RIIKKA MÄKELÄ

Role of -463G/A Promoter Polymorphism of Myeloperoxidase in the Development of Atherosclerosis

Clinical and Autopsy Studies

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ACADEMIC DISSERTATION
To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the Auditorium of Finn-Medi 1,
Biokatu 6, Tampere, on March 27th, 2009, at 12 o'clock.

UNIVERSITY OF TAMPERE

ACADEMIC DISSERTATION
University of Tampere, Medical School
Tampere University Hospital, Centre for Laboratory Medicine and Department of Clinical Chemistry, Laboratory of Atherosclerosis
Tampere Graduate School in Biomedicine and Biotechnology (TGSBB)
Finland

Supervised by
Professor Terho Lehtimäki
University of Tampere
Finland
Professor Pekka Karhunen
University of Tampere
Finland

Reviewed by
Professor Marja-Leena Kortelainen
University of Oulu
Finland
Docent Ken Lindstedt
University of Helsinki
Finland

Distribution
Bookshop TAJU
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Tel. +358 3 3551 6055
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Cover design by
Juha Siro

Acta Universitatis Tamperensis 1383
ISBN 978-951-44-7597-9 (print)
ISSN 1455-1616

Acta Electronica Universitatis Tamperensis 808
ISBN 978-951-44-7598-6 (pdf)
ISSN 1456-954X
http://acta.uta.fi

Tampereen Yliopistopaino Oy – Juvenes Print
Tampere 2009

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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications, refered to in the text by their Roman numerals I-V.

I Mäkelä R, Laaksonen R, Janatuinen T, Vesalainen R, Nuutila P, Jaakkola O, Knuuti J and Lehtimäki T (2004): Myeloperoxidase gene variation and coronary flow reserve in young healthy men. J Biomed Sci 11:59-64.

II Mäkelä R, Loimaala A, Nenonen A, Mercuri M, Vuori I, Huhtala H, Oja P, Bond G, Koivula T and Lehtimäki T (2008): The association of myeloperoxidase promoter polymorphism with carotid atherosclerosis is abolished in patients with type 2 diabetes. Clin Biochem 41:532-537.

III Mäkelä R, Karhunen PJ, Kunnas TA, Ilveskoski E, Kajander OA, Mikkelsson J, Perola M, Penttilä A and Lehtimäki T (2003): Myeloperoxidase gene variation as a determinant of atherosclerosis progression in the abdominal and thoracic aorta: an autopsy study. Lab Invest 83:919-925.

IV Mäkelä R, Dastidar P, Jokela H, Jaakkola O, Saarela M, Punnonen R and Lehtimäki T (2006): Relation of myeloperoxidase promoter polymorphism and long-term hormone replacement therapy to oxidized low-density lipoprotein autoantibodies in postmenopausal women. Scand J Clin Lab Invest 66:371-383.

V Mäkelä R, Dastidar P, Jokela H, Saarela M, Punnonen R and Lehtimäki T (2003): Effect of long-term hormone replacement therapy on atherosclerosis progression in postmenopausal women relates to myeloperoxidase promoter polymorphism. J Clin Endocrinol Metab 88:3823-3828.

ABBREVIATIONS

ACS	acute coronary syndrome
AN(C)OVA	analysis of (co)variance
apo	apolipoprotein
ASC	atherosclerosis severity score
BMI	body mass index
CAD	coronary artery disease
CFR	cononary flow reserve
CHD	coronary heart disease
Cl^-	chloride ion
CRP	c-reactive protein
CVD	cardiovascular disease
DM	diabetes mellitus
DNA	deoxyribonucleic acid
EC(s)	endothelial cell(s)
ELISA	enzyme-linked immunosorbent assay
EV	estradiol valerate
EVP	estradiol valerate and combinated levonorgestrel
ERα	estrogen receptor alpha
FGF	fibroblast growth factor
H_2O_2	hydrogen peroxide
HDL	high-density lipoprotein
HOCl	hypochlorous acid
HRT	hormone replacement therapy
HSDS	Helsinki Sudden Death Study
HTA	hypertension arterialis
IL	interleukin
IMT	intima-media thickness
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
LO	lipoxygenase
LOOH	lipid hydroperoxide
LSD	Least Significant Difference post-hoc test

LV	left ventricle
MBF	myocardial blood flow
MCP	monocyte chemotactic protein
MDA	malondialdehyde
MDA-LDL	malondialdehyde-modified LDL
MI	myocardial infarction
MMP	matrix metalloproteinase
MPO	myeloperoxidase
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
NAP	number of atherosclerotic plaques
NO	nitric oxide
O_2^-	superoxide
$^{\cdot}OH$	hydroxyl radical
oxLDL	oxidized LDL
oxLDL-abs	autoantibodies againts oxidized LDL
PCR	polymerase chain reaction
PET	positron emission tomography
PMNLs	polymorphonuclear leukocytes
PPAR-γ	peroxisome proliferator-activated receptor gamma
RNA	ribonucleic acid
ROS	reactive oxygen species
RPP	rate-pressure product
SD	standard deviation
SMC(s)	smooth muscle cell(s)
SNP	single-nucleotide polymorphism
SP1	SP1 nuclear transcription factor
SR	scavenger receptor
TF	tissue factor
TG(s)	triglyceride(s)
Th1	type 1 helper T-cell
Th2	type 2 helper T-cell
TNF-α	tumor necrosis factor alpha
VLDL	very low density lipoprotein
XO	xanthine oxidase

ABSTRACT

Background. Atherosclerosis results from a combination of environmental risk factors and genetic susceptibility which contribute individually in the clinical expression of cardiovascular disease. The atherosclerotic disease process is considered to be a chronic proliferative inflammatory response involving the extravasation and accumulation of blood inflammatory cells i.e., monocyte-macrophages and lymphocytes in the arterial intima. Myeloperoxidase (MPO) is a heme peroxidase found in neutrophils, monocytes and tissue macrophages. In addition to the essential role of MPO as a component of the innate immune responses, both MPO and its reactive oxidants are enriched in human atheroma. The MPO gene has a functional G/A promoter polymorphism (rs2333227) at position -463, which affects the transcription efficiency of the GG, AG and AA genotypes and creates an estrogen receptor α (ERα) binding site to the A-allele.

Objectives. To investigate the association between MPO -463G/A (rs2333227) genotypic variation and lipoprotein oxidation, coronary reactivity, the carotid artery intima-media thickness (IMT) and the development of early and advanced atherosclerotic lesions in the thoracic and abdominal aortas was analyzed. In addition, as the ERα is known to bind more effectively to A-allele than to G-allele, the association of MPO genotype dependent effect on atherosclerosis progression during long-term hormone replacement therapy (HRT) was assessed.

Subjects and Methods. The study was based on five different study populations (Studies **I-V**), comprising a total of 685 individuals. In Study **I**, the association of the MPO genotypes with the coronary blood flow was examined in 49 healthy but mildly hypercholesterolemic men. In Study **II,** the link between MPO genotypes and carotid IMT was studied in 37 men with type 2 diabetes mellitus (DM) and in 161 non-diabetic middle-aged men. In Study **III,** the relationship between the MPO genotypes and autopsy-confirmed areas of different types of atherosclerotic lesions in the abdominal and thoracic aorta was examined in 266 middle-aged men. In Study **IV**, the effect of the MPO genotype on lipoprotein oxidation was studied in 87 women with long-term HRT. In Study **V**, the effect of MPO genotypes on the progression of atherosclerosis was studied in the same study population.

Results. In Study **I,** the GG genotype carriers had 18.1% lower coronary flow reserve than A-allele carriers. In Study **II**, the non-diabetic subjects GG homozygotes had lower overall carotid IMT than A-allele carriers whereas no genotype dependent association was found among men with type 2 DM. In the autopsy Study **III,** the MPO GG genotype carriers had smaller area of fibrotic and calcified lesions in the abdominal aorta. The association weakened with advancing age. In Study **IV,** the MPO GG genotype carriers on long-term HRT were found to have higher values of

antibodies against low-density lipoprotein than women without treatment. In Study **V**, the progression of atherosclerosis was faster in non-treated controls than HRT users among the GG homozygotes whereas no such association was found among A-allele carriers.

