value of less than 0.05 was considered significant.

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

The expression of G-MCF1, which was constantly up-regulated by ox-LDL and down-regulated by ox-HDL after 1 and 3 days of incubation in cDNA microarray experiments, was verified by quantitative RT-PCR (QRT-PCR) using the LightCycler (Roche Diagnostics, Mannheim, Germany). RPS9 showed stabile expression between compared

samples in QRT-PCR experiments and was thus selected to a housekeeping gene. The primer sequences for G-MCF1 were from BD Biosciences and the primers from the Centre of Biotechnology (Helsinki, Finland). The primers for RPS9 were obtained from TIB MolBiol (Berlin, Germany). Total RNA (1 μg) was synthesized to cDNA with random primers using the First-Strand cDNA Synthesis Kit for RT-PCR (AMW) (Roche Diagnostics) following the manufacturer's instructions. PCR analyses were performed in a total volume of 20 μL using the LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics). The PCR reaction mixture included 0.5 μM primers, 4 mM $MgCl_2$ for G-MCF1 and 3 mM for RPS9, 2 μL LightCycler – FastStart Reaction Mix SYBR Green I and 2 μL of 1:10 diluted cDNA. Primer sequences, product lengths and cycling conditions are presented in Table I. Each sample was analyzed in duplicate. To verify the amplification specificity, melting curves were run at denaturing +95°C for 0 s, annealing at +55°C (G-MCF1) or +60°C (RPS9) for 30 s followed by denaturing of the samples to + 95°C at a ramping rate of 0.1°C s^{-1}. PCR products were further verified by running 1 % agarose gel electrophoresis. QRT-PCR analyses were done using LightCycler Relative Quantification Software with efficiency correction (Roche Diagnostics).

Results

Microarray

RNA extracted from cells that were incubated with native time-matched LDL and HDL was used as the control in microarray and QRT-PCR analyses. The genes that showed at least a 2-fold up- or down-regulation according to the ratio of time-matched oxidized lipoprotein to native lipoprotein-treated cells (e.g. ratio of oxidized to native LDL or HDL for 1 or alternatively for 3 days) were selected for statistical analysis. The number and the distribution of genes fulfilling this criterion are presented in Table II.

Ox-LDL and ox-HDL treatments of monocyte-macrophages for one day caused an up-regulation of 34 vs. 13 genes ($p=0.0018$) and for three days 52 vs. 99 genes ($p<0.0001$), respectively. For 1-day incubation the number of down-regulated genes was 1 versus 57 ($p<0.0001$) and for 3-day incubation the number was 7 versus 14 genes ($p=0.12$). Ox-LDL and ox-HDL 1-day incubations differed significantly for up- ($p=0.0295$) and

Table I. Primer sequences and cycling conditions in quantitative RT-PCR.

Primer sequences and product lengths	G-MCF1	RPS9
Forward and reverse primers	fw 5′ CggAgTACTgTAgCCACATgATTgg 3′ rev 5′ gATTggCggTgTTATCTCTgAAgCg 3′	fw 5′ TCCTgggCCTgAAgATAgAg 3′ rev 5′ gACAATgAAggACgggATgT 3′
Product length	210 bp	158 bp
Cycling conditions		
Initial denaturation	+95°C for 10 min	+ 95°C for 10 min
Amplification (50 cycles)		
Denaturation	+95°C for 1 s	+ 95°C for 1 s
Annealing	+50°C for 5 s	+ 55°C for 5 s
Elongation	+72°C for 17 s	+ 72°C for 12 s

*Abbreviations: G-MCF1=granulocyte-macrophage colony stimulating factor 1; RPS9=ribosomal protein S9; RT-PCR=reverse transcriptase-polymerase chain reaction.

Table II. Comparison of the effects of oxidized low- and high-density lipoproteins on the gene expression profile (up- and down-regulation) of cultured human macrophages measured by cardiovascular cDNA expression array. Total number of genes in the array for each functional group is shown in parentheses.

	Oxidized LDL		Oxidized HDL		*p*-value**	
Functional group	*Up %	*Down %	*Up %	*Down %	*Up	*Down
Lipid metabolism						
1-day	6 (5/89)	0 (0/89)	0 (0/89)	11.2 (10/89)	0.0295	0.0007
3-day	10.1 (9/89)	2.2 (2/89)	19.1 (17/89)	2.2 (2/89)	0.0895	1.0000
Growth and chemotaxis						
1-day	0 (0/46)	0 (0/46)	2.2 (1/46)	8.7 (4/46)	0.500	0.0584
3-day	6.5 (3/46)	2.2 (1/46)	10.9 (5/46)	6.5 (3/46)	0.3570	0.3083
Inflammation and coagulation						
1-day	15.6 (5/32)	0 (0/32)	9.4 (3/32)	6.3 (2/32)	0.3539	0.2460
3-day	25.0 (8/32)	3.1 (1/32)	46.9 (15/32)	0 (0/32)	0.0682	0.5000
Others						
1-day	5.7 (24/421)	0 (0/421)	2.1 (9/421)	9.7 (41/421)	0.008	0.0000
3-day	7.6 (32/421)	0.7 (3/421)	14.7 (62/421)	2.1 (9/421)	0.0010	0.0716
Total (all genes)						
1-day	5.8 (34/588)	0.2 (1/588)	2.2 (13/588)	9.7 (57/588)	0.0018	0.0000
3-day	8.8 (52/588)	1.2 (7/588)	16.8 (99/588)	2.4 (14/588)	0.0000	0.1232

Abbreviations: LDL=low-density lipoprotein; HDL=high-density lipoprotein. *At least 2-fold up- or down-regulation in cDNA microarray experiments as determined by the ratio of time-matched oxidized to native lipoprotein-treated cells; **chi-square test between oxidized LDL and HDL treated cells.

down- ($p=0.0007$) regulated genes in the group comprising lipid metabolism genes (Table II). For other non-classified genes this difference was also seen in the 1-day incubation, where ox-LDL seemed mainly to up-regulate genes and ox-HDL to down-regulate them. No such difference between ox-LDL and ox-HDL was seen during the 3-day incubations.

The most constantly expressed genes that showed altered expression in at least two of the four different lipoprotein treatments (ox-LDL 1-d, ox-LDL 3-d, ox-HDL 1-d, ox-HDL 3-d) were divided into groups based on their biological function (Table III). Groups were selected as genes involved in 1) lipid metabolism, 2) inflammation, growth and hemostasis, 3) matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases, 4) enzymes, 5) structural and binding proteins and 6) annexins. The genes were further classified according to the effects induced by ox-LDL and ox-HDL; opposite effects (A), parallel (B), only ox-LDL (C) or ox-HDL (D) induced change in expression. Some of the genes have previously been linked to atherosclerosis but, in addition, several new candidate genes, not previously shown to have altered expression in response to lipoprotein loading in macrophages, are presented.

QRT-PCR

Ox-LDL and ox-HDL incubations induced opposite effects on the expression of certain genes. Since we were interested in this phenomenon, we sought to verify the expression of G-MCF1 that was constantly up-regulated by ox-LDL and down-regulated by ox-HDL in both 1- and 3-day incubations in microarray experiments. Since RPS9 was found to have stabile expression in the microarray as well as in QRT-PCR experiments, it was used as a

Table III. Comparison of the effects of oxidized low and high density lipoproteins (LDL and HDL) on the gene expression of human cultured macrophages measured by BD Atlas™ Human Cardiovascular Array. The gene expression is shown as ratio of time-matched oxidized lipoprotein / native lipoprotein. Genes are divided into functional groups and organized into four different classes depending on the change of expression induced by oxidized LDL and HDL; A) Opposite, B) parallel, C) oxidized LDL or D) oxidized HDL alone caused a change. Minus (−) indicates down-regulation.

		Oxidized LDL		Oxidized HDL		
Functional group	Class	1 day	3 day	1 day	3 day	Gene Bank accession
Lipid metabolism (n=12)						
Apolipoprotein E precursor (APOE)	A	2.5	–	−3.1	–	M12529
Fatty acid-binding protein 3 (FABP3)	A	2.9	2.1	−4.3	−2.3	Y10255
Lysosomal acid lipase (LAL)	A	3.9	6.0	−4.4	–	M74775
LDL receptor-related protein-associated protein 1 (LRPAP1)	A	–	−6.3	–	44.1	M63959
Macrosialin precursor (CD68)	A	2.7	2.0	−2.4	–	S57235
Cholesterol acyltransferase (ACAT)	A	–	5.7	−3.2	–	L21934
Low-density lipoprotein receptor-related protein 1 (LRP1)	B	–	4.3	–	224	X13916
High-density lipoprotein-binding protein (HBP)	B	6.2	4.0	–	9.6	M83789
Apolipoprotein A precursor (APOA)	B	–	3.0	–	46.8	X06290
Macrophage scavenger receptor I (MSR1)	B	−4.5	3.4	–	3.0	D90187
Very-long-chain-specific acyl-CoA dehydrogenase precursor (VLCAD)	D	–	–	−14.3	−5.2	D43682
Phospholipase C, beta 2 (PLCB2)	D	–	–	−8.6	−4.0	M95678
Inflammation, growth and hemostasis (n=23)						
Macrophage colony stimulating factor 1 (MCSF1)	A	10.4	9.0	−5.2	−19.0	M37435
Monocyte chemotactic protein 1 (MCP1)	A	–	4.0	−5.6	−5.1	M24545
Retinoid acid receptor alpha (RAR)	A	–	2.3	−3.0	–	X06614
HLA class I histocompatibility antigen C-4 alpha subunit (HLAC)	A	2.0	−2.6	–	2.8	M11886
CD9 antigen (CD9)	A	2.7	3.4	−2.3	–	M38690
Intercellular adhesion molecule-1 precursor, CD54 antigen (ICAM1)	A	–	2.3	−4.4	–	J03132
Profilin 1 (PFN1)	A	3.2	3.1	−2.9	–	J03191
Calcium and integrin binding protein (CIB)	A	–	2.9	−3.1	–	U85611
Thrombospondin 2 (THBS2)	B	30.3	13.9	244.6	449.6	L12350
Fibrinogen B beta polypeptide (FGB)	B	115.3	66.9	2.2	9.4	J00129
Platelet-activating factor acetylhydrolase IB gamma subunit (PAF-AH)	B	33.0	11.0	5.5	51.8	D63391
Tissue-type plasminogen activator (TPA)	B	19.7	–	10.1	15.4	M18182
Vascular endothelial cell growth factor 165 receptor 2 (VEGF165R2)	B	–	2.6	–	35.8	AF016098
Rantes pro-inflammatory cytokine (RANTES)	B	–	2.8	2.8	–	M21121
Leukocyte-endothelial cell adhesion molecule 1 (LECAM1)	B	18.4	–	–	3.4	M25280

Table III. Continued.

Functional group	Class	Oxidized LDL 1 day	Oxidized LDL 3 day	Oxidized HDL 1 day	Oxidized HDL 3 day	Gene Bank accession
Integrin alpha L, CD11a antigen (ITGAL)	B	8.1	4.8	4.9	–	Y00796
Tumor necrosis factor receptor superfamily, member 5 (TNFRSF5)	B	–	6.9	–	2.1	X60592
Selectin P ligand (PSGL1)	B	–	2.7	–	13.8	U02297
Tissue factor pathway inhibitor (TFPI1)	B	–	3.1	–	13.1	J03225
Prothrombin precursor (PT)	B	–	3.3	–	6.1	V00595
Integrin beta 2, LFA-1, CD18, (ITGB2)	C	3.4	2.9	–	–	M15395
Platelet-derived growth factor A (PDGFA)	D	–	–	–15.4	3.1	X06374
Small inducible cytokine A1 (SCYA1)	D	–	–	−3.8	−8.9	M57502
Matrix metalloproteinases and inhibitors (n=5)						
Matrix metalloproteinase 9 (MMP9)	A	4.3	4.8	−3.6	–	J05070
Tissue inhibitor of metalloproteinase 1 precursor (TIMP1)	A	3.2	2.0	−2.2	–	X03124
Tissue inhibitor of metalloproteinase 2 precursor (TIMP2)	A	–	2.6	−3.3	–	J05593
Tissue inhibitor of metalloproteinase 3 (TIMP3)	B	20.5	10.0	12.6	77.0	U14394
Matrix metalloproteinase 12 (MMP12)	D	–	–	−16.3	−5.2	L23808
Enzymes (n=16)						
Superoxide dismutase 2 (SOD 2)	A	–	3.5	−2.3	–	M36693
Natriuretic peptide precursor B (NPPB)	A	3.2	2.1	−5.3	−2.2	M25296
Glucosamine-6 sulfatase (G6S)	A	2.5	3.5	−3.0	–	M23657
Angiotensin I converting enzyme (ACE)	A	4.0	2.6	−15.7	–	X14329
Protein disulfide isomerase precursor (PDI)	A	3.1	2.9	−2.9	–	X05130
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	A	2.1	–	−8.0	–	X01677
NADPH-cytochrome P450 reductase (P450)	A	–	3.2	−2.2		S90469
Cytochrome P450 IB1 hydroxylase (CYPB1)	A	–	2.9	−2.5	–	U03688
Protein disulfide isomerase (PDI)	A	3.1	2.9	−2.9	–	X05130
NADH-cytochrome B5 reductase (NADH)	A	–	2.6	−2.5	–	Y09501
Cytochrome P450 IA1 (CYP1A1)	B	24.5	–	23.0	311	M38504
Endothelin-converting enzyme 1	D	–	–	−9.6	−2.0	Z35307
Cytochrome P450 VIIA1 (CYP7A1)	D	–	–	52.0	182.0	X56088
Protein kinase, AMP-activated (AMPK)	D	–	–	−10.4	−2.6	U42412
Farnesyl diphosphate synthase (FDPS)	D	–	–	4.6	12.4	J05262
Oxoacyl-CoA thiolase (PTHIO)	D	–	–	2.7	6.2	AF035295
Structural and binding proteins (n=8)						
Endothelial actin-binding protein, nonmuscle filamin 1 (ABP-280)	A	6.3	5.4	−4.8	−2.4	X53416
Actin, beta (ACTB)	A	2.8	2.9	−2.5	–	X00351

Table III. Continued.

Functional group	Class	Oxidized LDL 1 day	Oxidized LDL 3 day	Oxidized HDL 1 day	Oxidized HDL 3 day	Gene Bank accession
Caveolin 3 (CAV-3)	B	3.7	4.0	2.0	9.9	AF043101
Amyloid-like protein 2 (APPH)	C	5.5	3.5	–	–	S60099
Vimentin (VIM)	C	2.7	2.0	–	–	M14144
Villin 2 (VIL2)	D	–	–	12.8	324.0	X51521
Junction plakoglobin (JUP)	D	–	–	4.0	20.5	Z68228
Lectin, galactoside binding (LGALS1)	D	–	–	3.6	2.6	J04456
Annexins (n=5)						
Annexin V (ANX5)	A	2.2	3.4	−3.8	–	X12454
Annexin IV (ANX4)	A	2.1	–	−3.0	–	M19383
Annexin I (ANX1)	A	3.2	2.9	−4.0	–	X05908
Annexin III (ANX3)	B	80.2	–	11.8	116.0	J03899
Annexin VI (ANX6)	D	–	–	−8.2	−3.2	D00510

housekeeping gene. A similar expression pattern was verified for G-MCF1 with QRT-PCR to that found in the microarray experiments. Incubation of monocyte-derived macrophages with ox-LDL for 1 and 3 days caused a more than 2-fold up-regulation of the G-MCF1 gene in QRT-PCR analyses, while incubation with ox-HDL had a converse effect, leading to down-regulation for both time-points studied.

Discussion

Effects of oxidized LDL and HDL on the gene expression profile of human macrophages

Modified lipoproteins have been suggested to modulate gene expression in the vascular wall. Therefore, in the present work we studied the effects of ox-LDL and ox-HDL on the gene expression of cultured human macrophages using a cDNA microarray including 588 genes related to cardiovascular diseases. The overall gene expression profiles were significantly affected by both ox-LDL and ox-HDL treatments. Ox-LDL and ox-HDL induced similar as well as different, even contrasting, effects on the gene expression of cultured human macrophages. Changes in gene expression involved in atherosclerosis have also previously been investigated using THP-1 cells [22,23,26] and atherosclerotic arteries [27,28] but not by using healthy human macrophages which are treated with both ox-LDL and ox-HDL. Most of the interest in foam cell formation has concerned the role of ox-LDL. However, all lipoprotein density fractions of advanced atherosclerotic lesions have been found to be substantially oxidized [29] and ox-HDL has been detected in atherosclerotic plaques [30]. Oxidation of lipoproteins is known to lead to a number of changes in their composition and function [31].

New candidate genes for foam cell formation found with the cDNA microarray

We found a large number of differentially expressed genes fulfilling our criterion (Table III). The genes were organized into six different groups based on their biological functions; 1) lipid metabolism, 2) inflammation, growth and hemostasis, 3) matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases, 4) enzymes, 5) structural and binding proteins and 6) annexins. Four of the studied genes, i.e. fatty

acid-binding protein 3 (FABP3), G-MCSF1, natriuretic peptide precursor B (NPPB) and endothelial actin-binding protein (ABP-280), showed the most constantly converse expression profiles between ox-LDL and ox-HDL incubations. Owing to our limited time resources, we selected G-MCF1 from these genes for confirmation in QRT-PCR. The G-MCF1 gene was selected since ox-LDL has previously been shown to induce monocyte differentiation by stimulating the release of this protein [11]. Another reason for the selection of G-MCF1 for confirmation was that it was most constantly up-regulated by ox-LDL and down-regulated by ox-HDL in both the 1- and 3-day incubations in the microarray experiments. Thus, with regard to this gene, our cDNA microarray and QRT-PCR experiments suggest that the effect of ox-HDL is the reverse of that of ox-LDL. Ox-LDL and ox-HDL might have a different effect on macrophage gene expression because of their different composition or possibly they use different scavenger receptors to enter these cells.

Lipid metabolism. Disturbances in lipid metabolism are essential for foam cell formation. Our focus was on genes that have not been previously connected to foam cell formation. We found that the expression of FABP3 is induced in human macrophages in response to incubations with oxidized lipoproteins. Incubations with ox-LDL increased the expression of FABP3, whereas incubations with ox-HDL decreased it. Cytoplasmic fatty acid-binding proteins are a family of proteins that bind fatty acid ligands with high affinity. They function in the shuttling of fatty acids to specific enzymes and cellular compartments, modulation of intracellular lipid metabolism and regulation of gene expression [32]. Another protein involved in lipid metabolism is LDL receptor-associated protein 1 (LRPAP1), which is required for normal processing of LDL receptor [33]. It has been suggested that LRPAP1 prevents association of newly synthesized receptors with their ligands during transport to the cell surface [34]. We report that 3 days of incubation with ox-LDL decreased the expression of LRPAP1 6-fold. If ox-LDL decreases the expression of LRPAP1 also *in vivo*, the secretion of mature LRP to the cell surface might get blocked. In contrast to ox-LDL, ox-HDL significantly increased the expression of LRPAP1. The initial rate-limiting step in mitochondrial β-oxidation of fatty acids is catalyzed by very-long-chain acyl-CoA dehydrogenase (VLCAD) [35]. VLCAD has previously been linked to mitochondrial fatty-acyl-CoA beta-oxidation disorders [35]. We report that both 1- and 3-day incubations with ox-HDL clearly decreased the expression of VLCAD. In all, it is clear that oxidized lipoproteins have effects on genes involved in lipid metabolism that are different from the effects of native lipoproteins.

Inflammation, growth and hemostasis. Atherosclerosis is considered as an inflammatory disease and growth factors as well as cytokines play a crucial role in its progression. Small inducible cytokine A1 (SCYA1) has been reported to be expressed in T lymphocytes and activated monocytes [36]. SCYA1 is also widely distributed in human atherosclerotic plaque [37] and has been suggested to be a novel modulator of angiogenesis [38]. One- and 3-day incubations with ox-HDL decreased the expression of SCYA1. One-day incubation with ox-HDL down-regulated SCYA1 3.8-fold and at 3 days of incubation down-regulation was already 8.9-fold.

Vascular endothelial growth factor 165 receptor 2 (VEGF165R2), also called neuropilin 2, is a receptor for predominant vascular endothelial cell growth factor (VEGF) isoform VEGF(165). VEGF(165) is a protein [39] that is secreted by a variety of normal and transformed cells [40]. The expression of VEGF165R2 was increased in 3 days of

incubation with ox-LDL and ox-HDL, suggesting that VEGF165R2 might affect the development of foam cells and thus the formation of early atherosclerotic lesions. This is the first time that VEGF165R2 expression has been found to be altered in human monocyte-macrophages in response to cholesterol loading.

Numerous studies have shown an association between plasma fibrinogen and cardiovascular diseases [41–43]. Synthesis of the B beta chain is the rate-limiting step in the formation of fibrinogen. Previously, the hepatocyte and megakaryocyte have been considered the sites for fibrinogen B beta polypeptide (FGB) expression [44,45] but interestingly, Haidaris suggested that the expression of FGB could also be induced in extrahepatic tissues in response to inflammatory stimuli [46]. We are the first to report a significant up-regulation of FGB induced by both ox-LDL and ox-HDL incubation of human monocyte-macrophages. Another gene involved in coagulation was also strongly up-regulated by ox-LDL and ox-HDL; thrombospondin 2 (THBS-2). THBS-2 is thought to be an adaptor and modulator of cell-matrix interactions through interaction with cell-surface receptors, cytokines, growth factors, proteases and structural proteins [47]. THBS-2 has also been speculated to be a potential candidate for genetic and functional studies on atherothrombosis [48].

Conclusions

Gene expression in cultured monocyte-macrophages was studied in response to native and oxidized lipoproteins. Several genes were found to have altered expression, including genes that have previously been linked to atherosclerosis, whereas the role of others in the development of the disease is not known. We conclude that cDNA microarray experiments are applicable in measuring the effects on human monocyte-macrophages induced by oxidized lipoproteins and for finding new genes potentially involved in foam cell formation. Based on our results, ox-LDL and ox-HDL seemed to have different effects on the gene expression of macrophage-derived foam cells *in vitro*. If this is also the case *in vivo*, it would be interesting to evaluate the role of ox-HDL, in addition to ox-LDL, in foam cell and early atherosclerotic plaque formation.

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References

[1] Stary HC, Chandler AB, Glagov S, Guyton JR, Insull W Jr, Rosenfeld ME, et al. A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation 1994;89:2462–78.

[2] Kruth HS. Macrophage foam cells and atherosclerosis. Front Biosci 2001;6:D429–55.

[3] Goldstein JL, Ho YK, Basu SK, Brown MS. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. Proc Natl Acad Sci USA 1979;76:333–7.

[4] Zha X, Tabas I, Leopold PL, Jones NL, Maxfield FR. Evidence for prolonged cell-surface contact of acetyl-LDL before entry into macrophages. Arterioscler Thromb Vasc Biol 1997;17:1421–31.

[5] Ross R. Atherosclerosis: an inflammatory disease. N Engl J Med 1999;340:115–26.
[6] Lusis AJ. Atherosclerosis. Nature 2000;407:233–41.
[7] Camejo G, Hurt-Camejo E, Wiklund O, Bondjers G. Association of apo B lipoproteins with arterial proteoglycans: pathological significance and molecular basis. Atherosclerosis 1998;139:205–22.
[8] Heinecke JW. Oxidants and antioxidants in the pathogenesis of atherosclerosis: implications for the oxidized low density lipoprotein hypothesis. Atherosclerosis 1998;141:1–15.
[9] Frei B, Stocker R, Ames BN. Antioxidant defenses and lipid peroxidation in human blood plasma. Proc Natl Acad Sci USA 1988;85:9748–52.
[10] Ylä-Herttuala S, Lipton BA, Rosenfeld ME, Särkioja T, Yoshimura T, Leonard EJ, et al. Expression of monocyte chemoattractant protein 1 in macrophage-rich areas of human and rabbit atherosclerotic lesions. Proc Natl Acad Sci USA 1991;88:5252–6.
[11] Rajavashisth TB, Andalibi A, Territo MC, Berliner JA, Navab M, Fogelman AM, et al. Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low-density lipoproteins. Nature 1990;344:254–7.
[12] Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993;362:801–9.
[13] Francis GA. High density lipoprotein oxidation: in vitro susceptibility and potential in vivo consequences. Biochim Biophys Acta 2000;1483:217–35.
[14] Fielding CJ, Fielding PE. Molecular physiology of reverse cholesterol transport. J Lipid Res 1995;36:211–28.
[15] Salonen JT, Malin R, Tuomainen TP, Nyyssönen K, Lakka TA, Lehtimäki T. Polymorphism in high density lipoprotein paraoxonase gene and risk of acute myocardial infarction in men: prospective nested case-control study. Br Med J 1999;319:487–9; discussion 90.
[16] Malin R, Rantalaiho V, Huang XH, Wirta O, Pasternack A, Leinonen JS, et al. Association between M/L55-polymorphism of paraoxonase enzyme and oxidative DNA damage in patients with type 2 diabetes mellitus and in control subjects. Hum Genet 1999;105:179–80.
[17] Maier JA, Barenghi L, Pagani F, Bradamante S, Comi P, Ragnotti G. The protective role of high-density lipoprotein on oxidized-low-density-lipoprotein-induced U937/endothelial cell interactions. Eur J Biochem 1994;221:35–41.
[18] Nishigaki I, Hagihara M, Tsunekawa H, Maseki M, Yagi K. Lipid peroxide levels of serum lipoprotein fractions of diabetic patients. Biochem Med 1981;25:373–8.
[19] Nagano Y, Arai H, Kita T. High density lipoprotein loses its effect to stimulate efflux of cholesterol from foam cells after oxidative modification. Proc Natl Acad Sci USA 1991;88:6457–61.
[20] Hurtado I, Fiol C, Gracia V, Caldu P. In vitro oxidised HDL exerts a cytotoxic effect on macrophages. Atherosclerosis 1996;125:39–46.
[21] Greilberger J, Jurgens G. Oxidation of high-density lipoprotein HDL3 leads to exposure of apo-AI and apo-AII epitopes and to formation of aldehyde protein adducts, and influences binding of oxidized low-density lipoprotein to type I and type III collagen in vitro 1. Biochem J 1998;331:185–91.
[22] Mikita T, Porter G, Lawn RM, Shiffman D. Oxidized low density lipoprotein exposure alters the transcriptional response of macrophages to inflammatory stimulus. J Biol Chem 2001;276:45729–39.
[23] Llaverias G, Noe V, Penuelas S, Vazquez-Carrera M, Sanchez RM, Laguna JC, et al. Atorvastatin reduces CD68, FABP4, and HBP expression in oxLDL-treated human macrophages. Biochem Biophys Res Commun 2004;318:265–74.
[24] Jaakkola O, Ylä-Herttuala S, Särkioja T, Nikkari T. Macrophage foam cells from human aortic fatty streaks take up beta-VLDL and acetylated LDL in primary culture. Atherosclerosis 1989;79:173–82.
[25] Lehtimäki T, Lehtinen S, Solakivi T, Nikkilä M, Jaakkola O, Jokela H, et al. Autoantibodies against oxidized low density lipoprotein in patients with angiographically verified coronary artery disease. Arterioscler Thromb Vasc Biol 1999;19:23–7.
[26] Shiffman D, Mikita T, Tai JT, Wade DP, Porter JG, Seilhamer JJ, et al. Large scale gene expression analysis of cholesterol-loaded macrophages. J Biol Chem 2000;275:37324–32.
[27] Hiltunen MO, Tuomisto TT, Niemi M, Brasen JH, Rissanen TT, Toronen P, et al. Changes in gene expression in atherosclerotic plaques analyzed using DNA array. Atherosclerosis 2002;165:23–32.
[28] Armstrong PJ, Johanning JM, Calton WC Jr, Delatore JR, Franklin DP, Han DC, et al. Differential gene expression in human abdominal aorta: aneurysmal versus occlusive disease. J Vasc Surg 2002;35:346–55.
[29] Niu X, Zammit V, Upston JM, Dean RT, Stocker R. Coexistence of oxidized lipids and alpha-tocopherol in all lipoprotein density fractions isolated from advanced human atherosclerotic plaques. Arterioscler Thromb Vasc Biol 1999;19:1708–18.
[30] Nakajima T, Origuchi N, Matsunaga T, Kawai S, Hokari S, Nakamura H, et al. Localization of oxidized HDL in atheromatous plaques and oxidized HDL binding sites on human aortic endothelial cells. Ann Clin Biochem 2000;37:179–86.

[31] Stocker R. Lipoprotein oxidation: mechanistic aspects, methodological approaches and clinical relevance. Curr Opin Lipidol 1994;5:422–33.
[32] Coe NR, Bernlohr DA. Physiological properties and functions of intracellular fatty acid-binding proteins. Biochim Biophys Acta 1998;1391:287–306.
[33] Willnow TE, Armstrong SA, Hammer RE, Herz J. Functional expression of low density lipoprotein receptor-related protein is controlled by receptor-associated protein in vivo. Proc Natl Acad Sci USA 1995;92:4537–41.
[34] Willnow TE. Receptor-associated protein (RAP): a specialized chaperone for endocytic receptors. Biol Chem 1998;379:1025–31.
[35] Aoyama T, Uchida Y, Kelley RI, Marble M, Hofman K, Tonsgard JH, et al. A novel disease with deficiency of mitochondrial very-long-chain acyl-CoA dehydrogenase. Biochem Biophys Res Commun 1993;191:1369–72.
[36] Miller MD, Wilson SD, Dorf ME, Seuanez HN, O'Brien SJ, Krangel MS. Sequence and chromosomal location of the I-309 gene. Relationship to genes encoding a family of inflammatory cytokines. J Immunol 1990;145:2737–44.
[37] Haque NS, Zhang X, French DL, Li J, Poon M, Fallon JT, et al. CC chemokine I-309 is the principal monocyte chemoattractant induced by apolipoprotein(a) in human vascular endothelial cells. Circulation 2000;102:786–92.
[38] Bernardini G, Spinetti G, Ribatti D, Camarda G, Morbidelli L, Ziche M, et al. I-309 binds to and activates endothelial cell functions and acts as an angiogenic molecule in vivo. Blood 2000;96:4039–45.
[39] Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 1989;246:1306–9.
[40] Dvorak HF, Brown LF, Detmar M, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am J Pathol 1995;146:1029–39.
[41] Meade TW, Mellows S, Brozovic M, Miller GJ, Chakrabarti RR, North WR, et al. Haemostatic function and ischaemic heart disease: principal results of the Northwick Park Heart Study. Lancet 1986;2:533–7.
[42] Wilhelmsen L, Svardsudd K, Korsan-Bengtsen K, Larsson B, Welin L, Tibblin G. Fibrinogen as a risk factor for stroke and myocardial infarction. N Engl J Med 1984;311:501–5.
[43] Salonen JT, Seppänen K, Rauramaa R, Salonen R. Risk factors for carotid atherosclerosis: the Kuopio Ischaemic Heart Disease Risk Factor Study. Ann Med 1989;21:227–9.
[44] Belloc F, Hourdille P, Fialon P, Boisseau MR, Soria J. Fibrinogen synthesis by megakaryocyte-rich human marrow cell concentrates. Thromb Res 1985;38:341–51.
[45] Uzan G, Courtois G, Stanckovic Z, Crabtree GR, Marguerie G. Expression of the fibrinogen genes in rat megakaryocytes. Biochem Biophys Res Commun 1986;140:543–9.
[46] Haidaris PJ. Induction of fibrinogen biosynthesis and secretion from cultured pulmonary epithelial cells. Blood 1997;89:873–82.
[47] Bornstein P. Diversity of function is inherent in matricellular proteins: an appraisal of thrombospondin 1. J Cell Biol 1995;130:503–6.
[48] Hamsten A, Eriksson P. Thrombospondins and premature coronary artery disease: time to go beyond genotype-phenotype association studies. Arterioscler Thromb Vasc Biol 2003;23:6–7.

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ORIGINAL ARTICLE

ADAM8 and its single nucleotide polymorphism 2662 T/G are associated with advanced atherosclerosis and fatal myocardial infarction: Tampere vascular study

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Abstract

Objective. Previously, we scanned all 23,000 human genes for differential expression between normal and atherosclerotic tissues and found the involvement of ADAM8.

Methods. We investigated the expression of ADAM8 mRNA and protein level in human atherosclerotic tissues and non-atherosclerotic internal thoracic arteries as well as the association of ADAM8 2662 T/G single nucleotide polymorphism (SNP) with the extent of coronary atherosclerosis and with the risk of fatal myocardial infarction.

Results. ADAM8 mRNA was up-regulated in carotid, aortic, and femoral atherosclerotic plaques ($n=24$) when compared with non-atherosclerotic arteries. ADAM8 protein expression was increased in advanced atherosclerotic plaques as compared to control vessels wherein it was localized to macrophages and smooth muscle cells. The G allele carriers of the ADAM8 2662 T/G SNP had significantly larger areas of fibrotic, calcified, and complicated plaques in coronary arteries ($P=0.027$, $P=0.011$, and $P=0.011$, respectively) and significantly higher occurrence of myocardial infarction (MI) ($P=0.004$) and fatal pre-hospital MI ($P=0.003$) than did the TT homozygotes.

Conclusion. ADAM8 is a promising candidate to be involved in atherosclerosis, and its 2662 T/G allelic variant significantly associates with advanced atherosclerotic lesion areas and MI.