Conclusions. We conclude that the MPO -463G/A (rs2333227) polymorphism is a genetic marker for atherosclerosis. Postmenopausal females carrying the A-allele may benefit less from long-term HRT.

TIIVISTELMÄ

Tausta. Ateroskleroosi on vuosikymmenien aikana kehittyvä sairaus, jonka ilmiasuun vaikuttavat sekä perinnöllinen sairastumisalttius sekä ympäristötekijät. Ateroskleroosia pidetään kroonisena inflammaatioprosessina jolle on tyypillistä rasvan ja tulehdussolujen, kuten monosyytti-makrofagien ja lymfosyyttien, kertyminen valtimon seinämään. Myeloperoksidaasi (MPO) on neutrofilien, monosyyttien ja makrofagien erittämä hemientsyymi ja osa immuunipuolustuksen hapettamisjärjestelmää. Sekä MPO entsyymiä että sen reaktiotuotteita on pystytty osoittamaan ateroskleroosimuutoksista. MPO geenin säätelijä- eli promoottorialueella esiintyy perinnöllistä vaihtelua, polymorfismia. Geenin aktiivisuuteen vaikuttavan -463G/A pistemutaation (rs2333227) seurauksena väestössä esiintyy kolme erilaista genotyyppiä GG, AG ja AA. Lisäksi pistemutaation seurauksena A-alleeliin syntyy estrogeenireseptori α (ERα) sitoutumiskohta.

Tavoite. Väitöskirjatyön tavoitteena oli selvittää MPO geenin perinnöllisen vaihtelun yhteyttä lipoproteiinien hapettumiseen, sepelvaltimoiden virtauksen reserviin, kaulavaltimoiden seinämäpaksuuteen sekä rinta- ja vatsa-aortan ateroskleroottisiin muutoksiin. Koska ERα tiedetään sitoutuvan tehokkaammin A-alleeliin, tutkittiin MPO genotyypin vaikutusta lipoproteiinien hapettumiseen sekä ateroskleroosin etenemiseen pitkäaikaisen hormonikorvaushoidon aikana.

Aineisto ja menetelmät. Tutkimus perustuu viiteen tutkimussarjaan, joihin kuului yhteensä 685 tutkittavaa. Ensimmäisessä osatyössä tutkittiin MPO genotyypin vaikutusta selpelvaltimokierron reserviin 49 lievästi hyperkolesterolemisten mutta muutoin terveiden miesten aineistossa. Toisessa osatyössä tutkittiin MPO genotyypin yhteyttä kaulavaltimoiden sisäseinämien ultraäänitutkimuksen tuloksiin ei-diabeetikoilla ja tyypin 2 diabetesta sairastavilla 196 keski-ikäisen miehen aineistossa. Kolmannessa osatyössä selvitettiin MPO genotyypin yhteyttä vatsa- ja rinta-aortan ateroskleroottisiin muutoksiin 266 keski-ikäisen miehen ruumiinavausaineistossa. Neljännessä osatyössä tutkittiin MPO genotyypin vaikutusta LDL-kolesterolin hapettumiseen hormonikorvaushoidon aikana 87 naisen aineistossa. Viidennessä osatyössä tutkittiin samassa aineistossa MPO genotyypin vaikutusta ateroskleroottisten muutosten etenemiseen hormonikorvaushoidon aikana.

Tulokset. Ensimmäisessä osatyössä GG genotyyppiä kantavilla nuorilla miehillä oli alhaisempi sepelvaltimokierron reservi kuin A-alleelin kantajilla. Toisessa osatyössä todettiin GG genotyypin kantajilla matalampi kaulasuonten seinämäpaksuus kuin A-alleelin kantajilla, mutta tyypin 2 diabeetikoilla eroa genotyyppien välillä ei tullut esiin. Kolmannessa osatyössä todettiin GG genotyypin kantajilla olevan pienemmät fibroottiset ja kalkkeutuneet ateroskleroottiset

muutokset vatsa-aortassa kuin A-alleelin kantajilla mutta yhteys heikkeni ikäriippuvaisesti. Neljännessä osatyössä todettiin hormonikorvaushoitoa saaneilla GG genotyypin kantajilla korkeammat hapettunutta LDL-kolesterolia vastaan muodostuneiden vasta-aineiden tasot kuin niillä GG genotyypin kantajilla jotka eivät saaneet hormonikorvaushoitoa. Viidennessä osatyössä todettiin ateroskleroosin etenemisen olevan hitaampaa niillä GG genotyypin kantajilla jotka saivat hormonikorvaushoitoa verrattuna lääkkeettömiin kontrolleihin, kun taas A-alleelin kantajilla ei vastaavaa yhteyttä todettu.

Johtopäätökset. Yhteenvetona väitöskirjatutkimuksen tuloksista voi todeta että MPO -463G/A (rs2333227) polymorfismi on yhteydessä ateroskleroosin ilmiasuun ja MPO genotyyppi toimii ateroskleroosin geneettisenä markkerina. MPO -463G/A polymorfismin A-alleelia kantavien naisten saama hyöty postmenopausaalisesta hormonikorvaushoidosta saattaa jäädä vähäisemmäksi kuin GG genotyypin kantajilla.

INTRODUCTION

Atherosclerosis of large and medium sized arteries is the main cause of cardiovascular disease (CVD) which manifests as coronary artery disease (CAD), acute myocardial infarction (MI), stroke and peripheral vascular disease (Ross 1993, Ross 1999, Lusis 2000). By the end of the 20th century, CAD has become the most common cause of death and premature invalidity in the world (Murray and Lopez 1997, Glass and Witztum 2001). Therefore, an intense discussion about atherosclerosis prevention has been ongoing in recent decades (LaRosa 1999) and several risk factors such as age, sex, hypertension, diabetes mellitus (DM), hypercholesterolemia and smoking have been investigated in epidemiological studies (Criqui 1986). Most of the risk scores for clinical use are based on these traditional risk factors; i.e. Framingham Study and the European SCORE –research (Wilson et al. 1998, Conroy et al. 2003). Moreover, a large number of studies have demonstrated an association of family history with CVD, suggesting that inherited genetic factors may play an important role in disease progression. There are rare high-risk single gene defects contributing to atherosclerosis but most commonly CVD is believed to be multifactorial and result from a combination of many genes which interact with the environment and other genes leading to individual phenotypes with a different risk of progression of the disease (Lloyd-Jones et al. 2004, Arnett et al. 2007, Parikh et al. 2007, Zhao et al. 2007). Twin studies have demonstrated that the heritability of CVD has an inverse relationship with age. According to these studies in older age environmental risk factors seem to influence disease progression more than genetic factors (Sorensen et al. 1988, Marenberg et al. 1994). In recent years several candidate genes have been evaluated and pooling this genetic risk data with environmental risk factors has led to a promising improvement in the prediction of CAD (Humphries et al. 2007, Morrison et al. 2007).

Several hypothese on atherosclerosis have been evolved ever since the first observations of the plaque morphology in the 19th century (Stocker and Keaney 2004, Langheinrich and Bohle 2005). The demonstration of oxidatively modificated low-density lipoprotein (oxLDL) in the atherosclerotic plaque in the late 1970s and 1980s (Ylä-Herttuala et al. 1989, Glass and Witztum 2001) resulted in the oxidative modification hypothesis of atherosclerosis in 1989 (Steinberg et al. 1989) which has contributed over the last decade to the theory of atherosclerosis as an inflammatory disease (Ross 1999). The cornerstone of the classification of atherosclerotic plaque development is a six-stage process based on the morphological and histological findings (Stary et al. 1992, Stary et al. 1994, Stary et al. 1995, Stary 2000). According to this model, the initial atherosclerotic lesion can be seen already in the fetal aortas and is characterized by the accumulation of low density lipoprotein (LDL) and lipid droplets containing macrophages in the

intima of the arterial wall (Pearson et al. 1983, Napoli et al. 1997). According to current knowledge, the event behind the earliest Type I and II atherosclerotic plaques is characterized by the presence of an excessive number of inflammatory cells as activated T lymphocytes and monocyte-derived macrophages. Inflammatory cellular and molecular events are involved in every stage of the atherosclerosis and the long-lasting inflammatory injury seems to be the basis for the development of mature type IV lesions into symptomatic type V and VI plaques which finally lead to clinical symptoms of CVD (Stary et al. 1995, Ross 1999, Mullenix et al. 2005).