Key words: *ADAM8, atherosclerosis, gene expression, myocardial infarction*

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Key messages

- ADAM8 mRNA expression and protein level are increased in human atherosclerotic arteries as compared to healthy internal thoracic arteries.
- The G allele carriers of the ADAM8 2662 T/G single nuclotide polymorphism had significantly larger areas of fibrotic, calcified, and complicated plaques in their coronary arteries and significantly higher occurrence of myocardial infarction (MI) and fatal pre-hospital MI than did the TT homozygotes.
- The up-regulated expression at transcriptional and protein level as well as the association of the 2662 T/G single nuclotide polymorphism indicate the ADAM8 as a candidate to be involved in multiple mechanisms underlying atherosclerosis and MI.

Abbreviations

2662 T/G	the allelic variant of ADAM8 (rs2995300)
ADAM8	a disintegrin and metalloprotease
AMI	acute myocardial infarction
ASO	atherosclerosis obliterans
CAD	coronary artery disease
CD156	human leukocyte antigen (ADAM8)
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GWEA	genome-wide expression array
HSDS	Helsinki Sudden Death Study
IL1	interleukin 1
ILR1	interleukin receptor 1
ITA	internal thoracic artery
LAD	left anterior descending coronary artery
LCX	left circumflex coronary artery
LDA	low-density array
LDL	low density lipoprotein
MI	myocardial infarction
OCL	osteoclast
OR	odds ratio
PPARG	peroxisome proliferator-activated receptor γ
RCA	right coronary artery
RT-PCR	reverse transcription polymerase chain reaction
SCD	sudden cardiac death
SELL	L-selectin
VCAM-1	vascular cell adhesion molecule

Introduction

ADAMs (a disintegrin and metalloprotease) are a family of transmembrane proteins implicated in a variety of processes including cell adhesion and proteolytic release of cell surface molecules, a process called ectodomain shedding (1). ADAM8, also known as human leukocyte antigen CD156 (2), was initially identified in mouse macrophages and macrophage cell lines (3). It was later found to be expressed in human immune cells, with the exception of T cells (2). ADAM8 has an intact metalloprotease active site consensus sequence, and it is activated through autocatalytic removal of its inhibitory pro-domain (4). It has also been suggested to interact with cytosolic regulatory proteins through putative proline-rich consensus SH3 binding sequences (3). ADAM8 may participate in the degradation of the vascular basement membrane or in the liberation of physiologically active molecules from their precursors harbored on leukocyte or endothelial cell surfaces (5). These liberated molecules include L-selectin (SELL) and vascular cell adhesion molecule 1 (VCAM-1) that both mediate leukocyte trafficking to inflamed tissues. Intriguingly, also peroxisome proliferator-activated receptor γ (PPARG), an important regulator of immune response in atherosclerosis, is found to up-regulate ADAM8 expression. It is suggested that ADAM8 might also be able to shed interleukin receptor 1 (ILR1) (4) that mediates the proinflammatory effects of interleukin 1 (IL1) (6).

In order to identify novel candidate factors possibly associated with the pathogenesis of atherosclerosis, we previously screened all known 23,000 human genes for differential expression in atherosclerotic and healthy arteries with a genome-wide expression array (GWEA) (7). Of the $\sim$200 genes up-regulated in atherosclerotic tissue, we selected ADAM8 for further characterization. Since atherosclerosis is a complex inflammatory disease (8), ADAM8 is a particularly relevant subject for closer examination as a member of the ADAM metalloprotease integrins implicated in diverse inflammatory processes (9). Here we report the up-regulation of ADAM8 in advanced human atherosclerosis and the association of 2662 T/G (rs2995300) with lesion areas and myocardial infarction (MI). No previous information is available for the expression of ADAM8 in normal or atherosclerotic human arteries nor the association of 2662 T/G with atherosclerosis or myocardial infarction.

Material and methods

The Helsinki Sudden Death Study (HSDS)

The HSDS material comprises 700 Finnish men subjected to a medico-legal autopsy in the Helsinki

region due to an out-of-hospital death of a previously healthy man, accidental death, suspected intoxication, or suicide. The majority of the men studied had died due to cardiac causes (41%, $n=288$). Other causes of death were unnatural deaths as the result of accidents or suicides (39%, $n=272$) and other diseases (20%, $n=140$). The study was approved by the Ethics Committee of the Department of Forensic Medicine, University of Helsinki, and all the medico-legal autopsies were performed at the same department (10).

Vascular samples—Tampere vascular study

Tampere vascular study (TVS) material for genome-wide expression analysis consists of 4 femoral arteries, 9 carotid arteries, and 7 abdominal aortas together from 20 patients. All the patients had polyvascular disease. Six control samples were taken from internal thoracic arteries (ITA) from patients obligated to by-pass surgery due to coronary heart disease. The sample from internal thoracic artery was removed from the distal end of the artery at the beginning of dissection. From these patients, only two had polyvascular disease, and all the rest had monovascular disease. For the relative gene expression analysis, 24 atherosclerotic tissue samples were used, and similarly the 6 ITA vessels were used as controls. The study has been approved by the Ethics Committee of Tampere University Hospital. The samples were taken from patients subjected to open vascular surgical procedures at the Division of Vascular Surgery, Tampere University Hospital. All the patients gave informed consent. The vascular samples were classified according to American Heart Association (AHA) classification (11).

RNA isolation and genome-wide expression analysis

The fresh tissue samples were soaked in RNALater solution (Ambion Inc., Austin, TX, USA) and isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and the RNAEasy Kit (Qiagen, Valencia, CA, USA). The concentration and quality of the RNA was evaluated spectrophotometrically (BioPhotometer, Eppendorf, Wesseling-Berzdorf, Germany). More than 23,000 known and candidate genes were analyzed using Sentrix Human-8 Expression BeadChips, according to the manufacturer's instructions (Illumina, San Diego, CA, USA). In brief, a 200 ng aliquot of total RNA from each sample was amplified to cDNA using the Ambion's Illumina RNA Amplification kit according to the instructions (Ambion, Inc., Austin, TX, USA). Each sample cRNA (1500 ng) was hybridized to Illumina's Sentrix Human-8 Expression BeadChip arrays (Illumina). Hybridized biotinylated cRNA was detected with 1 μg/mL Cyanine3-streptavidine (Amersham Biosciences, Pistacataway, NJ, USA). BeadChips were scanned with the Illumina BeadArray Reader. The method has been described in more detail in our previous work (7).

Quantitative RT-PCR

Gene expression analyses were performed with TaqMan low-density arrays (LDAs) (Applied Biosystems, Foster City, CA, USA) using TaqMan Gene expression assay Hs00174246_m1 for ADAM8 detection. Total-RNA (500 ng) was transcribed to cDNA using the High Capacity cDNA Kit (Applied Biosystems) according to the manufacturer's instructions. After the cDNA synthesis, the LDA cards were loaded with 8 μL undiluted cDNA, 42 μL H_2O, and 50 μL PCR Universal Master Mix (Applied Biosystems) and run according to the manufacturer's instructions. Samples were analyzed as duplicates, and both cDNA synthesis and PCR reactions were validated for inhibition according to manufacturer's recommendations. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. The results were analyzed using SDS 2.2 Software (Applied Biosystems).

Immunohistochemistry

Immunohistochemistry was performed using the ABC-method (Vestastain Elite kit, Vector Laboratories, Burlingame, CA, USA) and paraffin-embedded vascular samples. ADAM8 in the vascular wall was detected with a goat anti-human ADAM8 antibody (AF1031, R&D Systems, Minneapolis, MN, USA). The following primary antibodies were used to detect vascular cell markers in adjacent sections. Muscle actin (mouse anti-human muscle actin, clone HHF35 (DakoCytomation, Glostrup, Denmark)) was used to detect smooth muscle cells. CD68 (mouse anti-human CD68, clone PG-M1 (DakoCytomation)) was used as marker of monocytes and macrophages. For the detection of endothelial cells, CD31 antibody (mouse anti-human CD31, clone JC70A (DakoCytomation)) was employed. Sections were subjected to microwave antigen retrieval treatment as described earlier (12). Subsequently, the sections were incubated overnight with the primary antibodies, followed by biotinylated horse anti-goat (1:500, Vector Laboratories) or sheep anti-mouse (1:300, Amersham Int., Buckinghamshire, UK) and ABC-complex for 30 min. Diaminobenzidine was used as a chromogen.

The co-localization of CD68 and ADAM8 in carotid arteries was studied with double-staining immunofluorescence. The samples were fixed with 4% paraformaldehyde (in 0.1 M phosphate buffered saline (PBS), pH 7.3) for 6 h at +4°C and cryoprotected with 20% sucrose in PBS. Frozen sections (6 μm) were cut with Micron HM560 cryostat and thaw-mounted onto Polysine glass slides (Menzel, Braunschweig, Germany). The sections were incubated overnight with mouse monoclonal anti-CD68 (dilution 1:10) and goat anti-ADAM8 (dil. 1:10) followed by a mixture of biotinylated horse anti-mouse antibody (dil. 1:300, Vector Labs) and rhodamine-conjugated donkey anti-goat (dil. 1:50, Jackson Immunoresearch, West Grove, PA, USA) for 30 min at +37°C. Subsequently, the sections were incubated with fluorescein isothiocyanate (FITC)-conjugated avidin (dil. 1:100, Vector Labs, Peterborough, England) for 30 min. All antibodies were diluted in PBS containing 1% bovine serum albumin (BSA) and 0.3% of Triton X-100. Sections were mounted in a mixture of glycerol and PBS (3:1) containing 0.1% paraphenylenediamine and examined in Nikon Microphot FXA microscope equipped with a proper fluorescence filter. Photographs were obtained with PCO Sensicam digital camera (PCO, Kelheim, Germany). Alternatively the co-distribution of ADAM8 and CD68 was studied in 5 μm paraffin sections (mirror-image sections). Sections were stained with ABC-method as described above.

DNA extraction and ADAM8 genotyping

The samples for genotyping were collected in two series during 1981–1983 (A series, $n=400$) and 1991–1992 (B series, $n=300$). In the A series, DNA was extracted from paraffin-embedded samples of cardiac muscle by the method of Isola et al. (13). In the B series, DNA was isolated from frozen (−70°C) cardiac samples by the standard phenol-chloroform method (10). The samples were genotyped using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA), which is based on the 5′-nuclease assay for allelic discrimination. The nucleotide sequences of the primers and the fluorogenic allele-specific oligonucleotide probes used in the PCR reaction were deduced from published sequences in the GenBank database and were chosen and synthesized in conjugation with Applied Biosystems. PCR reaction was performed according to standard protocol for TaqMan MBG probes in a total volume of 5 μL. After cycling, end-point fluorescence was measured, and the allelic discrimination analysis module carried out the genotype calling, which was also checked also manually. Genotyping was successful in 539 cases of the 700 samples (298 cases in the A series and 241 in the B series). As a quality control method, we genotyped random blind duplicates from the A and B series.

Measurements of atherosclerotic plaque area

The relative areas of different types of atherosclerotic plaques in the left anterior descending coronary artery (LAD), right coronary artery (RCA), and left circumflex coronary artery (LCX) were measured with the standard computer-assisted planimetric technique, which has been described in detail by Ilveskoski et al. (10). Silicon rubber models of arteries were used to measure the stenosis of the three main epicardial coronary arteries (LAD, RCA, and LCX) (14). Maximal stenosis was used to define the extent of coronary narrowing for each coronary artery. These data are available for 670 men. To confirm acute myocardial infarction (AMI), the myocardium was examined. The presence of neutrophil granulocytes was used as a definitive marker of AMI, and scar tissue in the myocardium was a marker of previous myocardial infarction (MI). Furthermore, the presence of thrombosis in coronary arteries was studied during the autopsy.

Collection of risk factor data

Data on coronary artery disease (CAD) risk factors were collected by interviewing the spouse, relative, or a close friend of the deceased. An informant providing interview data was available for 500 (71%) of the cases. The interview consisted of more than 50 detailed questions on smoking and alcohol consumption habits, hypertension, diabetes, or other previous illnesses (15).

Statistical analyses

Statistical analysis was performed using SPSS version 14.0. (SPSS Inc., Chicago, IL, USA). The non-parametric Mann-Whitney U-test was used for comparison of mRNA expression between atherosclerotic and control tissues. For statistical analyses, the genotypes were merged into two groups: TT homozygotes and carriers of the G allele (i.e. TG heterozygotes and GG homozygotes), because of the small number of GG homozygotes ($n=6$). Categorical variables were compared with Pearson chi-square test and continuous variables using analysis of variance (ANOVA). The relative surface areas of different types of atherosclerotic plaques

and total atherosclerotic surface area were square-root transformed for the analyses because of their skewed distributions. The Mann-Whitney U-test was used to analyze non-normally distributed variables. To determine adjusted odds ratios for the occurrence of MI, sudden cardiac death (SCD), and different subtypes of SCD by ADAM8 genotype groups, logistic regression analysis was employed. The logistic regression analyses were adjusted for age and body mass index (BMI) and with hypertension because these covariates significantly associate with the risk of MI and fatal AMI in the present study. Smoking, alcohol consumption, and diabetes were not used as covariates because these data were missing for several cases and including them would have caused too many cases to be excluded from the analysis. There were no significant associations between ADAM8 genotype group and the occurrence of smoking or diabetes and with daily alcohol consumption. The combined group of men who had died of other diseases or by unnatural causes was used as a reference group when analyzing the association between ADAM8 genotype group and lethal AMI. The ADAM8 genotype group did not associate with the occurrence of death either by other diseases or by unnatural causes. A *P*-value less than 0.05 was considered statistically significant.

Results

Characteristics of the subjects and vascular samples studied

Table I shows the characteristics of the HSDS study population according to atherosclerosis risk factors, cause of death, and genotype groups. Of the 539 subjects included in the study, 422 had the TT genotype (78.3%), 111 had the TG genotype (20.6%), and 6 had the GG genotype (1.1%). The allele frequencies of T and G were 0.89 and 0.11, respectively. The genotype distribution was in Hardy-Weinberg equilibrium, and allele frequencies were similar to those published by Applied Biosystems for the Caucasian population on their web site. ADAM8 was not significantly associated with the risk factors for atherosclerosis in the present study. In a histological study of atherosclerotic vessels, the carotid and femoral artery samples were type V or VI, and all aorta samples were type VI. All control vessels were healthy.

ADAM8 expression in the atherosclerotic tissue

We compared the ADAM8 mRNA expression examined with GWEA between atherosclerotic arteries (types V–VI) and non-atherosclerotic internal thoracic arteries, as well as ADAM8 expression between different vessel types. The median ADAM8 expression level was significantly higher in atherosclerotic tissue artery specimens ($n=20$) than in control tissue ($n=6$, $P<0.000$ Mann-Whitney U-test, interquartile range q1–q3: 263–617) (Figure 1A). The ADAM8 gene expression was elevated 2.6-fold ($P=0.000$, q1–q3: 263–1127), 3.0-fold ($P=0.005$, q1–q3: 263–617), and 2.7-fold ($P=0.038$, q1–q3: 263–617) in the carotid arteries, aortas, and femoral arteries, respectively. The Illumina array results were verified with relative quantitative PCR. Similarly, the median ADAM8 expression level was significantly higher in atherosclerotic tissue artery specimens ($n=20$) than in control tissue ($n=6$, $P<0.000$, q1–q3: 5.2–23.9) (Figure 1A). The ADAM8 expression was increased 8.2-fold ($P=0.000$, q1–q3: 5.2–23.9), 10.0-fold ($P=0.002$, q1–q3: 5.2–23.9),

Table I. General characteristics of the study population.

			ADAM8 genotype groups		
	Valid *n*	All ($n=539$)	TT ($n=422$)	TG/GG ($n=117$)	P[a]
Age (years)[b]	539	53.0±9.6	53.2±9.4	52.2±10.1	0.32
Body mass index (kg/m^2)[b]	538	24.7±4.7	24.6±4.7	24.9±4.9	0.56
Alcohol use (< 60 g/d/ ≥ 60 g/d)	351	179/172	140/140	39/32	0.49
Hypertensive (*n* (%))	539	79	62 (15)	17 (15)	0.97
Diabetic (*n* (%))	366	88	65 (23)	23 (29)	0.23
Smokers[c] (*n* (%))	381	319	253 (83)	66 (86)	0.60
Cause of death (*n* (%)):	539				
Cardiac causes		211	160 (38)	51 (44)	0.27
Other disease		119	101 (24)	18 (15)	0.05
Intoxication or violence		209	161 (38)	48 (41)	0.57

[a]*P*-values have been derived from analysis of variance (normally distributed continuous variables), Mann Whitney U-test (non-normally distributed continuous variables), and chi-square test for categorical variables.
[b]Mean±standard deviation.
[c]Current and former smokers.

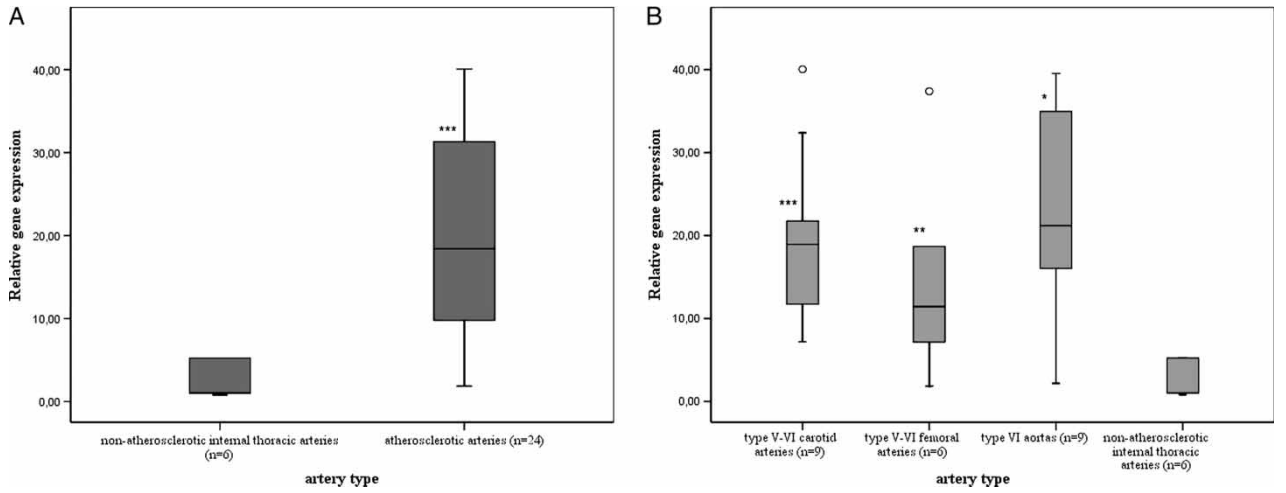

Figure 1. A: The relative ADAM8 mRNA expression in atherosclerotic arteries and non-atherosclerotic arteries (controls) measured with TaqMan Low Density Array (*** $P=0.000$, Mann-Whitney U-test). B: Relative gene expression of the ADAM8 in the non-atherosclerotic internal thoracic arteries (controls), carotid arteries, aortas, and femoral arteries measured with TaqMan Low Density Array (***$P=0.000$; **$P=0.002$; *$P=0.009$; Mann-Whitney U-test) (o = individual case).

and 6.3-fold ($P=0.009$, q1–q3: 5.24–23.9) in the carotid arteries, aortas, and femoral arteries, respectively (Figure 1B), when compared to controls. Furthermore, the expression of ADAM8 was higher in type VI vessels than in type V plaques. Immunohistochemistry of the atherosclerotic lesions revealed that ADAM8 protein is present in both healthy arteries and advanced atherosclerotic lesions but there is a distinct increase in the ADAM8 protein in atherosclerotic plaques. ADAM8 protein was observed in macrophages and smooth muscle cells. However, endothelial cells did not show immunoreactivity to the ADAM8. The ADAM8 protein was most prominent in macrophages (Figure 2). The ADAM8 protein presence in macrophages was further verified with double-immunofluorescence and staining adjacent mirror-image section that clearly demonstrated the localization of ADAM8 in macrophages in carotid arteries. The ADAM8 protein was seen mainly in the cell membranes of macrophages (Figure 3).

The ADAM8 2662 T/G polymorphism and the area of different types of atherosclerotic plaques in coronary arteries

According to the coronary measurements of our study, the carriers of the ADAM8 2662 G allele had significantly more atherosclerosis (measured as the relative surface area covered by atherosclerotic lesions) in their coronary arteries than the TT homozygotes ($P=0.020$ in ANOVA adjusted with age and BMI). When the areas of specific plaque types were compared separately, only the areas of fatty streaks were not significantly associated with this polymorphism ($P=0.229$ in ANOVA adjusted with age and BMI).

In line with the results concerning total plaque areas, the G allele carriers had significantly larger relative areas of fibrotic plaques in their coronary arteries ($P=0.027$ in ANOVA adjusted with age and BMI). A large proportion (23.4%) of the autopsied men were not found to have calcified plaques in their coronary arteries, and, therefore, we first studied whether the ADAM8 polymorphism would affect the occurrence of such plaques. According to logistic regression analysis adjusted with age and BMI, there was no difference in the occurrence of calcified plaques. However, in the group of men who were found to have calcified plaques in their coronary arteries, the relative areas of such plaques were significantly larger among the G allele carriers than among the TT homozygotes ($P=0.011$ by ANOVA adjusted with age and BMI). The proportion of men without complicated plaques was also very high (53.7%), and the same protocol was therefore followed as with analyzing the occurrence and surface areas of calcified plaques. The G allele carriers a had higher occurrence rate for complicated plaques (age and BMI adjusted odds ratio (OR) 1.75 with 95% confidence interval (CI) 1.04–2.96, $P=0.037$), and among the men who had complicated plaques the G allele carriers had significantly larger areas covered by such plaques than the TT homozygotes ($P=0.011$ by ANOVA adjusted with BMI and age) (Table II).

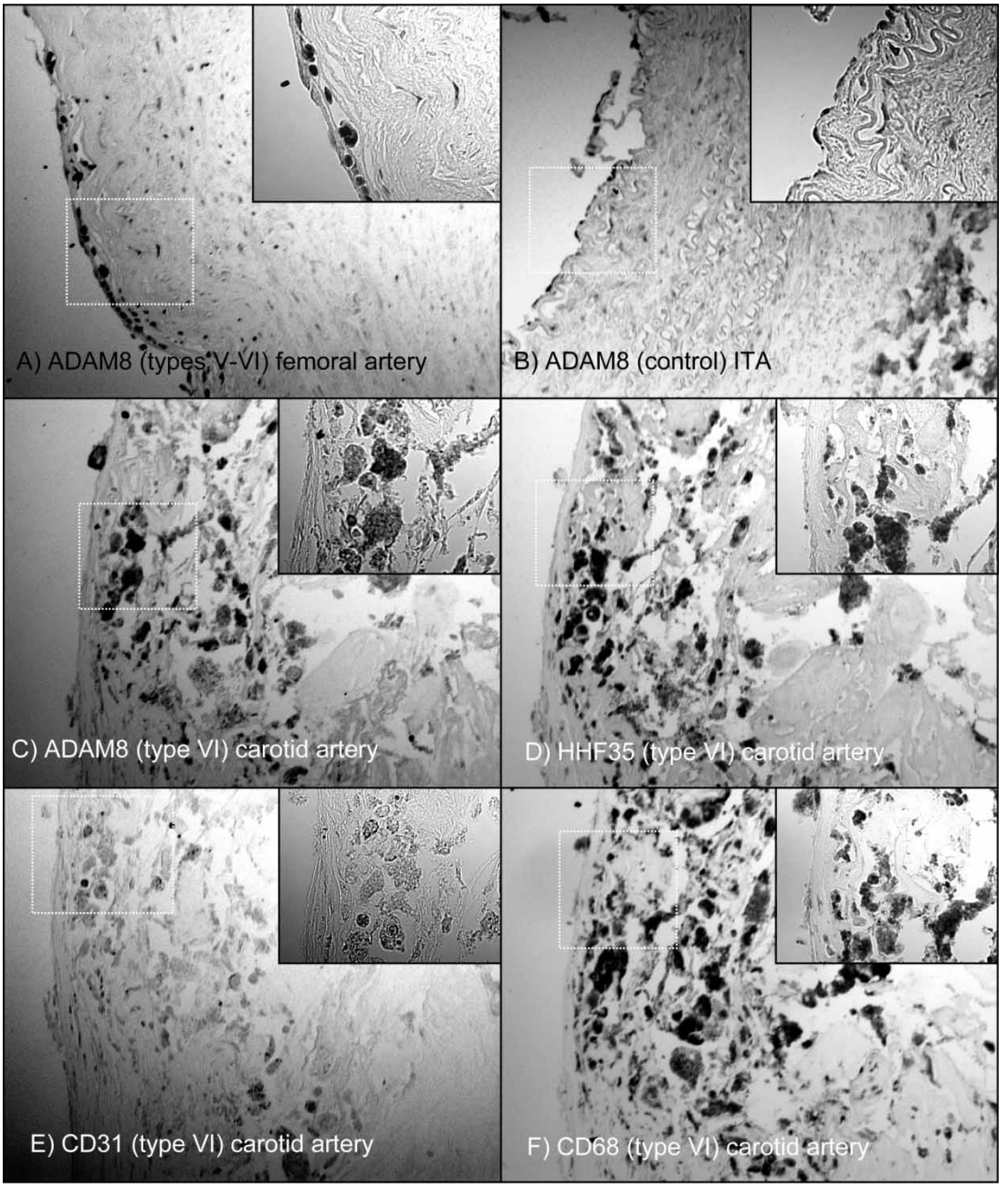

Figure 2. A: ADAM8 protein in type V–VI femoral artery; B: ADAM8 expression in internal thoracic artery (control); C: ADAM8 expression in type VI carotid artery; D–F: smooth muscle cell, endothelial cell, and monocyte/macrophage stainings in type VI carotid artery (ITA = internal thoracic artery).

ADAM8 2662 T/G polymorphism and the occurrence of autopsy-verified myocardial infarctions and fatal pre-hospital coronary events

In the whole Helsinki Sudden Death Study population, the G allele carriers had a higher occurrence rate for autopsy-verified MI (old or acute) compared to T allele carriers: 23.9% ($n = 101$) of the TT homozygotes and 35.0% ($n = 41$) of the G allele carriers had suffered an MI (adjusted OR 2.03 with 95% CI 1.25–3.31, $P = 0.004$). Correspondingly, the G allele carriers had a significantly higher risk of SCD by acute MI (AMI): 12.3% ($n = 52$) of all TT homozygotes and 23.9% ($n = 28$) of all G allele carriers had suffered a fatal pre-hospital AMI (adjusted OR 2.41 with 95% CI 1.34–4.32, $P = 0.003$). When the same analysis was limited to men whose death was caused by autopsy-verified

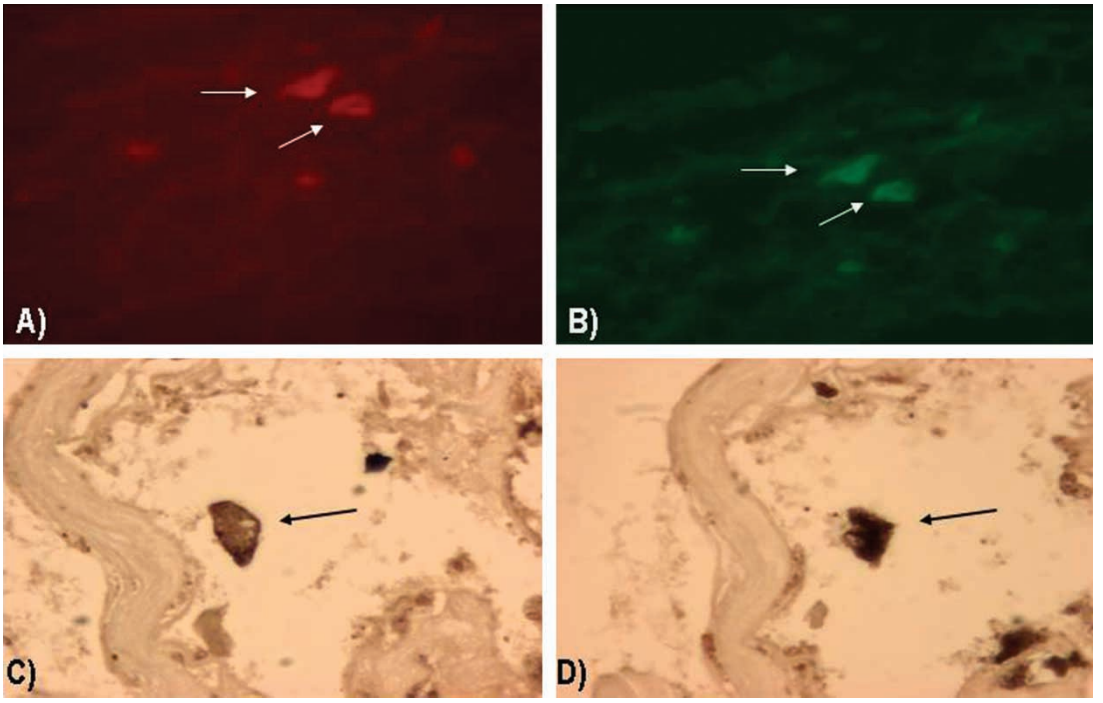

Figure 3. Double immunofluorescence images demonstrating co-distribution of (A) ADAM8 and (B) CD68. Arrows indicate the double-labeled cells. Adjacent mirror-image sections showing a macrophage (arrows) heavily stained for (C) ADAM8 and (D) CD68. ADAM8 immunoreactivity is strongest in the cell membrane.

coronary thrombus, the association became more significant: 6.9% ($n=29$) versus 17.9% ($n=20$) (adjusted OR 3.18 with 95% CI 1.59–6.34, $P=0.001$).

In our material the relative areas of complicated plaques significantly associated with the occurrence of fatal AMI. We found that the ADAM8 genotype group associated with both the surface areas of complicated plaques and the occurrence of fatal AMI. Therefore, we tested whether the higher susceptibility of the G allele carriers to fatal AMI was due to the larger surface areas of complicated plaques in their coronary arteries. When the surface areas of complicated plaques were included as a covariate into the regression analyses, the effect of the G allele on the occurrence of fatal AMI was weakened markedly and was no longer statistically significant (adjusted OR 1.56 with 95% CI 0.69–3.5, $P=0.284$).

Discussion

For the first time, the present study implicates ADAM8 in the pathogenesis of human atherosclerosis. ADAM8 has previously been suggested to have a

Table II. Autopsy-verified surface areas of different atherosclerotic plaques and of total atherosclerosis in coronary arteries by ADAM8 genotype group in the Helsinki Sudden Death Study material. Values are expressed as percentages (%) ±standard error (SE) of the total vessel surface of the three main coronary arteries (LAD, LCX, and RCA).

			ADAM-8 genotype group		
	Valid n	All	TT (n=422)	TG/GG (n=117)	*P*-value[a]
Plaque type:					
Fatty streaks	399	5.43±0.23	5.26±0.24	6.14±0.60	0.229
Fibrotic plaques	399	3.89±0.21	3.61±0.22	5.02±0.56	0.027
Calcified plaques	516	2.59±0.19	2.38±0.20	3.35±0.48	0.011
Complicated plaques	399	1.53±0.16	1.30±0.18	2.46±0.38	0.011
Total atherosclerosis	399	9.33±0.33	8.87±0.34	11.16±0.86	0.020

[a]Significance calculated by ANOVA adjusted with age and body mass index after square-root transformation.
LAD =left anterior descending; LCX =left circumflex; RCA =right coronary artery.

role, e.g. in allergic response (16) and in pathological neuron-glia interactions (17). Another member of the same family, ADAM15, has been shown to be up-regulated in atherosclerotic lesions in monkeys (18). Here we show that ADAM8 mRNA and protein levels are up-regulated in advanced atherosclerotic lesions wherein ADAM8 was co-distributed to monocyte-macrophages and smooth muscle cells. Furthermore, we found that the 2662 T/G SNP of ADAM8 gene is associated with advanced atherosclerotic lesion areas and risk of MI and fatal acute MI.

ADAM8 has previously been found to be up-regulated by PPARG activation of macrophages (19). PPARG is an important regulator of macrophage gene expression that is found in atherosclerotic lesions (20). PPARG activation decreases inflammatory cytokines produced by macrophages (21) and promotes cholesterol efflux from macrophages (22). In addition, PPARG agonists have been shown to block the proliferation and increase the apoptosis of vascular smooth muscle cells (23,24). Given the present results showing increased ADAM8 mRNA and protein in the atherosclerotic arteries and the localization of ADAM8 protein to the monocyte-macrophages and smooth muscle cells, it is intriguing to speculate that the ADAM8 up-regulation could be involved in the PPARG mediated anti-inflammatory mechanisms.

The G allele carriers of the ADAM8 2662 T/G allelic variant had significantly more atherosclerosis as well as increased areas of fibrotic, calcified, and complicated plaques in their coronary arteries, than did the TT homozygotes. The observed SNP appears to be related to the extent of the atherosclerotic plaque area, suggesting that ADAM8 is somehow involved in the plaque formation.

Patients with the G variant of 2662 T/G (TG and GG genotypes) appeared to have higher rates of MI and fatal acute MI, possibly explained by the association with the increased areas of complicated lesions in the coronary arteries of the G allele carriers. However, when the MI prevalence was adjusted with the areas of complicated lesions, the correlation disappeared. This suggests that the G allele might have an effect on the emergence of MI at the level of complicated lesion areas rather than directly affecting the thrombus formation. Even though the 2662 T/G is located in the 3′-UTR region of the gene, it has been suggested that the 3'-UTR can affect gene expression by influencing mRNA stability and translation (25). Since the 2662 T/G belongs to bin (SNPs in high linkage disequilibrium) with four other SNPs it is also possible that the 2662 T/G is a neutral marker of some other SNP belonging to the same subset.