Myeloperoxidase (MPO), an abundant oxidative hemoprotein compound is expressed in activated neutrophils, monocytes and macrophages in human atheroma (Daugherty et al. 1994, Hazen and Heinecke 1997). MPO is part of the host defense system of the phagocytes and responsible for microbicidal activity against a wide range of organisms. The main function of MPO enzyme is to generate oxidants and contribute to the immune defence system (Nauseef et al. 1988, Hurst and Barrette 1989, Klebanoff 1999). On the other hand, MPO has also been suggested to have an important role in the propagation of CVD (Hoy et al. 2002, Nicholls and Hazen 2005) and the oxidative compounds as well as the reaction remnants produced by MPO are found in atherosclerotic lesion (Daugherty et al. 1994, Hazen and Heinecke 1997). Elevated blood levels of MPO have been found to predict the presence of CAD (Zhang R et al. 2001). In chest pain patients, initial plasma MPO levels significantly predict the risk of MI, even in patients who are negative for troponin at baseline (Brennan et al. 2003). MPO also plays a role in the vascular signaling and vasodilatory function of nitric oxide (NO) (Eiserich et al. 2002). MPO gene promoter area has a functional G to A single nucleotide polymorphism (SNP, rs2333227) at site -463 (Piedrafita et al. 1996, Reynolds et al. 1997). The G-allele promotes binding by SP1 nuclear transcription factor (SP1) and is associated with stronger promoter activity and gene expression, whereas the A-allele creates a binding site for an estrogen receptor α (ERα) (Piedrafita et al. 1996, Reynolds et al. 1997). The association of MPO -463G/A polymorphism (rs2333227) with MPO activity is age and gender-dependent (Rutgers et al. 2003) and several studies also imply that this also has an effect on the development of CVD (Nikpoor et al. 2001, Pecoits-Filho et al. 2003b). A considerable number of SNPs similar to -463G/A are scattered in the human genome and they appear to be a useful tool in the study of polygenic disorders such as CVD. As in the case of our candidate gene, many SNPs have functional consequences if they occur in gene coding or regulatory site and make possible to directly test for association between a phenotype and a functional variant (Pecoits-Filho et al. 2003a).

In this thesis, the effect of MPO -463G/A promoter polymorphism (rs2333227) on atherosclerotic biomarkers and atherosclerotic development was investigated in five different

study series, representing different developmental stages and backrounds of atherosclerosis. In the first of these studies positron emission tomography (PET) was used to study the effect of MPO polymorphism on the early markers of atherosclerosis i.e., coronary function and reactivity. Also, the effect of MPO polymorphism on autoantibodies against oxLDL (oxLDL-abs) was studied. In the second study, the interaction effect of type 2 DM and MPO polymorphism on carotid intima-media thickness (IMT) was studied. In the third study, the effect of MPO genotypes on atherosclerotic lesion areas in the abdominal and thoracic aortas was evaluated in an autopsy series of the Helsinki Sudden Death Study (HSDS). In the fourth study, the effect of MPO polymorphism and long-term hormone replacement therapy (HRT) on oxLDL-abs was studied. In the last study, the effect of MPO polymorphism and long-term HRT on ultrasonographically measured atherosclerosis progression was assessed.

REVIEW OF THE LITERATURE

1. Pathogenesis of atherosclerosis

1.1. Structure of the artery wall

The normal muscular and elastic arteries consist of three morphologically distinct layers; namely the intima, media and adventitia. In the primary situation, the arteries have a very simple tissue structure; the cell types appearing in the intima and media are the endothelial cells (ECs) smooth muscle cells (SMCs) and in some individuals isolated macrophages. In the adventitia, fibrocytes are also found (Geer et al. 1961).

Intima. The intima is the innermost, narrow region on the luminal side of the artery. Most of the pathological changes of atherosclerosis develop in this layer. It is covered with a single continuous layer of ECs which are bound to the basement membrane. ECs synthesize and secrete the extracellural matrix components such as fibronectin, type IV and type V collagen, laminin and proteoglycans and they regulate the permeability of macromolecules, thrombolysis, vascular tone and immune responses. The subendothelium is divided into the proteoglycan and musculoelastic layers. The proteoglycan-rich layer consists of connective tissue, macrophages and isolated cells of synthesizing type of SMCs. The musculoelastic layer underlies the proteoglycan layer and contains more SMCs, elastic fibers and collagen than the inner layer. SMCs are involved in the contractility, structural maintenance and lipid metabolism of the intima. The intima is separated from the media by internal elastic lamina (Geer et al. 1961, Ross and Glomset 1973, Wight and Ross 1975, Ross and Glomset 1976, Stary 1987, Stary et al. 1992).

Media. The media layer is situated under the internal elastic lamina and is manifested as diagonally oriented SMCs attached to each other surrounded by collagen, small elastic fibers and proteoglycans. The synthesizing SMC type produces collagen whereas the contractile SMC type is involved in vasodilatation and vasoconstriction of the artery. The media layer is surrounded by the external elastic lamina, which forms an elastic border with the third outermost layer, called the adventitia (Geer and Haust 1972, Ross and Glomset 1976).

Adventitia. The adventitia is separated from the media by the external elastic lamina and consists of fibroblasts and SMCs surrounded by proteoglycans and type I collagen. The vasa vasorum provide blood supply to the wall structures of in large arteries, where the media has several layers of SMCs (Ross and Glomset 1973, Ross and Glomset 1976, Gulbenkian et al. 1993).

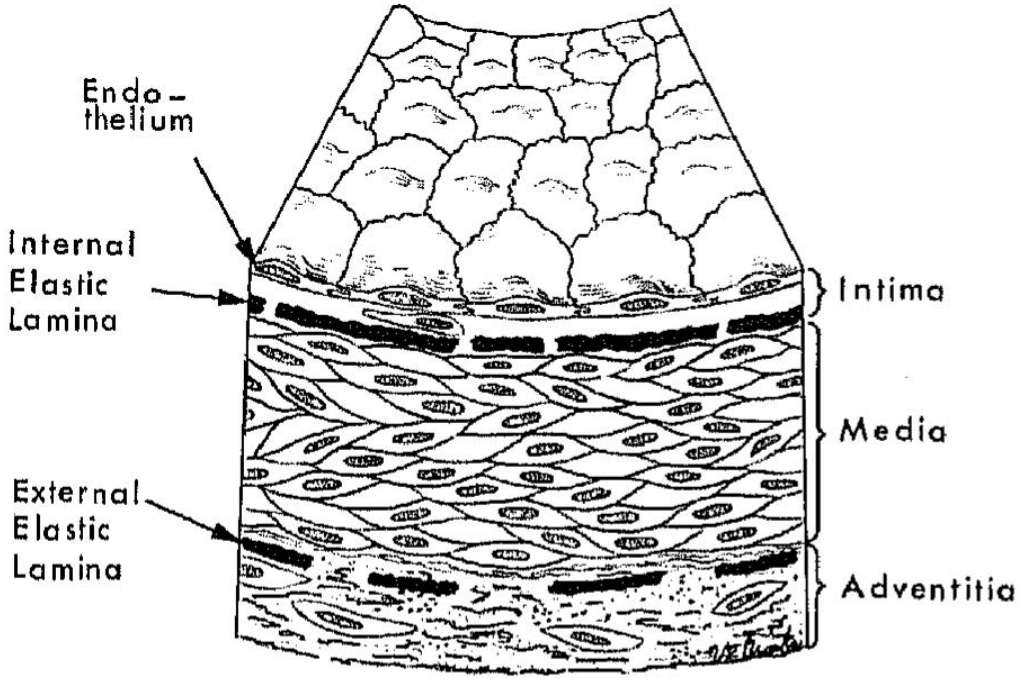

Figure 1. Structure of the artery wall.

See text for details. Modified from Ross and Glomset (1976): The pathogenesis of atherosclerosis (First of two parts). N Engl J Med 295:369-377.