An obvious possibility is that ADAM8 might contribute to the pathogenesis of atherosclerosis and acute coronary syndromes through its proteolytic potential. ADAM8 has been found to act as L-selecting sheddase. Transgenic mice over-expressing the soluble ADAM8 ectodomain showed attenuated leukocyte infiltration and down-regulation of L-selectin (26). L-selectin is a leukocyte-specific adhesion molecule involved in the initial rolling of leukocytes on the endothelium and, indeed, ADAM8 has been shown to be capable of cleaving L-selectin from the cell surface (27). Interestingly, inhibition of L-selectin shedding has been shown to alter leukocyte rolling over activated endothelium and to enhance leukocyte recruitment (28). ADAM8 also mediates the shedding of VCAM-1 (29), another mediator of the attachment of monocytes and lymphocytes (30). Thus, ADAM8 might have a protective role in atherosclerosis by its ability to suppress leukocyte trafficking into the vessel wall. Besides shedding other molecules to the blood-stream, ADAM8 is itself processed by metalloprotease domain removal (31). There is evidence suggesting that soluble ADAM8 might be active (31) and thus elicit multiple events either in the vascular wall or blood-stream. For example, ADAM8 can interact with alpha9beta1 integrin (32) that is also a ligand for coagulation factor XIII and von Willebrand factor (33). Thus it is intriguing to speculate whether soluble ADAM8 could prevent the thrombotic events in atherosclerosis by blocking the binding site for these essential factors in blood coagulation.

ADAM8 has also previously been shown to be an important factor in osteoclast (OCL) formation (34), which could be an important finding considering that the monocytotic line of hematopoietic cells have been reported to undergo osteoclastic differentiation and OCL-like cells have also been observed in atherosclerotic plaques (35).

The vascular samples were collected from carotid, aorta, and femoral arteries, where the local hemodynamic conditions are different as compared to coronary arteries. The majority of the samples, however, were from histologically advanced plaques (types V-VI), which allows us to suggest that they reflect the systemic nature of atherosclerosis. The pattern of ADAM8 expression was similar in the different atherosclerotic arteries with different hemodynamic conditions, also supporting the role of ADAM8 in systemic atherosclerosis. We compared the expression of ADAM8 mRNA and protein levels between atherosclerotic plaques obtained during

open vascular surgical procedures and non-atherosclerotic internal thoracic arteries taken during coronary artery by-pass grafting. Even though comparison of diseased and healthy arteries from the same source would be more accurate, we were unfortunately not able to receive any corresponding healthy arteries from carotid, aortic, and femoral regions due to ethical issues. Still, we believe that comparison of atherosclerotic plaques to ITA vessels provides sufficient information to reveal the biological processes going on in the diseased vessels. By combining data from the two study material sets (the vascular sample series and the HSDS material), we were able to demonstrate the ADAM8 expression in vascular samples and the association of ADAM8 2662 T/G polymorphism with the advanced atherosclerotic lesion areas and MI.

Although there are several classical factors for atherosclerosis and cardiac disease (elevated plasma (LDL), cigarette smoking, hypertension, diabetes mellitus) (36) which confer a high probability of future pathogenic events, additional prognostic markers are required for a more accurate prediction of the risk. In addition, clarifying the inflammatory mechanisms in the atherosclerotic plaque formation may result in pharmacological applications for disease prevention or treatment. In conclusion, the present results emphasize that ADAM8 is a promising candidate to be involved in multiple mechanisms of atherosclerosis and MI. The observed increase of ADAM8 mRNA protein in atherosclerotic plaques might elicit multiple anti-inflammatory mechanisms. On the other hand, the SNP significantly associated with plaque areas and myocardial infarction, suggesting ADAM8 is also involved in these processes. The present study takes the first step towards understanding the role of ADAM8 in atherosclerosis, and more work is needed to elucidate the exact pathogenic mechanism and to further define its prognostic and therapeutic potential.

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References

1. Huovila AP, Turner AJ, Pelto-Huikko M, Karkkainen I, Ortiz RM. Shedding light on ADAM metalloproteinases. Trends Biochem Sci. 2005;30:413–22.
2. Yoshiyama K, Higuchi Y, Kataoka M, Matsuura K, Yamamoto S. CD156 (human ADAM8): expression, primary amino acid sequence, and gene location. Genomics. 1997;41:56–62.
3. Yoshida S, Setoguchi M, Higuchi Y, Akizuki S, Yamamoto S. Molecular cloning of cDNA encoding MS2 antigen, a novel cell surface antigen strongly expressed in murine monocytic lineage. Int Immunol. 1990;2:585–91.
4. Amour A, Knight CG, English WR, Webster A, Slocombe PM, Knauper V, et al. The enzymatic activity of ADAM8 and ADAM9 is not regulated by TIMPs. FEBS Lett. 2002;524:154–8.
5. Yamamoto S, Higuchi Y, Yoshiyama K, Shimizu E, Kataoka M, Hijiya N, et al. ADAM family proteins in the immune system. Immunol Today. 1999;20:278–84.
6. Bevilacqua MP, Pober JS, Wheeler ME, Cotran RS, Gimbrone MA Jr. Interleukin-1 activation of vascular endothelium. Effects on procoagulant activity and leukocyte adhesion. Am J Pathol. 1985;121:394–403.
7. Oksala N, Levula M, Airla N, Pelto-Huikko M, Ortiz RM, Jarvinen O, et al. ADAM-9, ADAM-15, and ADAM-17 are upregulated in macrophages in advanced human atherosclerotic plaques in aorta and carotid and femoral arteries–Tampere vascular study. Ann Med. 2009;41:279–90.
8. Ross R. Atherosclerosis–an inflammatory disease. N Engl J Med. 1999;340:115–26.
9. Reiss K, Ludwig A, Saftig P. Breaking up the tie: disintegrin-like metalloproteinases as regulators of cell migration in inflammation and invasion. Pharmacol Ther. 2006;111 :985–1006.
10. Ilveskoski E, Perola M, Lehtimaki T, Laippala P, Savolainen V, Pajarinen J, et al. Age-dependent association of apolipoprotein E genotype with coronary and aortic atherosclerosis in middle-aged men: an autopsy study. Circulation. 1999;100:608–13.
11. Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W Jr, et al. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation. 1995;92:1355–74.
12. Shi SR, Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem. 1991;39:741–8.
13. Isola J, DeVries S, Chu L, Ghazvini S, Waldman F. Analysis of changes in DNA sequence copy number by comparative

genomic hybridization in archival paraffin-embedded tumor samples. Am J Pathol. 1994;145:1301–8.
14. Weman SM, Salminen US, Penttila A, Mannikko A, Karhunen PJ. Post-mortem cast angiography in the diagnostics of graft complications in patients with fatal outcome following coronary artery bypass grafting (CABG). Int J Legal Med. 1999;112:107–14.
15. Karhunen PJ, Penttila A. Validity of post-mortem alcohol reports. Alcohol Alcohol. 1990;25:25–32.
16. King NE, Zimmermann N, Pope SM, Fulkerson PC, Nikolaidis NM, Mishra A, et al. Expression and regulation of a disintegrin and metalloproteinase (ADAM) 8 in experimental asthma. Am J Respir Cell Mol Biol. 2004;31:257–65.
17. Schlomann U, Rathke-Hartlieb S, Yamamoto S, Jockusch H, Bartsch JW. Tumor necrosis factor alpha induces a metalloprotease-disintegrin, ADAM8 (CD 156): implications for neuron-glia interactions during neurodegeneration. J Neurosci. 2000;20:7964–71.
18. Herren B, Raines EW, Ross R. Expression of a disintegrin-like protein in cultured human vascular cells and in vivo. FASEB J. 1997;11:173–80.
19. Hodgkinson CP, Ye S. Microarray analysis of peroxisome proliferator-activated receptor-gamma induced changes in gene expression in macrophages. Biochem Biophys Res Commun. 2003;308:505–10.
20. Marx N, Sukhova G, Murphy C, Libby P, Plutzky J. Macrophages in human atheroma contain PPARgamma: differentiation-dependent peroxisomal proliferator-activated receptor gamma(PPARgamma) expression and reduction of MMP-9 activity through PPARgamma activation in mononuclear phagocytes in vitro. Am J Pathol. 1998;153:17–23.
21. Jiang C, Ting AT, Seed B. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. Nature. 1998;391:82–6.
22. Chinetti G, Lestavel S, Bocher V, Remaley AT, Neve B, Torra IP, et al. PPAR-alpha and PPAR-gamma activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. Nat Med. 2001;7:53–8.
23. Bruemmer D, Yin F, Liu J, Berger JP, Kiyono T, Chen J, et al. Peroxisome proliferator-activated receptor gamma inhibits expression of minichromosome maintenance proteins in vascular smooth muscle cells. Mol Endocrinol. 2003;17:1005–18.
24. Bruemmer D, Yin F, Liu J, Berger JP, Sakai T, Blaschke F, et al. Regulation of the growth arrest and DNA damage-inducible gene 45 (GADD45) by peroxisome proliferator-activated receptor gamma in vascular smooth muscle cells. Circ Res. 2003;93:e38–47.
25. Misquitta CM, Iyer VR, Werstiuk ES, Grover AK. The role of 3'-untranslated region (3'-UTR) mediated mRNA stability in cardiovascular pathophysiology. Mol Cell Biochem. 2001;224:53–67.
26. Higuchi Y, Yasui A, Matsuura K, Yamamoto S. CD156 transgenic mice. Different responses between inflammatory types. Pathobiology. 2002;70:47–54.
27. Gomez-Gaviro M, Dominguez-Luis M, Canchado J, Calafat J, Janssen H, Lara-Pezzi E, et al. Expression and regulation of the metalloproteinase ADAM-8 during human neutrophil pathophysiological activation and its catalytic activity on L-selectin shedding. J Immunol. 2007;178:8053–63.
28. Kishimoto TK, Jutila MA, Berg EL, Butcher EC. Neutrophil Mac-1 and MEL-14 adhesion proteins inversely regulated by chemotactic factors. Science. 1989;245:1238–41.
29. Matsuno O, Miyazaki E, Nureki S, Ueno T, Kumamoto T, Higuchi Y. Role of ADAM8 in experimental asthma. Immunol Lett. 2006;102:67–73.
30. Li H, Cybulsky MI, Gimbrone MA Jr, Libby P. An atherogenic diet rapidly induces VCAM-1, a cytokine-regulatable mononuclear leukocyte adhesion molecule, in rabbit aortic endothelium. Arterioscler Thromb. 1993;13:197–204.
31. Schlomann U, Wildeboer D, Webster A, Antropova O, Zeuschner D, Knight CG, et al. The metalloprotease disintegrin ADAM8. Processing by autocatalysis is required for proteolytic activity and cell adhesion. J Biol Chem. 2002;277:48210–9.
32. Rao H, Lu G, Kajiya H, Garcia-Palacios V, Kurihara N, Anderson J, et al. Alpha9beta1: a novel osteoclast integrin that regulates osteoclast formation and function. J Bone Miner Res. 2006;21:1657–65.
33. Takahashi H, Isobe T, Horibe S, Takagi J, Yokosaki Y, Sheppard D, et al. Tissue transglutaminase, coagulation factor XIII, and the pro-polypeptide of von Willebrand factor are all ligands for the integrins alpha 9beta 1 and alpha 4beta 1. J Biol Chem. 2000;275:23589–95.
34. Choi SJ, Han JH, Roodman GD. ADAM8: a novel osteoclast stimulating factor. J Bone Miner Res. 2001;16:814–22.
35. Jeziorska M, McCollum C, Wooley DE. Observations on bone formation and remodelling in advanced atherosclerotic lesions of human carotid arteries. Virchows Arch. 1998;433:559–65.
36. Lusis AJ. Atherosclerosis. Nature. 2000;407:233–41.

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ORIGINAL ARTICLE

ADAM-9, ADAM-15, and ADAM-17 are upregulated in macrophages in advanced human atherosclerotic plaques in aorta and carotid and femoral arteries—Tampere vascular study

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Abstract

Background and aims. The expression of disintegrin and metalloprotease ADAM-9, ADAM-15, and ADAM-17 has been associated with cell-cell, cell-platelet, and cell-matrix interactions and inflammation. They are possibly implicated in the pathophysiology of atherosclerosis.

Methods and results. Whole-genome expression array and quantitative real-time polymerase chain reaction (PCR) analysis confirmed that ADAM-9, ADAM-15, and ADAM-17 are upregulated in advanced human atherosclerotic lesions in samples from carotid, aortic, and femoral territories compared to samples from internal thoracic artery (ITA) free of atherosclerotic plaques. Western analysis indicated that the majority of these ADAMs were in the catalytically active form. ADAM-9, ADAM-15, and ADAM-17-expressing cells were shown to co-localize with CD68-positive cells of monocytic origin in the atherosclerotic plaques using immunohistochemistry and double-staining immunofluorescence analysis. Co-localization was demonstrated in all vascular territories. In the carotid territory, cells expressing the ADAMs co-distributed also with smooth muscle cells and, in femoral territory, with CD31-positive endothelial cells, indicating that the ADAM expression pattern depends on vascular bed territory.

Conclusions. Present findings provide strong evidence for the involvement of catalytically active ADAM-9, ADAM-15, and ADAM-17 in advanced atherosclerosis, most notably associated with cells of monocytic origin.

Key words: *ADAM, atherosclerosis, gene expression, immunohistochemistry, whole genome*

Introduction

The inflammatory and chronic nature of atherosclerosis (1) makes it compelling to identify genes involved in this process. To better understand the alterations in gene expression in advanced atherosclerosis, we utilized genome-wide expression array

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Key messages

- Upregulated expression of ADAM-9, ADAM-15, and ADAM-17 and the presence of their catalytically active forms are implicated in advanced human atherosclerosis and suggest roles in the monocyte homing, migration, or proliferation in aorta, carotid, and femoral arteries.
- As the therapeutic intervention of ADAMs in human diseases is actively pursued, our findings underline the potential of targeting the development of atherosclerosis through modulation of ADAM function.
- A precise picture of ADAMs expression is a prerequisite for understanding the contribution of their intricate interplay in atherosclerosis, towards which the present study takes an important step.

(GWEA), encompassing all the known 23,000 genes to study a unique sample material of advanced plaques in aorta, carotid and femoral arteries, and control samples from internal thoracic artery (ITA) classified according to the American Heart Association classification (2). Over 200 genes were found to be upregulated, including members of the disintegrin and metalloprotease (ADAM) family (3). Of these, ADAM-9, ADAM-15, and ADAM-17 were selected for more detailed analysis because they have been associated with cellular and physiological functions pertinent to atherosclerosis, such as cell-cell, cell-platelet, and cell-matrix interactions, as well as with inflammation (4,5).

ADAM metalloprotease disintegrins are transmembrane proteins mediating targeted proteolysis, cell adhesion, and signal transduction (3,6). ADAM proteinases can release and activate cytokines, growth factors, and other bioactive proteins from their membrane-bound precursors and remove receptors from the cell surface, in the process called ectodomain shedding (4,5). However, not all ADAMs are active proteinases as half of them do not contain all the essential amino acids at the active site (5). ADAMs have also been implicated in cell-cell and cell-matrix interactions through binding to integrins and other adhesion molecules (6). Their diverse involvement in cell interactions suggests that alterations in the expression of ADAMs may play roles in complex pathologies such as atherosclerosis.

ADAM-9 has been implicated both in ectodomain shedding and cell interactions through integrin binding. The potential ADAM-9 sheddase substrates include growth factors and cytokines (5) which have been linked to atherosclerosis (7,8). ADAM-9 is capable of binding to $\alpha_6\beta_1$ and $\alpha_v\beta_5$ (9,10), and also these interactions might be relevant in atherosclerosis. ADAM-9 interaction with $\alpha_6\beta_1$ was shown to regulate fibroblast motility, and $\alpha_6\beta_1$ can induce the mobility of several cell types including macrophages (11–13). The interaction with $\alpha_v\beta_5$ was shown to stimulate proinflammatory interleukin-6 production in cultured osteoblasts (10). ADAM-9 has indeed been shown to be expressed in human atherosclerotic plaques, in conjunction with increased levels of $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins which also are associated with the development and progression of atherosclerosis (14). No ADAM-9 nor $\alpha_v\beta_3$ or $\alpha_5\beta_1$ mRNA and only weak ADAM-9 immunostaining were detected from human thyroid artery without atherosclerosis (14). Altogether, these interactions and expression data are consistent with a potential role of ADAM-9 in atherosclerosis.