1.2. Definition and classification of atherosclerosis

Atherosclerosis is a disease characterized by focal thickening of the intimal layer of the artery with accumulated fatty deposits. It affects particularly large arteries such as the aorta and iliac, femoral, coronary and cerebral arteries where it is distributed to diffuse plaques (Ross and Glomset 1973, Ravensbergen et al. 1998). The initiation, speed of progression and the phenotype of atherosclerotic plaques are artery-related. Foam cell lesions are frequent in the carotid arteries probably explaining the dynamics in carotid IMT. In the femoral arteries, the atherosclerosis development is slow and dominated by fibrous plaques. High prevalence of lipid core plaques is typical for coronary arteries in subjects dying of CAD (Dalager et al. 2007).

In the 1960's the International Atherosclerosis Project (IAP) launched an international survey aiming to describe the various types of atherosclerotic lesions. Methodologically, the

involvement of atherosclerosis was based on visual evaluation after a standard staining process and was graded visually as fatty streaks, fibrous plaques, complicated lesions and calcification (Guzman et al. 1968).

In the 1990's a new classification of atherosclerotic lesions was presented by the Committee on Vascular Lesions of the Council on Atherosclerosis, American Heart Association (AHA). The classification was based on cross-sectional microscopic examination of the histology and the histochemical composition of the cell and the matrix components of the lesion. The first report provided a definition of the arterial intima and atherosclerosis-prone regions (Stary et al. 1992). Initial lesions were further defined as type I lesions and fatty streaks as type II lesions (Stary et al. 1994). Intermediate lesions (type III) were followed by advanced lesions which were classified by the numerals IV (atheroma), V (fibroateroma) and VI (complicated lesion) (Stary et al. 1995). In 2000, the classification was updated and the type II lesion was subdivided into progression-prone (IIa) and progression-resistant (IIb) phenotypes (Stary 2000).

Table 1. Classification of atherosclerotic lesions.

	Histological classification of atherosclerotic lesions by the AHA (Stary et al. 1994, Stary et al. 1995)	Macroscopic classification by the International Atherosclerosis Project (IAP) (Guzman et al. 1968)
Intimal Thickening		Usually not visible or may be mistaken for a raised lesion
Early lesions		
Type I	Intial lesion	Usually not visible, fatty dots
Type IIa	Progression-prone type II	Fatty streak, fatty dots
Type IIb	Progression-resistant type II	
Intermediate lesions		
Type III	Preatheroma	Fatty streak or fibrous plaque
Advanced lesions		
Type IV	Atheroma	Fibrous plaque
Type Va	Fibroatheroma (type V lesion)	Fibrous plaque
Type Vb	Calcified lesion (type VII lesion)	Calcified plaque
Type Vc	Fibrotic lesion (type VIII lesion)	Fibrous plaque
Type VI	Lesion with surface disruption, hematoma/hemorrhage or thrombosis	Complicated lesion

Abbreviations: AHA, American Heart Association.

1.3. Pathogenesis and development of atherosclerotic lesions

1.3.1. Hypothesis of atherogenesis

During decades of research on atherosclerosis many hypotheses have been evinced to explain the complex events associated with disease development. During early atherogenesis, several processes are present simultaneously including endothelial injury or activation including shear stress-related events, local adherence of platelets, lipoprotein oxidation and aggregation, macrophage chemotaxis and foam cell formation, likewise SMC migration, proliferation and phenotypic alteration. However, each of these involves inflammation as a crucial component of atherosclerosis (Williams and Tabas 1995).

The response-to-injury hypothesis. The very early hypotheses regarding atherosclerosis suggested the disease progression to be a rather a passive deposition with no active cellular component (Stocker and Keaney 2004). In the 1970s the hypothesis of atherogenesis was augmented by the theory of compensatory mechanisms following the physical endothelial injury. The mechanisms included the migration of SMCs and the recruitment of macrophages into the vessel wall and furthermore the continuing inflammatory processes (Ross and Harker 1976). However, the endothelium may be intact in most stages of lesion progression (Stary et al. 1994).

The response-to-retention hypothesis. In 1995, the response-to-retention hypothesis was evinced suggesting lipoprotein retention as the triggering event in atherosclerosis development. The key event is the retention of lipoproteins within the vessel wall (Williams and Tabas 1995, Williams and Tabas 1998, Skalen et al. 2002). Retained lipoproteins are then modified into enzymatic, oxidative and other pathways (Tabas et al. 1993, Schissel et al. 1996, Zhang et al. 2000, Guyton 2001, Pentikäinen et al. 2002, Stocker and Keaney 2004, Öörni et al. 2004). According to this hypothesis, the role of shear stress in early atherogenesis is mediated through the synthesis of lipoprotein retention promoting molecules which is necessary to cause lesion in the normal artery (Munro and Cotran 1988).

The oxidative modification hypothesis. The oxidative modification theory is based on the observation that the native LDL itself is not atherogenic but needs to be chemically modified to enter the macrophages through the scavenger receptor (SR). While LDL accumulates in the subendothelial space of lesion-prone arterial sites it is subject to oxidation which leads to negatively charged lipoprotein particles (Ylä-Herttuala et al. 1989). Recent data on failure of antioxidant supplements to lower CAD events has led to a hypothesis that oxidation includes both

positive and negative actions dependent in cell type and location (Fuster et al. 1999, Ross 1999, Libby 2001, Kritharides and Stocker 2002, Corti et al. 2004, Williams and Fisher 2005).

1.3.2. Endothelial dysfunction in the development of early atherosclerotic lesions

The ECs of normal muscular and elastic arteries form a continuous layer of flattened and elongated cells. With the exception of areas of turbulent flow and reduced shear the ECs are oriented in the direction of flow (Vane et al. 1990, Stary et al. 1992). The normal endothelium does not support the attachment of circulating immune cells (Libby 2002). The endothelium produces a number of vasodilator substances such as NO which, besides vasodilatation, also inhibits monocyte adhesion and platelet aggregation (Ogita and Liao 2004). The disruption of endothelial homeostasis is still considered to be the crucial event in the inflammatory process, which further evolves into plaque progression and degeneration (Landmesser et al. 2004). Physiological stress includes both local and systemic factors such as lipid accumulation, mechanical denudation, oxidative stress, genetic variability and shear stress. Endothelial dysfunction is functionally and morphologically well characterized. The main elements of the endothelial response to injury are adhesiveness, permeability, proliferation and thrombogenesis, each of which has typical mediators, cellular elements, inflammatory responses and biological effects (Meidell 1994, Landmesser et al. 2004, Mullenix et al. 2005).

Adhesion. Adhesion of leukocytes to the endothelium is one of the most important events in the response to injury. Vascular injury induces the upregulation of endothelium-derived adhesion molecules which mediate the attachment and accumulation of monocytes, macrophages, T lymphocytes and platelets on the vessel wall (Jonasson et al. 1986, Mullenix et al. 2005). These include intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and platelet-endothelial cell adhesion molecule-1 (PECAM-1), integrins and selectins. As the inflammation proceeds, proinflammatory cytokines, membrane receptors and enzymes are released. These include interleukins (IL) 1, 2, 6, 7, 8 and 18, tumor necrosis factor α (TNF-α), interferon- γ (IFN- γ), monocyte chemotactic proteins (MCPs), CD40 ligand (CD40L), parathyroid hormone-related protein (PTHrP), osteopontin, cyclo oxygenase-2 (COX-2) and matrix metalloproteinases (MMPs). A proinflammatory positive feedback recruits additional cytokine releasing immune cells, promotes SR expression and aggregation of oxLDL particles in the endothelium and promotes the release of hepatic acute-phase reactants such as C-reactive protein

(CRP) with attendant activation of the systemic inflammatory cascade (Libby 2002, Ito and Ikeda 2003, Mullenix et al. 2005).

Permeability. The permeability of the endothelium increases in response to injury. Endothelial passage and deposition of oxLDL results in the influx of circulating macrophages to further modify and phagocytose the accumulating lipid-derived antigens. Resultant lipid-laden foam cells are representative of the histology of the early lesions. The protective inflammatory response may lead to a disproportionate recruitment of additional monocytes, macrophages, T lymphocytes and mast cells with attendant cytokine and chemokine release. This activates the classical and alternative complement pathways of the immune system and stimulates the local proliferation of vascular SMCs. Elaborated chemokines and MCP-1 promote the retention of recruited leukocytes and monocytes in the plaque itself (Ross 1999, Leskinen et al. 2003, Mullenix et al. 2005).

Proliferation. Activated endothelium functions in many ways as an endocrine tissue. The production of platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), fibroblast growth factor (FGF), transforming growth factor β (TGF-β), IL-1 and TNF-α all promote SMC proliferation, migration, local vasoconstriction, FGF-mediated collagen synthesis and matrix deposition, fibrous cap production and additional immune and platelet cell recruitment and activation (Ross 1999, Mullenix et al. 2005).

Thrombogenesis. Dysfunctional ECs lose their intrinsic anticoagulant properties. The factors contributing to the thrombogenic microenvironment are the local inflammatory activity such as platelet adherence, activation and degranulation, as well as disordered NO metabolism, increased phospholipase A_2 and plasminogen activator inhibitor-1 (PAI-1) activities, the release of vasoactive agents and the structural denudations in the endothelial layer itself with attendant collagen exposure, tissue factor (TF) release and MMP activity. MMP-related areas of fissuring or ulceration in advanced atherosclerotic plaques are particularly vulnerable to platelet-associated vascular haemorrhage, rupture, thrombosis, embolization and occlusion (Libby et al. 2002).

1.3.3. The role of inflammatory cells and infections during atherosclerosis

The fundamental event in the inflammatory response of atherosclerosis is the localized and restricted recruitment of blood leukocytes to tissues and organs through the endothelium-dependent mechanisms (Ross 1999, Osterud and Bjorklid 2003). It is enhanced by the presence of LDL which undergoes oxidative modification, as well as elevated arterial pressure, DM, chronic infections and acute activation of the immune system. These cells are mostly monocyte-

macrophages but also include activated T-cells, dendritic cells and activated degranulating mast cells all of which both accelerate lesion development and trigger the acute plaque rupture (Arbustini et al. 1991, Hansson 2005, Shah 2007, Lindstedt et al. 2007).

Monocyte-Macrophages. The accumulation of leukocytes in the lesion-prone areas is primarily of mononuclear origin (Osterud and Bjorklid 2003). Monocytes are the precursors of macrophages in all tissues and present in every stage of atherogenesis (Langheinrich and Bohle 2005). Macrophages are mobile phagocytic cells specialized in the endocytosis of cellular and extracellular debris and microorganisms. Monocyte recruitment into the arterial wall is a cascade involving leukocyte and EC adhesion molecules that support leukocyte rolling, firm adhesion and transmigration. The newly arrived monocytes then undergo transformation into macrophages, by expanding and becoming active in endocytosis and producing lysosomes (Rao et al. 2007). The subendothelial, modified LDL is hypothesised to provide an initiating ligand for macrophages which is a critical step for the development of atherosclerosis and is associated with the upregulation for innate immunity, including SRs and toll-like receptors (Langheinrich and Bohle 2005). Macrophages produce many immunoregulatory molecules which influence the activity of SMCs, ECs and macrophages themselves (Hansson 2005). Macrophages are also able to release a range of proteolytic and oxidizing agents including superoxide (O_2^-), hydrogen peroxide (H_2O_2), lipid peroxides, lipoxygenases (LOs) and possibly hypochlorite (HOCl) (Chisolm et al. 1999).

Macrophage activation can be either pro-inflammatory or anti-inflammatory (Martinez et al 2008). Cytokines of the type 1 helper T-cells (Th1) promote monocyte differentiation into proatherogenic M1 macrophages while the type 2 helper T-cell (Th2) cytokines lead to anti-inflammatory M2 macrophage phenotype (Bouhlel et al. 2007). Both M1 and M2 macrophages are present in atherosclerotic lesions (Charo 2007). The relative appearance of these two types of macrophages may change dynamically with the recruitment of polarized monocytes from the blood or through the effects of local cytokines on macrophages in the tissues (Charo 2007).

T lymphocytes. A T-cell infiltrate is always present in atherosclerotic lesions, mostly CD4+ T-cells but a subpopulation of natural killer cells and CD8+ cells are also found. Antigens, such as oxLDL, are presented to the T-cells by macrophages and dendritic cells to activate the antigen-specific T-cells in the artery. The cytokines presented in atherosclerotic lesion promote mostly Th1 instead of Th2 response (Frostegård et al. 1999, Hansson 2005). The Th1 response activates an inflammatory response similar to delayed hypersensitivity reaction, whereas Th2-mediated response resembles allergic inflammation. The Th1 pathway tends to stimulate atherosclerosis progression and Th1 type cytokines also dominate in human atherosclerotic plaques. The cytokines of Th2 pathway are able to inhibit the Th1 cell responses and may therefore promote

antiatherosclerotic immune reactions (Binder et al. 2004, Hansson 2005, Hansson and Libby 2006).

B lymphocytes. The belief about B-cell involvement in atherosclerosis has been based on the presence of circulating autoantibodies against oxLDL and immunoglobulins in atherosclerotic lesions (Ylä-Herttuala et al. 1994, Langheinrich and Bohle 2005). Antibody-producing B-cells are not numerous in lesions but may contribute to the atherosclerotic activity. Spleen B-cells may be particularly effective inhibitors of atherosclerosis due to the natural antibodies which some of the cells produce against oxLDL and apoptic cell membranes. This may lead to the elimination of oxLDL and dead cells. Accordinlgy, individuals who have undergone splenectomy have increased susceptility to CAD (Witztum 2002, Hansson 2005).

Granulocytes. Activated neutrophils release several proteolytic enzymes which are potent for tissue destruction. Granulocytes are rarely detected in atherosclerotic lesions but during acute MI neutrophils may infiltrate human culprit lesions (Naruko et al. 2002, Langheinrich and Bohle 2005).

Mast cells. Chronic activation of mast cells in the atherosclerotic lesion may predispose to plaque rupture. Mast cells are found to accumulate in the shoulder region of coronary atherosclerotic plaques, especially in the segments of plaque rupture. In sites of plaque erosion, the number of degranulating mast cells is also increased in the adventitia. Mast cells, when stimulated, degranulate and release their neutral proteases and histamine into the surrounding microenvironment where they may contribute the acute coronary events (Kovanen et al. 1995, Laine et al. 1999, Lindstedt et al. 2007).

Infections. Some pathogens and clinical infections have been linked to atherosclerosis and CAD, such as Chlamydia Pneumoniae, herpes and cytomegalo viruses. In theory, several types of pathogens and the total burden of varying infections may contribute to the progression of atherosclerosis and elicit clinical manifestations (Saikku et al. 1988, Moreno et al. 1994, Hansson 2005). Bacterial deoxyribonucleic acid (DNA) has been identified in the coronary arteries (Lehtiniemi et al. 2005, Ott et al. 2006) and abdominal aorta of atherosclerotic patients (Renko et al. 2008). Among the several types of pathogens identificated from the lesions are bacterial sequences similar to those found in human periodontitis (Renko et al. 2008). Oral infections are also considered a risk factor for coronary heart disease (CHD) (Mattila et al. 2000), MI (Mattila et al. 1989) and sudden cardiac death (Karhunen et al. 2006) and various oral bacteria have been detected in coronary plaques (Lehtiniemi et al. 2005). However, these speculations need to be studied further because the firm findings for this theory are still lacking.

1.3.4. Development of atherosclerotic lesion and plaque rupture

Fatty streak. As the accumulation of LDL in the vessel wall proceeds the dysfunctional endothelium expresses several adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intracellural adhesion molecule-1 (ICAM-1), in response to the proinflammatory stimulus (Glass and Witztum 2001). Monocytes adhere to the vessel wall and invade the subendothelial space in an attempt to protect the arterial wall from the cytotoxic oxLDL. The transformation of circulating monocytes to local macrophages is triggered by several inflammatory mediators such as macrophage colony stimulating factor (M-CSF). As the SRs become expressed in macrophages they uptake oxLDL in an unregulated manner. This leads to the formation of lipid-laden macrophages, foam cells, which form the basis of the fatty streak (Ross 1993, Hegele 1996). In addition to macrophages, B and T lymphocytes also enter the intima during lesion evolution. The interaction between these cells results in the production of a wide range of cytokines and growth factors (Glass and Witztum 2001).