The first direct indication of a potential role of an ADAM in atherosclerosis was, indeed, the demonstration of upregulated ADAM-15 in monkey atherosclerotic lesions (1). Also ADAM-15 has been shown to mediate both ectodomain shedding and binding to integrins. The potential sheddase substrates of ADAM-15 include epidermal growth factor (EGF) family growth factors (5) with possible association with atherosclerosis (7). Human ADAM-15, but remarkably not rodent adam-15, contains the 'classical' arginine-glycine-aspartate (RGD) sequence in its putative integrin-binding motif which appears to mediate binding to $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_{(IIb)}\beta_3$ integrins (9,15,16). ADAM-15 is highly expressed in mouse vascular cells and endocardium (17), and in cultured human aortic smooth muscle and umbilical vein endothelial cells (1). Similarly to ADAM-9, weak ADAM-15 immunostaining was detected from normal human arteries while the mRNA level was below detection, but atherosclerotic plaques from carotid arteries showed upregulated ADAM-15 (and ADAM-9) expression in conjunction with elevated $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins (14). ADAM-15 seems to be an important mediator of inflammation (18). Hence, also ADAM-15 is an intriguing candidate to be involved in the pathology of atherosclerosis.

ADAM-17, besides ADAM-10, is the most studied sheddase ADAM, originally identified as the tumor necrosis factor alpha (TNF-α) converting-enzyme (TACE) (19,20). In addition to TNF-α, ADAM-17 can activate several cytokines and growth factors implicated in atherosclerosis and remove their receptors from cell surfaces (5), and the number of putative novel substrates continues to grow. Since ADAM-17 has been implicated in

atherosclerosis (21,22), and since it can modulate leukocyte adhesion by shedding L-selectin (23,24) and modulating macrophage activity (25), ADAM-17 is an interesting potential target for the treatment of inflammatory diseases such as atherosclerosis (8). Further investigations of ADAM-17 will also advance our understanding of the molecular basis of atherosclerosis.

The current view of possible roles of ADAMs in atherosclerosis is based mainly on animal and *in vitro* experiments, and on a single report of arterial location but without classification of the degree of atherosclerosis or verification of gene expression data (14,22). Based on our genome-wide expression array data on ADAMs, we hypothesized that their expression is upregulated in advanced atherosclerosis and might co-distribute with endothelial, smooth muscle, or monocytic cells in atherosclerotic areas. Thus we performed detailed analysis of atherosclerotic plaques in different arterial beds, i.e. carotid region, aorta, and femoral region, to find out whether ADAMs associate with global progression of atherosclerosis in human subjects.

Methods

Vascular samples

Vascular sample series from femoral arteries, carotid arteries, and abdominal aortas were obtained during open vascular procedures from 30 patients under surveillance of a senior consultant vascular surgeon (NO). Six control samples were taken from internal thoracic arteries (ITA) obtained during coronary artery bypass grafting. The study has been approved by the Ethics Committee of Tampere University Hospital. All clinical investigations were conducted according to declaration of Helsinki principles. The samples were taken from patients subjected to open vascular surgical procedures in the Division of Vascular Surgery and Heart Centre, Tampere University Hospital. The vascular samples were classified according to recommendation of American Heart Association (AHA) (2).

RNA isolation and genome-wide expression analysis (GWEA)

The fresh tissue samples ($n = 26$) were immediately soaked in RNALater solution (Ambion Inc., Austin, TX, USA), and total-RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and RNAEasy Kit (Qiagen, Valencia, CA, USA). The concentration and quality of RNA were evaluated spectrophotometrically (BioPhotometer, Eppendorf, Wesseling-Berzdorf, Germany). The GWEA microarray experiments were performed by using Sentrix® Human-8 Expression BeadChips analyzing over 23,000 known genes and gene candidates (Illumina, San Diego, CA, USA) according to given instructions by the manufacturer. In brief, a 200 ng aliquot of total-RNA from each sample was amplified to cDNA using Ambion's Illumina RNA Amplification kit following the instructions (cat. no I1755, Ambion, Inc., Austin, TX, USA). *In vitro* transcription (IVT) reaction of cDNA to cRNA was performed overnight (14 h) including biotin-11-deoxy uridine triphosphate (dUTP) (PerkinElmer Life And Analytical Sciences, Inc., Boston, MA, USA) for labeling the cRNA product. Both before and after the amplifications the RNA/cRNA concentrations were checked with Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), and RNA/cRNA quality was controlled by BioRad's Experion Automated Electrophoresis System and RNA StdSens Analysis Kit (BioRad Laboratories, Inc., Hercules, CA, USA). Each sample cRNA (1500 ng) was hybridized to Illumina's Sentrix® Human-8 Expression BeadChip arrays (Illumina) at 55°C overnight (18 h) following the Illumina Whole-Genome Gene Expression Protocol for BeadStation. Hybridized biotinylated cRNA was detected with 1 μg/mL cyanine3-streptavidine (Amersham Biosciences, Pistacataway, NJ, USA). BeadChips were scanned with Illumina BeadArray Reader. The accuracy of Illumina Sentrix® Human-8 Expression BeadChips microarray methodology to measure the gene expression was earlier verified by real-time quantitative TaqMan PCR by quantitating the expression of 20 genes with both methods (26).

Quantitative real-time PCR (qPCR)

From the 30 tissue samples, gene expression analyses were done with TaqMan low-density arrays (LDAs) (Applied Biosystems, Foster City, CA, USA). Total-RNA (500 ng) was reverse-transcribed to cDNA using High Capacity cDNA Kit according to the manufacturer's instructions (Applied Biosystems). For the PCR, LDAs were loaded with 8 μL undiluted cDNA, 42 μL H_2O, and 50 μL PCR Universal Master Mix and run according to the manufacturer's instructions (Applied Biosystems). Samples were analyzed as duplicates, and both cDNA synthesis and PCR reactions were validated for inhibition. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene control, and the qPCR results were analyzed using SDS 2.2 Software (Applied Biosystems).

Western analysis (WB)

Four randomly selected samples of atherosclerotic plaques from carotid (two patients) and femoral artery, and aorta were homogenized (100 mg/350 μL) in ice-cold lysis buffer (10 mM Tris-HCl pH 7.4, 1% (w/v) Triton X-100, 0.1% (w/v) sodium dodecyl sulphate (SDS), 0.1% (w/v) sodium deoxycholate, 150 mM NaCl) (27) containing Roche Complete protease inhibitors (Roche Diagnostics, Mannheim, Germany) using a Potter-Elvehjem homogenizer with a teflon pestle. The amount of protein was not measured but the samples (each 100 mg wet weight) were prepared in equal volumes of homogenization and sample buffers. Insoluble debris was removed with centrifugation (16.2 k $\times g$, 10 min) in a refrigerated microcentrifuge at 4°C. Laemmli SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (5x) with dithiothreitol (DTT) was added to 1x concentration (0.1 M DTT final). The samples were heated at 99°C for 5 minutes, cooled to room temperature and centrifuged 16.2 k $\times g$ for 10 minutes prior to loading 100 μL/well onto a 10% SDS-polyacrylamide gel. Molecular weight markers (Chemichrome Ultimate, C2117, Sigma, St. Louis, MI, USA) were run along the samples. Following the SDS-PAGE, the proteins were electrotransferred onto a polyvinylidene fluoride (PVDF) membrane (BioRad, Hercules, CA, USA) in a semi-dry blotter. The membrane was then treated with 0.1% (w/v) Ponceau S in 5% acetic acid to verify the successful transfer, to ensure an even loading, and to fix the proteins onto the membrane.

The primary antibodies against human ADAM protein ectodomains were from R&D Systems (Abingdon, UK). Goat-anti-ADAM-9 (AF939), goat-anti-ADAM-15 (AF935), and goat-anti-ADAM-17 (AF2129) were diluted 1/1000 in tris-buffered saline with Tween-20 (TBST) containing 5% non-fat milk (TBS with 0.1% (w/v) Tween-20). The peroxidase-coupled secondary antibody, anti-goat/sheep (A9452, Sigma), was diluted 1/5000 in TBST/milk.

For the immunodetection, the blots were blocked with 5% (w/v) non-fat milk in TBST, followed with 1 h incubation at room temperature (RT) with the primary antibodies. After several TBST washes and the secondary antibody incubation (1 h, RT), the blots were washed with TBST, rinsed in TBS, and the bound secondary antibody was visualized with enhanced chemiluminescence (ECL) and a digital imager. Just prior to the ECL reagent incubation, the blots were rinsed in water. The ECL was done with SuperSignal West Dura reagents according to the manufacturer's protocol (Pierce, Rockford, USA). The ECL was visualized in a ChemiDoc XRS digital imager using Quantity One 4.5.2 software (BioRad).

Immunohistochemistry (IH)

Immunohistochemistry was performed using the avidin biotin complex (ABC) method (Vectastain Elite kit, Vector Laboratories, Burlingame, CA, USA) and paraffin-embedded vascular samples without any counterstain. ADAMs in vascular wall were detected with rabbit anti-human ADAM-9 (AF1031), mouse anti-human ADAM-15 (MAB935), and chicken anti-human ADAM-17 (AF930) ectodomain antibodies (R&D Systems, Minneapolis, MN, USA). The following primary antibodies were used to detect vascular cell markers in adjacent sections: Muscle actin (mouse anti-human muscle actin, clone HHF35; DakoCytomation, Glostrup, Denmark) was used to detect smooth muscle cells. CD68 (mouse anti-human CD68, clone PG-M1; DakoCytomation) was used as marker of monocytes and macrophages. For the detection of endothelial cells, CD31 antibody (mouse anti-human CD31, endothelial cell, clone JC70A; DakoCytomation) was used. The sections were subjected to microwave antigen retrieval treatment as described earlier (28). Endogenous peroxidase activity was extinguished by treating the section with 0.3% H_2O_2 for 30 min. Subsequently the sections were incubated overnight with the primary antibodies followed by biotinylated horse anti-goat, goat anti-chicken (1:500, Vector Laboratories), or sheep anti-mouse (1:300, Amersham Int., Buckinghamshire, UK) and ABC complex for 30 min. Diaminobenzidine was used as the chromogen. All antibodies were diluted in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.3% of Triton X-100. Controls included omitting the primary antibody or replacing it with non-immune sera. No staining was seen in the controls.

The co-localization of CD68 and ADAM-9 and ADAM-17 in carotid arteries was studied with double-staining immunofluorescence (IF). The samples were fixed with 4% paraformaldehyde (in 0.1 M PBS, pH 7.3) for 6 h at +4°C and cryoprotected with 20% sucrose in PBS. Frozen sections (6 μm) were cut with Micron HM560 Cryostat and thaw-mounted onto Polysine glass slides (Menzel, Braunschweig, Germany). The sections were incubated overnight with mouse monoclonal anti-CD68 (1:10) and rabbit anti-ADAM-9 (1:100), or with the mixture of CD68 antibody and chicken anti-ADAM-17 followed by a mixture of biotinylated sheep anti-mouse antibody (1:200, Amersham) and rhodamine-conjugated goat anti-rabbit antibody (1:50, Boehringer Mannheim),

or with a mixture of fluorescein-labeled sheep anti-mouse antibody (1:10, Amersham) and biotinylated goat anti-chicken (Vector Labs, Peterborough, England) for 30 min at +37°C, respectively. Subsequently, the sections were incubated with fluorescein isothiocyanate (FITC)-conjugated avidin or rhodamine-conjugated avidin (1:100, Vector Labs) for 30 min, respectively. Sections were mounted in a mixture of glycerol and PBS (3:1) containing 0.1% paraphenylenediamine and examined in Nikon Microphot FXA microscope equipped with proper fluorescence filters. Photographs were obtained with a PCO Sensicam digital camera (PCO, Kelheim, Germany). Alternatively the co-localization of ADAM-9, ADAM-15, ADAM-17, and CD68 was studied in 5 μm paraffin sections (mirror image sections). Sections were stained with ABC method as described above.

Statistical analyses

Statistical analyses were performed using SPSS version 14.0 (SPSS Inc., Chicago, IL, USA). The non-parametric Mann-Whitney U-test was used for comparison of mRNA expression between atherosclerotic and control tissues and between different arterial regions. Data are presented as median and range.

Results

Characteristics of the subjects and studied vascular samples

The median age was 70.0 years (45–93 years) and 75.9% of the subjects were men. The prevalence of risk factors was as follows: dyslipidemia 40.7%, hypertension 77.8%, diabetes 18.5%, history of smoking 86.2%, alcohol usage more than once a week 42.8%. A total of 73.3% of the samples were classified as type 5–6 advanced plaques.

ADAM mRNA expression in the atherosclerotic tissue

We compared the ADAM-9, ADAM-15, and ADAM-17 expression level between atherosclerotic tissue (types 5–6) and control tissue from internal thoracic artery (ITA) (type 0), and expression levels between different vessel types. In GWEA, the median ADAM-9 expression was elevated relative to ITA in the carotid arteries (1.2-fold, $P=0.012$), aortas (1.3-fold, $P=0.001$), and femoral arteries (1.5-fold, $P=0.010$), respectively (Figure 1). ADAM-15 expression was elevated relative to ITA in the carotid arteries (2.2-fold, $P<0.001$), aortas (2.7-fold, $P<0.001$), and femoral arteries (3.0-fold, $P=0.010$), respectively (Figure 1). ADAM-17 expression was elevated relative to ITA in the carotid arteries (1.2-fold, $P<0.001$), aortas (1.2-fold, $P=0.001$), and femoral arteries (1.3-fold, $P=0.01$), respectively (Figure 1).

According to GWEA, the median ADAM-9 expression level in atherosclerotic tissues ($n=20$) was 233 (153–338) and 174 (173–185) in ITA tissues (type 0, $n=6$) (1.3-fold relative to ITA, $P=0.001$) (Figure 2). The median ADAM-15 expression level in atherosclerotic tissues ($n=20$) was 635 (366–834), and 239 (219–263) in ITA (type 0, $n=6$) (2.7-fold relative to ITA, $P<0.001$) (Figure 2). The median ADAM-17 expression level in atherosclerotic tissues ($n=20$) was 285 (239–395), and 232 (231–234) in ITA (type 0, $n=6$) (1.2-fold relative to ITA, $P<0.001$) (Figure 2).

The Illumina GWEA array results were verified with quantitative PCR. ADAM-9 expression was elevated in the carotid arteries (1.8-fold, $P<0.001$), aortas (1.7-fold, $P<0.001$), and there was trend of increase in femoral arteries (1.4-fold, $P=0.065$), respectively (Figure 3). ADAM-15 expression was not elevated in the carotid arteries (1.4-fold, $P=0.607$), but there was a significant increase in aortas (2.1-fold, $P=0.012$), and a trend of increase in femoral arteries (2.2-fold, $P=0.065$), respectively (Figure 3). ADAM-17 expression was elevated in the carotid (1.1-fold, $P<0.001$), aorta (1.4-fold, $P<0.001$), and it was not elevated in femoral artery (1.3-fold, $P=0.132$), respectively (Figure 3). No apparent difference in gene expression profiles was found between early (type 5) and advanced plaques (type 6).

ADAM protein expression in the atherosclerotic tissue

Immunohistochemistry of atherosclerotic lesions revealed that ADAM-9, ADAM-15, and ADAM-17 proteins were expressed in the atheromatous core and to some extent in all the vessel layers in early and advanced plaques in all the vessel beds while only sparse cells were positive in ITA vessels (Figure 4). In aortas ADAM-9, ADAM-15, and ADAM-17-staining cells co-distributed mainly with CD68-positive cells in the plaque but did not co-distribute with CD31-positive cells (Figure 4A). In femoral arteries, however, ADAM-9, ADAM-15, and ADAM-17-positive cells co-distributed with endothelial cells and in addition with macrophages (Figure 4B). In the carotid arteries, ADAM-9, ADAM-15, and ADAM-17-positive cells co-distributed with both macrophages and smooth muscle cells (Figure 4C). Immunofluorescence double-staining microscopy of a representative carotid plaque confirmed that

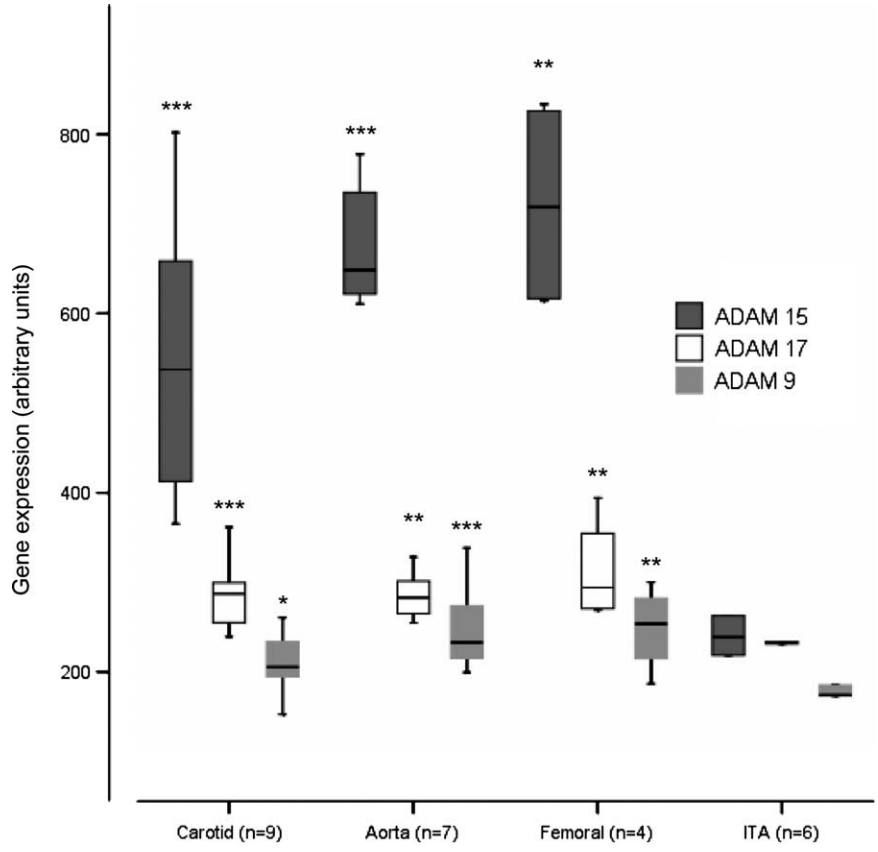

Figure 1. ADAM-9, ADAM-15, and ADAM-17 expression in control samples from internal thoracic artery (ITA), and carotid, aortic, and femoral artery samples. Gene expression value is the normalized average gene intensity for each group measured in the Illumina Expression BeadChips. *$P<0.05$, **$P<0.01$, ***$P<0.001$ relative to ITA, Mann-Whitney U-test.