Lesion progression. Inflammatory proteins such as TNF-α, IL-6 and MCP-1 activate different cell types in the atherosclerotic lesion, including ECs, platelets, SMCs and leukocytes (Murry et al. 1997, Gerthoffer 2007). The migratory and proliferative activities of vascular SMCs are regulated by growth promoters such as platelet-derived growth factor (PDGF), endothelin-1 (ET-1), thrombin, FGF, IL-1 and inhibitors such as heparin sulfates, NO, transforming growth factor β (TGF-β) and the matrix MMPs. These secreted growth factors and cytokines induce a phenotype change in SMCs from the quiescent contractile phenotype stage to the active synthetic stage in which they are capable of migrating and proliferating (Ross 1993, Glass and Witztum 2001). SMCs express specialized enzymes that can degrade the elastin and collagen in response to inflammatory stimulation. Thus, the SMCs penetrate through the elastic laminae and collagenous matrix of the growing plaque. SMCs then migrate and proliferate from the media to the intimal space, where they become the principal source of collagens. This response continues uninhibited and is accompanied by the accumulation of a new extracellular matrix. Apoptosis, proliferation and migration of SMCs are essential to the pathogenesis of atherosclerosis and finally, to plaque rupture (Libby et al. 1997, Glass and Witztum 2001).

Plaque rupture. The total amount of collagen in the fibrous cap consists of the biosynthesis by SMCs and coexistent degradative processes. The balance between these factors determines the plaque strength (Libby et al. 1997). Matrix depletion in fibrous cap results from the increased matrix breakdown induced by inflammatory activity and reduced matrix synthesis by the SMCs. The hemodynamic or other triggering event leads to plaque rupture and thrombosis (Shah 2003).

The resulting thrombosis causes an arterial occlusion which may in coronary arteries cause acute MI, unstable angina pectoris and even a sudden cardiac death (Stary et al. 1995, Shah 2007). Angiographically characterized symptomatic lesions are commonly eccentric stenoses which account for half of plaque ruptures (Levin and Fallon 1982, von Birgelen et al. 2001).

Plaque rupture is associated with increased number of fibrous cap macrophages, SMC apoptosis, and reduced number of fibrous cap SMC. Ruptured plaques have several histomorphological features that are different from intact plaques. The large lipid core, thin fibrous cap, neovascularity and infiltration of inflammatory cells are thought to indicate vulnerability to plaque rupture. The inflammatory cells are mostly monocyte-macrophages, in addition to activated T-cells and mast cells. They are located near the sites of cap rupture and also in the adventitia around areas of neovascularization. These cells produce several types of molecules such cytokines, proteases and radicals which can destabilize lesions and inhibit the formation of stable fibrous cap and accelerate the thrombus formation (van der Wal et al. 1994, Kovanen et al. 1995, Shah 2003).

TF is widely expressed in atherosclerotic plaques, especially in macrophages, foam cells, and the extracellular matrix and is considered to be the main contributor to the thrombogenicity of atherosclerotic plaques (Tremoli et al. 1999). After fibrous cap disruption, TF triggers thrombus formation leading to arterial lumen occlusion and embolization. Even though plaque rupture and thrombosis may remain clinically silent the thrombosis leads to accumulating plaque progression (Rauch et al. 2001, Croce and Libby 2007).

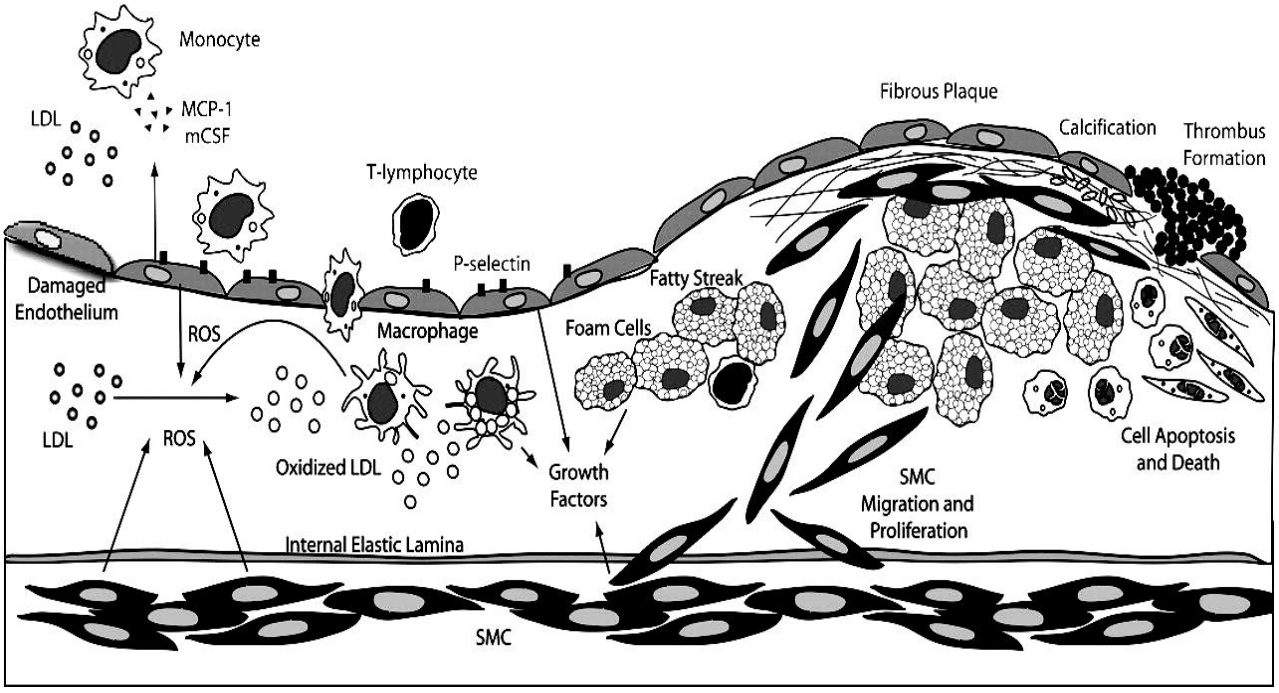

Figure 2. Initiation and progression of atherosclerotic lesions.

The atherosclerotic procress consists of a number of different events. The endothelial injury allows an induction of the adhesion molecules, adherence of platelets and the recruitment of leukocytes. Monocytes and lymphocytes permeate the arterial wall alongside the appearance of proinflammatory cytokines, infiltration and oxidation of LDL. The fibrous plaque is formed by the deposition of fibrous tissue and glycosaminoglycans in the intima and by the activation of the SMCs as well as the formation of foam. By the elaboration of hydrolytic enzymes, accumultation of lipids and necrotic debris in the plaque the unstable fibrous cap may rupture with ensuing ulcer or hemorrhage, possible thrombosis and occlusion of the artery. See text for details. Modified from Madamanchi et al (2005). Oxidative stress and vascular disease. Arterioscler Thromb Vasc Biol 25:29-38.

Abbreviations: LDL; low density lipoprotein, MCP-1; monocyte chemotactic protein, MCSF; macrophage colony stimulating factor, ROS; reactive oxygen species, SMC smooth muscle cell.

2. Oxidative stress, oxidative modification of LDL and their role in atherosgenesis

2.1. Free radicals

Reactive oxygen species (ROS) are products of a normal cellular metabolism involved in several physiological events such as in immunological defense or cellular signaling. The term oxidative stress is used when the increased formation of oxidants is accompanied by a loss of antioxidants or

accumulation of the oxidized forms of the antioxidants (Stocker and Keaney 2004). The production of ROS is regulated by many of the cytokines whose expression is increased after endothelial injury, shear stress and mechanical disruption. According to current knowledge, the pathology of atherosclerosis is due in part to excessive oxidative damage. Although low levels of ROS are necessary for normal vascular function, enhanced production of ROS stimulates the cellular responses to injury including monocyte adhesion, platelet aggregation, induction of inflammation and apoptosis, vascular SMC proliferation and migration, matrix degradation and impaired endothelium-dependent vascular tone regulation (Ferrari et al. 1998, Rosenfeld 1998, Halliwell 2000, Papaharalambus and Griendling 2007).