ADAM-9 and ADAM-17-positive cells co-localized with CD68-positive cells (Figure 5). The staining of ADAM-9 and ADAM-17 was localized at the plasma membrane while CD68 was cytoplasmic (Figure 5). ADAM-15 staining co-localized with CD68 in the mirror sections (Figure 5). The representative figure shows expression of ADAM-9 in macrophages invading the endothelial cell layer in a neovascularization capillary within the atherosclerotic plaque (Figure 5).

The majority of ADAM-9, ADAM-15, and ADAM-17 protein was in the catalytically active form in the atherosclerotic plaques of carotid and femoral arteries and aorta (Figure 6). A putative negligible signal of unprocessed pro-form at *c.* 110 kDa was seen only after very long over-exposure (1 h) for ADAM-9 and ADAM-15 (data not shown). Hence, virtually all ADAM-9, ADAM-15, and ADAM-17 protein appears to be in the processed mature, presumably catalytically competent form.

Discussion

The present study provides novel evidence suggesting that the metalloprotease disintegrins ADAM-9, ADAM-15, and ADAM-17 (4,5) are associated with the pathophysiology of human atherosclerosis. The results corroborate those of the earlier animal model and *in vitro* studies (1) indicating roles for these ADAMs in atherosclerosis, in addition to the first demonstration of upregulation of ADAM-9 and ADAM-15 in human atherosclerotic carotid arteries (14). The combined examination with GWEA, qPCR, immunohistochemistry (IHC), and IF showed that ADAM-9, ADAM-15, and ADAM-17 expression was upregulated in advanced atherosclerosis at the transcriptional and protein levels. The results are novel as four different vascular territories were examined, and they suggest that the upregulated expression of these ADAMs is likely to be associated with monocytic cells in the atherosclerotic plaques and with smooth muscle and endothelial cells in the carotid and femoral territories, respectively. A precise picture of ADAMs expression is a prerequisite for understanding the contribution of their intricate interplay in atherosclerosis, towards which the present study takes an important step.

In the previous study by Al-Fakhri et al., the mRNA levels of ADAM-9 and ADAM-15 in human

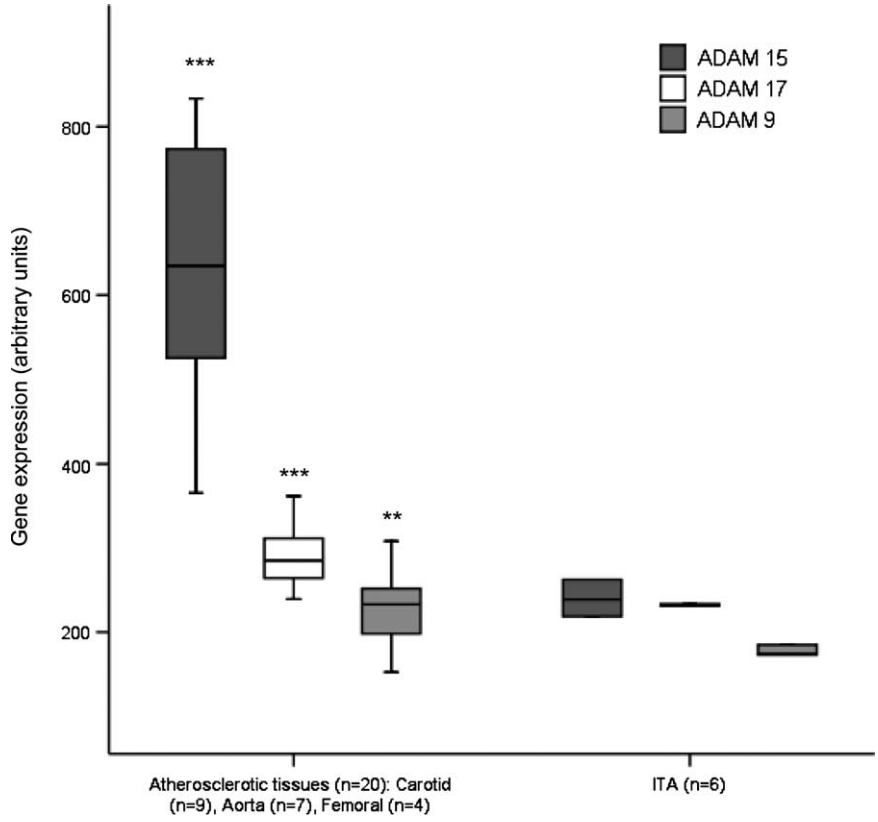

Figure 2. ADAM-9, ADAM-15, and ADAM-17 expression in atherosclerotic samples and control samples from internal thoracic artery (ITA). Gene expression value is the normalized average gene intensity for each group measured in the Illumina Expression BeadChips. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ relative to ITA, Mann-Whitney U-test.

carotid artery plaques were increased 9- and 7-fold, respectively, as compared to thyroid artery samples from subjects free of atherosclerotic disease (14). In the present study, the fold-change was lower, which might be explained by the fact that our control samples from internal thoracic artery were from subjects with coronary artery atherosclerosis and, albeit there was no histological evidence of atherosclerosis, it is obvious that the systemic nature of atherosclerosis might be reflected in the levels of ADAMs in all the vascular beds narrowing the difference in expression levels. According to the previous study, ADAM-9 and ADAM-15 were expressed in smooth muscle cells in the neointima of atherosclerotic arteries (14). Co-localization of ADAM-9, ADAM-15, and ADAM-17-positive cells with those labeled with CD68 antibody indicates that these cells are of monocytic origin, although these ADAMs were localized also to smooth muscle cells in the carotid samples. Whether the rheologic properties of different vascular beds have a different effect on expression of ADAMs in specific cell types in the plaque remains to be elucidated.

Proteolytic processing, the detachment of the pro-domain, has been associated with the maturation of at least some ADAMs, including ADAM-9 and ADAM-17, to a catalytically active form (5,6). Western analysis indicated that virtually all ADAM-9, ADAM-15, and ADAM-17 protein was in the catalytically active form in atherosclerotic lesions in carotid and femoral arteries and in aorta and hence in the catalytically competent state.

The present findings support a role of ADAM-9 in the pathology of atherosclerosis through ectodomain shedding, cell-cell, or cell-matrix interactions. ADAM-9 has been shown to be able to cleave several substrates, including heparin binding-EGF (HB-EGF) (29), and thus its upregulation might result in misregulated shedding of factors effecting atherogenesis (30). The ability of ADAM-9 to mediate cell interactions through $\alpha_6\beta_1$ and $\alpha_v\beta_5$ integrins (10,31) may also contribute to atherosclerosis since these interactions can regulate cell motility and the production of interleukin-6 (9,10).

The co-localization of ADAM-15-expressing cells with CD68 and co-distribution with CD31-positive cells in atherosclerotic areas strongly suggest that ADAM-15 on monocytic or endothelial cells might play a role in monocyte-macrophage migration, endothelial-platelet adhesion, or matrix remodeling in atherosclerosis. ADAM-15 on endothelial cells might interact with $\alpha_v\beta_3$ integrin on leukocytes,

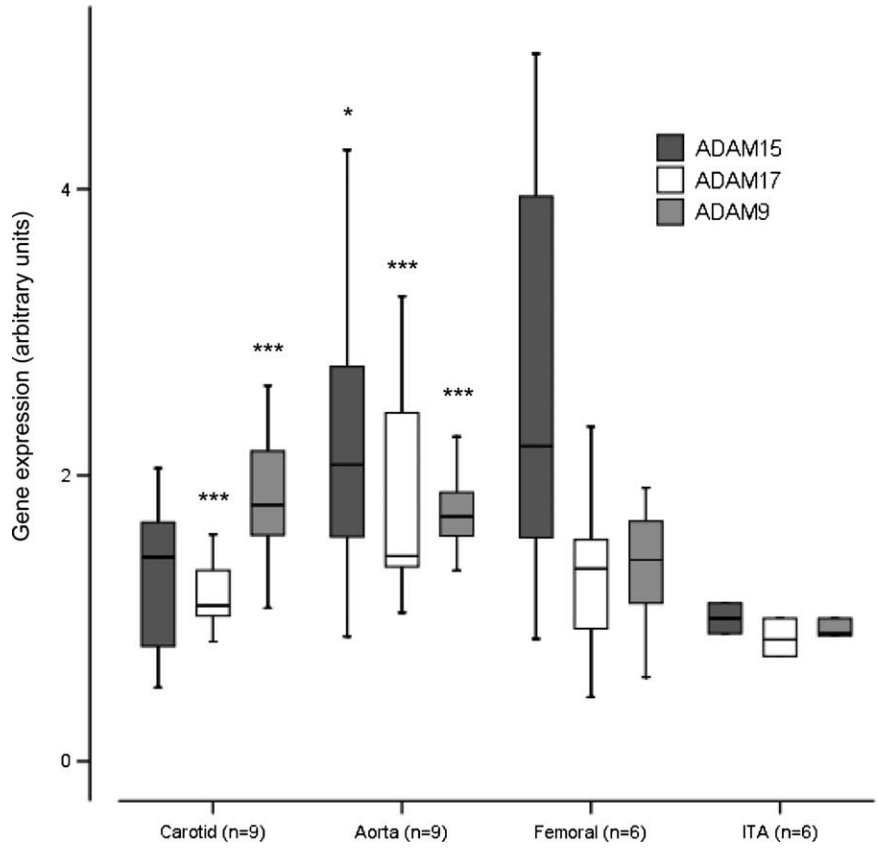

Figure 3. Expression of ADAM-9, ADAM-15, and ADAM-17 mRNA in control samples from internal thoracic artery (ITA), and carotid, aortic, and femoral artery samples measured with TaqMan Low-Density Array. $*P<0.05$, $**P<0.01$, $***P<0.001$ relative to ITA, Mann-Whitney U-test.

especially monocytes, during atherogenesis. Consistently cultured hematopoietic cells of monocytic lineage express $\alpha_v\beta_3$ integrin which was shown to bind recombinant ADAM-15 (31). Also, ADAM-15 binding to $\alpha_v\beta_3$ or $\alpha_5\beta_1$ on smooth muscle cells in atherogenic areas could modulate their migration/proliferation properties (32); however, we did not observe co-distribution of ADAM-15 with the smooth muscle cells. Furthermore, ADAM-15-mediated interactions between the endothelial cells and platelets are potentially important since the initiation, inflammation, atherogenesis, and plaque formation, including the recruitment of progenitor and dendritic cells, require adherence of platelets to the endothelial cells. This would be consistent with the platelet adhesion to ADAM-15-expressing endothelial-like cells and subsequent platelet activation and microthrombus formation in a flow chamber model (16). Given the inflammatory nature of atherosclerosis, it is interesting that in inflammatory bowel diseases, ADAM-15 is upregulated in epi- and endothelial cells in close contact with $\alpha_5\beta_1$-expressing leukocytes, suggesting a role in leukocyte migration (33). This also corroborates our hypothesis of ADAM-15 possibly contributing to monocyte-macrophage migration in atherosclerosis.

To our knowledge, the present study provides the first comprehensive immunohistochemical examination of ADAM-17 expression in human atherosclerosis. The results agree with and extend those of the earlier investigations implicating ADAM-17 in the pathology of atherosclerosis. Previously, Canault and co-authors have demonstrated elevated ADAM-17 protein levels in the lipidic core of human atherosclerotic plaques and showed with a detailed analysis of an apolipoprotein E-deficient mouse model that ADAM-17 immunostaining increased along with the development of atherosclerotic lesions (21). In a subsequent study they provided convincing evidence indicating the pathophysiological role of ADAM-17 sheddase activity in human atherosclerosis (22). The contribution of ADAM-17 in atherosclerosis is also corroborated by the studies showing increased TNF-α levels after acute myocardial infarction (34,35) and localization of TNF-α expression in atheromas (36). The ability of ADAM-17 to downregulate macrophage colony-stimulating factor receptor (M-CSFR) from macrophages undergoing activation enables

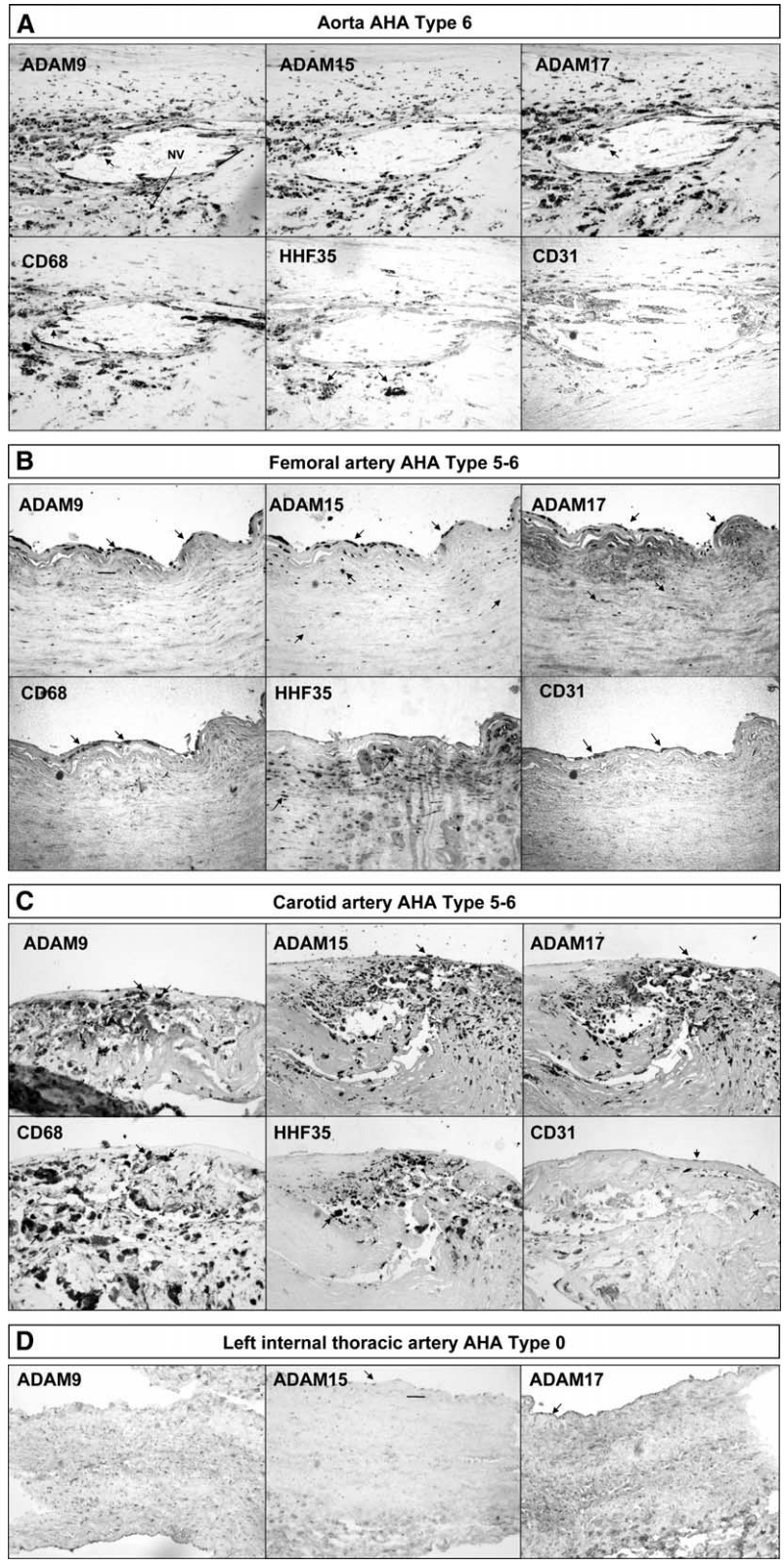

Figure 4. Expression of ADAM-9, ADAM-15, and ADAM-17 in human atherosclerotic plaques. Serial staining of monocyte marker CD68 and smooth muscle cell marker HHF35 in human aortic (A), femoral (B), and carotid (C) plaques. Samples from internal thoracic artery (ITA) served as controls (D). NV indicates a neovascularized vessel. A straight line indicates the transition between the intima and media. Arrows indicate typical positively stained cells. The stage of atherosclerosis was classified according to American Heart Association (AHA) classification (type 1–6). 100 × magnification.