2.1.1. Reactive oxygen and nitrogen species

Molecules or their fragments containing one or more unpaired electrons are called free radicals and they are usually highly reactive. Therefore, free radicals are likely to take part in chemical reactions. The two most important oxygen-centered free radicals are O_2^- and hydroxyl radical ($^{\cdot}OH$). O_2^- is derived from molecular oxygen under reducing conditions (Valko et al. 2007). A radical may join onto a nonradical molecule or abstract a hydrogen atom from a C-H, O-H or S-H bond of nonradical molecules. Such radical reactions are common in biological systems where most of the molecules are nonradical. The affected molecules include low-molecular-weight compounds such as antioxidants, cofactors of enzymes, lipids, proteins, nucleic acids and sugars (Stocker and Keaney 2004).

Superoxide (O_2^-). O_2^- is considered the primary oxygen -centered radical produced by the mitochondria and microsomal membranes of the cell. The mitochondrial electron transport chain is the main source of ATP in the mammalian cells and during the electron transport chain a small number of the electrons are able to form the O_2^- anion. O_2^- radical has been linked not only to atherogenesis but also to the pathology of a variety of diseases (Cadenas and Davies 2000, Valko et al. 2007).

Hydrogen peroxide (H_2O_2). O_2^- and hydrogen peroxide (H_2O_2) are the products of the univalent and bivalent reduction of oxygen (O_2) resulting from normal aerobic metabolism. H_2O_2 is a weak oxidizing agent and is generally poorly reactive. It may participate in cellular signaling and, in the presence of transition metals, can give rise to $^{\cdot}OH$ (Stocker and Keaney 2004).

Hydroxyl radical ($^{\cdot}OH$). $^{\cdot}OH$ has a high reactivity with all biomolecules and has a very short half-life reacting therefore close to its site of formation. The redox state of the cell is largely linked to an iron redox couple and is maintained in strict physiological limits. Iron can promote

peroxidation of biological macromolecules with ROS and has a toxic potential for cells although inactivated by specific molecules. Under stress conditions the excess O_2^- is able to oxidize the iron cluster containing enzymes and facilitate $^{\cdot}OH$ production from H_2O_2 by enabling the released iron (Fe^{2+}) to participate in a Fenton reaction (Valko et al. 2007).

Nitric oxide (NO). NO is formed from the amino acid L-arginine catalyzed by NO synthases (NOSs) and plays an important role in the regulation of vascular tone. NO is a relatively stable radical but when it reacts with O_2^- it generates peroxynitrite ($ONOO^-$), a powerful oxidizing agent. It is considered that NO inhibits the membrane-bound oxidase responsible for generating O_2^- radicals in activated neutrophils. Reactive nitrogen species may promote LDL oxidation in vivo. However, it has been suggested that NO may protect LDL from oxidation by several mechanisms (Rice-Evans and Gopinathan 1995, Heinecke 1998, Stocker and Keaney 2004).

Peroxynitrite ($OONO^-$). Like hydroperoxide, $ONOO^-$ is a relatively weak oxidant in alkaline pH. However, its protonated form, peroxynitrous acid (ONOOH) is extremely reactive. In biological systems the formation of $ONOO^-$ anion is very likely to result in a powerfully oxidizing environment comparable to that resulting from the generation of $^{\cdot}OH$. Nonradical oxidants like peroxynitrous acid (ONOOH) and HOCl appear to react preferentially with proteins rather than lipids (Stocker and Keaney 2004).

Hypochlorous acid (HOCl). HOCl is a weak acid but a strong oxidant which reacts preferably with protein rather than lipids and gives rise to secondary reactive species including chloramines and amino-acid derived aldehydes. Hypochlorite (ClO^-) is cytotoxic and reacts with many biological compounds including heme proteins, porphyrins, ascorbic acid and many protein constituents (Rice-Evans and Gopinathan 1995, Stocker and Keaney 2004).

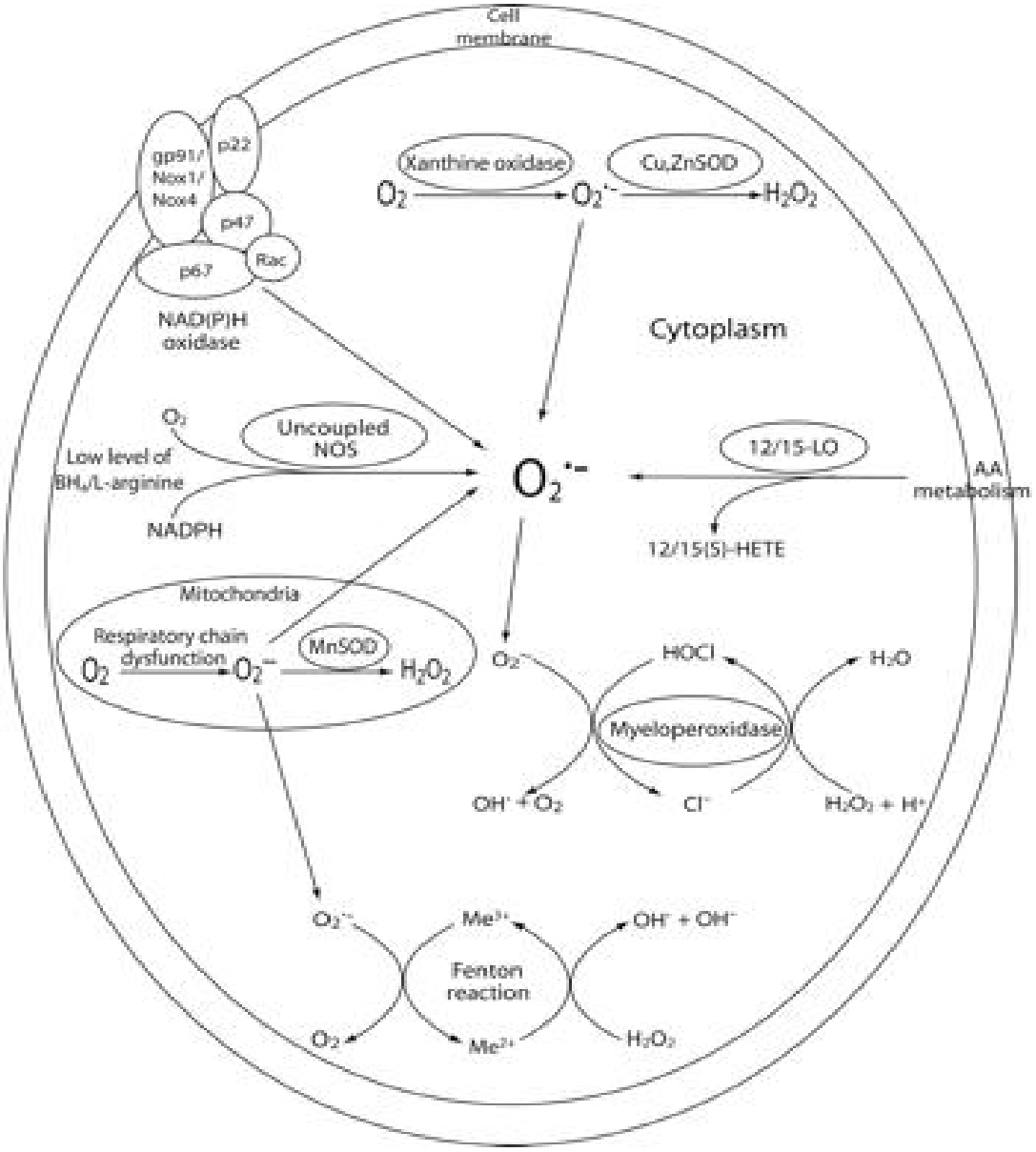

Figure 3. Sources of ROS in phagocytic cells and interactions between different oxidative enzyme systems.