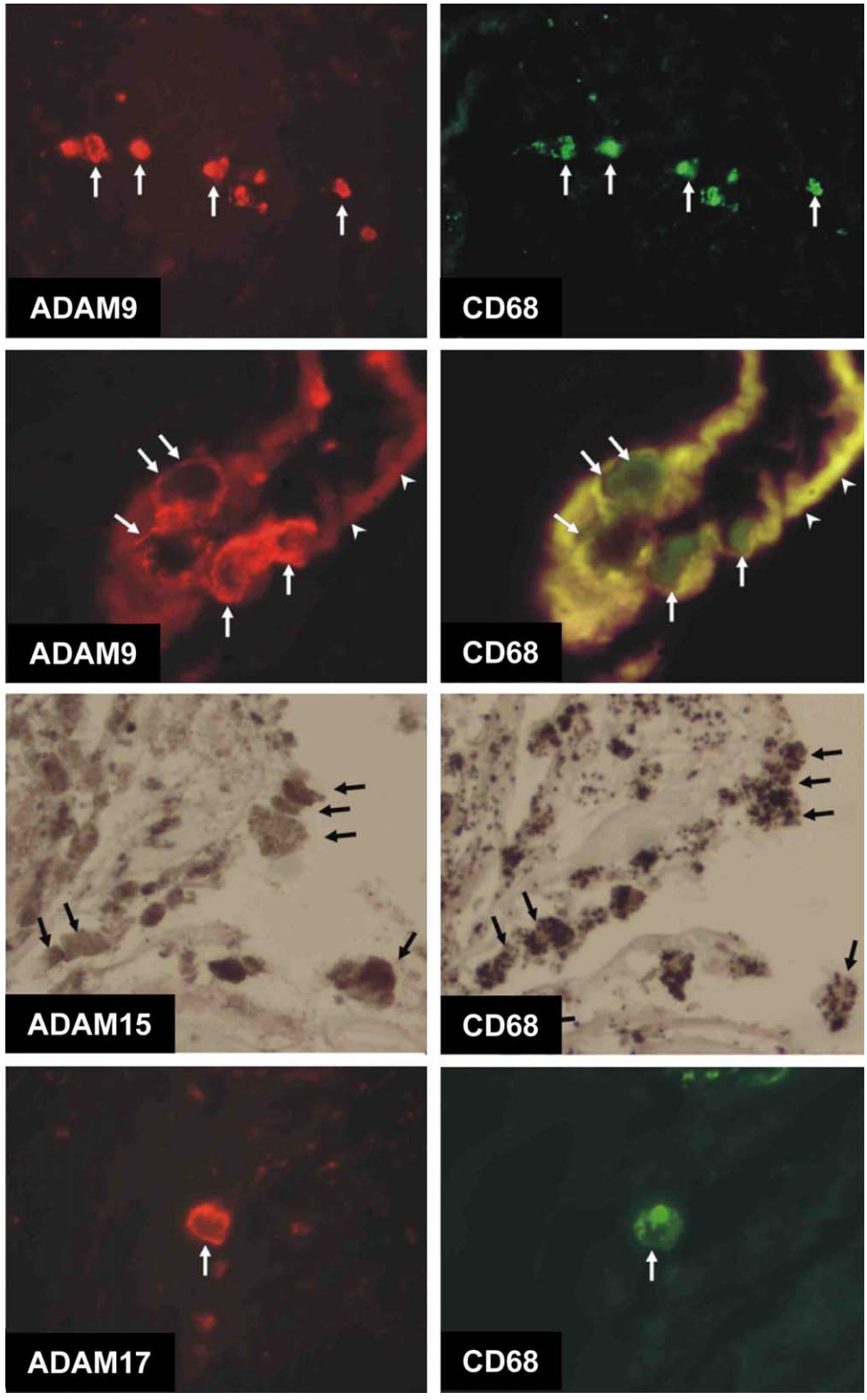

Figure 5. Double immunofluorescence images demonstrating co-localization of ADAM-9 and ADAM-17 and CD68, and mirror image sections showing the co-localization of ADAM-15 and CD68. ADAM-9-immunoreactive cells at the border of adventitia and media (arrows) are also labeled with CD68. High-magnification image showing ADAM-9/CD68-immunoreactive cells (arrows) adhered to the intima of neovascularization capillary (arrowheads point to the intima exhibiting red/yellow autofluorescence). ADAM-9 labeling is very strong in the cell membrane. Mirror image sections indicating co-localization of ADAM-15 and CD68. Arrows point to the cells immunoreactive to both antigens. Double immunofluorescence photographs demonstrating the co-localization of ADAM-17 and CD68. ADAM-17 immunoreactivity is very strong in the cell membrane whereas CD68 labeling is seen throughout the cytoplasm.

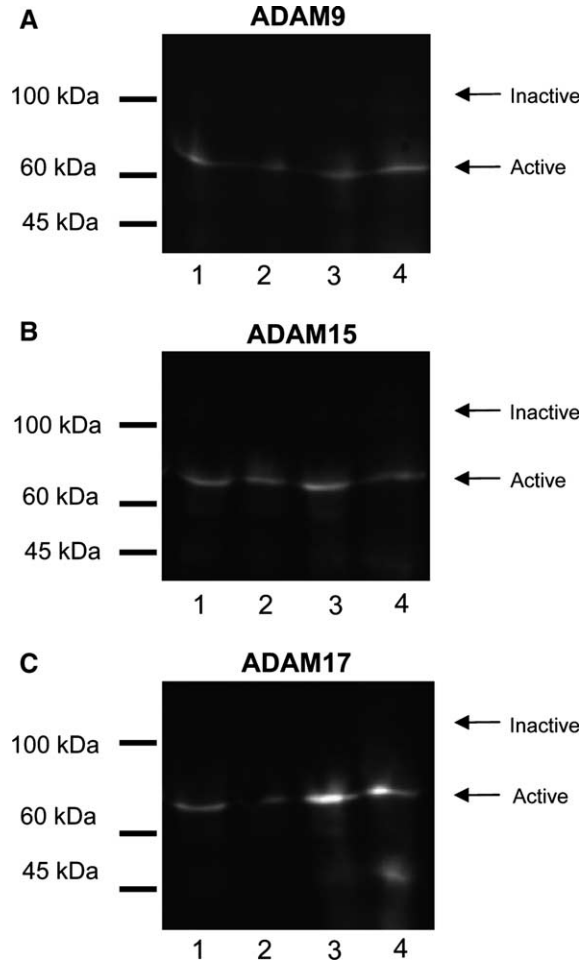

Figure 6. Western detection of (A) ADAM-9, (B) ADAM-15, and (C) ADAM-17 in atherosclerotic patient samples from carotid (1,3), femoral (2), and aortic (4) plaques, visualized with enhanced chemiluminescence. The positions of the molecular weight markers and active/inactive forms are indicated.

fully activated phenotype in mononuclear macrophages by rapidly preventing M-CSF-mediated signaling (25), suggesting that also ADAM-17 might effect macrophage function in atherogenesis. To sum up, our results combined with other studies suggest that ADAM-17 might contribute to atherosclerosis by activating and inactivating pro- and anti-inflammatory factors, respectively, and thus regulating inflammation, leukocyte adhesion, migration, and proliferation.

In conclusion, our results implicate ADAM-9, ADAM-15, and ADAM-17 in advanced atherosclerosis and suggest roles in the monocyte homing, migration, or proliferation in all major vascular beds. As the therapeutic intervention of ADAMs in human diseases is actively pursued (5,37), our findings underline the potential of targeting the development of atherosclerosis through modulation of ADAM function.

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References

1. Herren B, Raines EW, Ross R. Expression of a disintegrin-like protein in cultured human vascular cells and in vivo. FASEB J. 1997;11:173–80.
2. Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W Jr, et al. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation. 1995;92:1355–74.
3. Wolfsberg TG, Straight PD, Gerena RL, Huovila AP, Primakoff P, Myles DG, et al. ADAM, a widely distributed and developmentally regulated gene family encoding membrane proteins with a disintegrin and metalloprotease domain. Dev Biol. 1995;169:378–83.
4. Blobel CP. ADAMs: key components in EGFR signalling and development. Nat Rev Mol Cell Biol. 2005;6:32–43.
5. Huovila AP, Turner AJ, Pelto-Huikko M, Karkkainen I, Ortiz RM. Shedding light on ADAM metalloproteases. Trends Biochem Sci. 2005;30:413–22.
6. Seals DF, Courtneidge SA. The ADAMs family of metalloproteases: multidomain proteins with multiple functions. Genes Dev. 2003;17:7–30.
7. Dreux AC, Lamb DJ, Modjtahedi H, Ferns GA. The epidermal growth factor receptors and their family of ligands: their putative role in atherogenesis. Atherosclerosis. 2006;186:
38–53.
8. Popa C, Netea MG, van Riel PL, van der Meer JW, Stalenhoef AF. The role of TNF-alpha in chronic inflammatory conditions, intermediary metabolism, and cardiovascular risk. J Lipid Res. 2007;48:751–62.
9. Nath D, Slocombe PM, Webster A, Stephens PE, Docherty AJ, Murphy G. Meltrin gamma (ADAM-9) mediates cellular adhesion through alpha(6)beta(1)integrin, leading to a marked induction of fibroblast cell motility. J Cell Sci. 2000;113(Pt 12):2319–28.
10. Karadag A, Zhou M, Croucher PI. ADAM-9 (MDC-9/meltrin-gamma), a member of the a disintegrin and

metalloprotease family, regulates myeloma-cell-induced interleukin-6 production in osteoblasts by direct interaction with the alpha(v)beta5 integrin. Blood. 2006;107:3271–8.
11. Jasiulionis MG, Chammas R, Ventura AM, Travassos LR, Brentani RR. alpha6beta1-Integrin, a major cell surface carrier of beta1-6-branched oligosaccharides, mediates migration of EJ-ras-transformed fibroblasts on laminin-1 independently of its glycosylation state. Cancer Res. 1996;56:1682–9.
12. Gimond C, Baudoin C, van der Neut R, Kramer D, Calafat J, Sonnenberg A. Cre-loxP-mediated inactivation of the alpha6A integrin splice variant in vivo: evidence for a specific functional role of alpha6A in lymphocyte migration but not in heart development. J Cell Biol. 1998;143:253–66.
13. Shaw LM, Mercurio AM. Regulation of cellular interactions with laminin by integrin cytoplasmic domains: the A and B structural variants of the alpha 6 beta 1 integrin differentially modulate the adhesive strength, morphology, and migration of macrophages. Mol Biol Cell. 1994;5:679–90.
14. Al-Fakhri N, Wilhelm J, Hahn M, Heidt M, Hehrlein FW, Endisch AM, et al. Increased expression of disintegrin-metalloproteases ADAM-15 and ADAM-9 following upregulation of integrins alpha5beta1 and alphavbeta3 in atherosclerosis. J Cell Biochem. 2003;89:808–23.
15. Zhang XP, Kamata T, Yokoyama K, Puzon-McLaughlin W, Takada Y. Specific interaction of the recombinant disintegrin-like domain of MDC-15 (metargidin, ADAM-15) with integrin alphavbeta3. J Biol Chem. 1998;273:7345–50.
16. Langer H, May AE, Bultmann A, Gawaz M. ADAM 15 is an adhesion receptor for platelet GPIIb-IIIa and induces platelet activation. Thromb Haemost. 2005;94:555–61.
17. Horiuchi K, Weskamp G, Lum L, Hammes HP, Cai H, Brodie TA, et al. Potential role for ADAM15 in pathological neovascularization in mice. Mol Cell Biol. 2003;23:5614–24.
18. Charrier-Hisamuddin L, Laboisse CL, Merlin D. ADAM-15: a metalloprotease that mediates inflammation. FASEB J. 2008;22:641–53.
19. Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, et al. A metalloprotease disintegrin that releases tumour-necrosis factor-alpha from cells. Nature. 1997;385:729–33.
20. Moss ML, Jin SL, Milla ME, Bickett DM, Burkhart W, Carter HL, et al. Cloning of a disintegrin metalloprotease that processes precursor tumour-necrosis factor-alpha. Nature. 1997;385:733–6.
21. Canault M, Peiretti F, Kopp F, Bonardo B, Bonzi MF, Coudeyre JC, et al. The TNF alpha converting enzyme (TACE/ADAM17) is expressed in the atherosclerotic lesions of apolipoprotein E-deficient mice: possible contribution to elevated plasma levels of soluble TNF alpha receptors. Atherosclerosis. 2006;187:82–91.
22. Canault M, Leroyer AS, Peiretti F, Leseche G, Tedgui A, Bonardo B, et al. Microparticles of human atherosclerotic plaques enhance the shedding of the tumor necrosis factor-alpha converting enzyme/ADAM17 substrates, tumor necrosis factor and tumor necrosis factor receptor-1. Am J Pathol. 2007;171:1713–23.
23. Condon TP, Flournoy S, Sawyer GJ, Baker BF, Kishimoto TK, Bennett CF. ADAM17 but not ADAM10 mediates tumor necrosis factor-alpha and L-selectin shedding from leukocyte membranes. Antisense Nucleic Acid Drug Dev. 2001;11:107–16.
24. Gomez-Gaviro M, Dominguez-Luis M, Canchado J, Calafat J, Janssen H, Lara-Pezzi E, et al. Expression and regulation of the metalloprotease ADAM-8 during human neutrophil pathophysiological activation and its catalytic activity on L-selectin shedding. J Immunol. 2007;178:8053–63.
25. Rovida E, Paccagnini A, Del Rosso M, Peschon J, Dello Sbarba P. TNF-alpha-converting enzyme cleaves the macrophage colony-stimulating factor receptor in macrophages undergoing activation. J Immunol. 2001;166:1583–9.
26. Laaksonen R, Katajamaa M, Paiva H, Sysi-Aho M, Saarinen L, Junni P, et al. A systems biology strategy reveals biological pathways and plasma biomarker candidates for potentially toxic statin-induced changes in muscle. PLoS ONE. 2006;1:e97.
27. Huovila AP, Eder AM, Fuller SD. Hepatitis B surface antigen assembles in a post-ER, pre-Golgi compartment. J Cell Biol. 1992;118:1305–20.
28. Shi SR, Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem. 1991;39:741–8.
29. Izumi Y, Hirata M, Hasuwa H, Iwamoto R, Umata T, Miyado K, et al. A metalloprotease-disintegrin, MDC9/meltrin-gamma/ADAM9 and PKCdelta are involved in TPA-induced ectodomain shedding of membrane-anchored heparin-binding EGF-like growth factor. EMBO J. 1998;17:7260–72.
30. Miyamoto S, Yagi H, Yotsumoto F, Kawarabayashi T, Mekada E. Heparin-binding epidermal growth factor-like growth factor as a novel targeting molecule for cancer therapy. Cancer Sci. 2006;97:341–7.
31. Nath D, Slocombe PM, Stephens PE, Warn A, Hutchinson GR, Yamada KM, et al. Interaction of metargidin (ADAM-15) with alphavbeta3 and alpha5beta1 integrins on different haemopoietic cells. J Cell Sci. 1999;112(Pt 4):579–87.
32. Moiseeva EP. Adhesion receptors of vascular smooth muscle cells and their functions. Cardiovasc Res. 2001;52:372–86.
33. Mosnier JF, Jarry A, Bou-Hanna C, Denis MG, Merlin D, Laboisse CL. ADAM15 upregulation and interaction with multiple binding partners in inflammatory bowel disease. Lab Invest. 2006;86:1064–73.
34. Maury CP, Teppo AM. Circulating tumour necrosis factor-alpha (cachectin) in myocardial infarction. J Intern Med. 1989;225:333–6.
35. Heinisch RH, Zanetti CR, Comin F, Fernandes JL, Ramires JA, Serrano CV Jr. Serial changes in plasma levels of cytokines in patients with coronary artery disease. Vasc Health Risk Manag. 2005;1:245–50.
36. Barath P, Fishbein MC, Cao J, Berenson J, Helfant RH, Forrester JS. Detection and localization of tumor necrosis factor in human atheroma. Am J Cardiol. 1990;65:297–302.
37. Moss ML, Bartsch JW. Therapeutic benefits from targeting of ADAM family members. Biochemistry. 2004;43:7227–35.