Activated NAD(P)H oxidase, 12/15-LO and XO generate $O_2^{\cdot-}$. $O_2^{\cdot-}$ can dismute spontaneously to H_2O_2. MnSOD, and CuZnSOD dismutate $O_2^{\cdot-}$ to produce H_2O_2. Activated NAD(P)H oxidase produces $O_2^{\cdot-}$ by phosphorylation of one of its subunits. 12/15-LO oxidize polyunsaturated fatty acids to hydroperoxy fatty-acids as 12(S)-HETE and 15(S)-HETE. XO generate $O_2^{\cdot-}$ by catalyzing hypoxanthine and xanthine to uric acid. Redox cycling of Fe^{2+} and Fe^{3+} through Haber-Weiss and Fenton reaction formats $OH^{\cdot}$ from H_2O_2. NOS catalyze the oxidation of L-arginine to L-citrulline and the potent vasodilator NO. If L-arginine or BH_4 is reduced, the NOS may become uncoupled and reduce molecular oxygen rather resulting in $O_2^{\cdot-}$generation. MPO can catalyze the formation of HOCl from H_2O_2 in the presence of Cl^- ions. Also, dysfunctional mitochondrial respiratory chain serves as a source of $O_2^{\cdot-}$ generation. See text for details.

Modified from Madamanchi et al (2005). Oxidative stress and vascular disease. Arterioscler Thromb Vasc Biol 25:29-38.

Abbreviations: AA; arachidonic acid, BH_4; tetrahydrobiopterin, Cl^-; chloride ion, Cu/ZnSOD; Copper/Zinc superoxide dismutase, H_2O_2; hydrogen peroxide, HETE; hydroxyeicosatetraenoic acid, HOCl; hypochlorous acid, LO; lipoxygenase, MnSOD; manganese superoxide dismutase, MPO; myeloperoxidase, NAD(P)H; nicotinamide adenine dinucleotide (phosphate), NO; nitric oxide, NOS; nitric oxide synthase, O_2^-; superoxide, OH^-; hydroxide radical; ROS; reactive oxygen species, XO; Xanthine oxidase.

2.2. Sources of oxidants in vascular cells

There are several factors controlling the endogenous release of the free radicals during tissue injury. Alongside phagocyte recruitment, the activation of the membrane-bound oxidase of neutrophils, monocytes, macrophages and eosinophils produces O_2^- radicals. Activated macrophages can produce ROS via the membrane associated nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) oxidase which is able to create O_2^- and H_2O_2 as a part of respiratory burst – a reaction activated for killing of foreign organisms (Rice-Evans and Gopinathan 1995). In addition to this, macrophages isolated from rabbit atherosclerotic lesions actively produce significant amounts of O_2^-, H_2O_2 and NO (Rosenfeld 1998).

2.2.1. Oxidants

Nicotinamide adenine dinucleotide (phosphate) (NADH/NAD(P)H) oxidases. The NADH/NADPH oxidases are membrane-associated enzymes that catalyze the 1-electron reduction of oxygen using NAD(P)H as the electron donor. NAD(P)H oxidase is considered to be the major source of O_2^- generation in vascular cells. It is expressed in the phagocytes and also to a lesser extent in vascular SMCs, ECs and adventitial fibroblasts. The vascular NAD(P)H oxidases are essential in the physiological response of vascular cells but have also been linked to the inflammatory processes in atherosclerosis (Harrison et al. 2003, Madamanchi et al. 2005). For example, when incubated with LDL, NAD(P)H oxidase activation of the macrophages is induced (Aviram et al. 1996).

Xanthine oxidase (XO). In addition to NAD(P)H oxidase, XO is another important source of O_2^-. It has a unique capability to reduce O_2 to form O_2^- and H_2O_2. XO is generated in ECs but is also found from circulation where it binds to the endothelial matrix. XO generates O_2^- by catalyzing hypoxanthine and xanthine to uric acid (Harrison et al. 2003, Madamanchi et al. 2005).

The XO is activated in the coronary arteries of CAD patients but XO activity is also expressed in asymptomatic subjects with familiar hypercholesterolemia (Madamanchi et al. 2005).

Nitric oxide synthase (NOS). NOS, and especially its vascular isoform endothelial NOS (eNOS), uses 5,6,7,8-tetrahydrobiopterin (BH_4) as a cofactor for the transfer of electrons from a heme group within the oxygenase domain to L-arginine to form L-citrulline and NO. If either BH_4 or L-arginine is absent, the electrons from the heme reduce O_2 to form O_2^-. Thus, an oxidative depletion of BH_4 can lead to a marked increase in O_2^- from the NOSs (Vasquez-Vivar et al. 1998, Harrison et al. 2003).

Lipoxygenases (LOs). LOs are intracellular non-heme enzymes that peroxidize polyunsaturated fatty acids to hydroxyperoxy fatty-acid derivatives. Regarding atherosclerosis, 12/15-LO and 5-LO have received the most attention because of their expression patterns in ECs and inflammatory cells (Lötzer et al. 2005, Funk 2006). The number of 5-LO expressing leukocytes increases during atherosclerosis progression (Spanbroek et al. 2003). LO-15 protein is found in atherosclerotic plaques and 12/15-LO enzymatic pathway may promote LDL oxidation in vivo (Ylä-Herttuala et al. 1990, Heinecke 1998, Funk 2006). In addition, leukocyte-type 12/15-LO activation induces SMC growth, hypertrophy and inflammatory gene expression (Droge 2002, Madamanchi et al. 2005).

Mitochondrial respiration. Oxygen (O_2) can also be formed as O_2^- nonenzymatically by a reaction with the active compounds of the mitochondrial electron transport chain, such as semi-ubiquinone (Droge 2002, Madamanchi et al. 2005). The extent of atherosclerosis correlates with mitochondrial ribonucleic acid (RNA) damage in atherosclerosis-prone mice (Ballinger et al. 2002).

Transition metals (Fenton reaction). H_2O_2 can react with transition metals as Fe^{2+} to produce highly reactive $^{\cdot}OH$, a reaction known as the Fenton reaction. Fe^{2+} initiates the Fenton reaction and Fe^{3+} is regenerated, which in turn maintains the production of $^{\cdot}OH$ (Rice-Evans and Gopinathan 1995, Kehrer 2000, Madamanchi et al. 2005). Metal ions are the most studied pathway for LDL oxidation. Cultured SMCs oxidize LDL if iron or copper are present in the incubation medium. High concentrations of iron or copper oxidize LDL independently of the presence of cells (Heinecke et al. 1984, Steinbrecher et al. 1984). Instead of involvement in the early atherosclerosis, the number of free radical ions may be increased around regions of cell death, leading to necrosis in the advanced plaque (Gaut and Heinecke 2001). However, the exact mechanism of LDL oxidation by metal ions is not understood and the evidence of the relationship between plasma iron levels and atherosclerosis is the reverse (Heinecke 1998).

Myleoperoxidase (MPO). MPO is a heme protein secreted by phagocytotic cells. It has an ability to produce HOCl and other oxidants. HOCl modified proteins are found in atherosclerotic lesions and therefore, MPO is thought to be implicated in the macrophage-mediated oxidation of LDL (Daugherty et al. 1994, Hazell et al. 1996, Jacob et al. 1996).

2.3. Consequences of ROS

2.3.1. Cellular nucleic acid and protein damage, cell proliferation and cell death

In general, oxidized DNA exhibits an increased propensity for genetic mutations and alterations in transcription by several mechanisms. ROS may damage the DNA directly, interfere with the DNA repair and affect the cell division, thus disrupting the cell functions during non-carcinogenic toxicity events (Kehrer 2000, Marnett 2000, Stocker and Keaney 2004). Mitochondrial DNA is also prone to oxidative damage and ROS formed in mitochondria are associated with enhanced susceptibility to atherosclerosis (Madamanchi et al. 2005).

Oxidative stress has several effects on the progression of the cell cycle. Exposure of the cells to low doses of ROS usually results in activation of mitogenic signal transduction pathways leading to cellular proliferation. ROS are able to alter signal transduction pathways and affect cellular processes essential for cyclin functions and proteosomal degradation. A wide range of growth factors become activated by ROS and lead to cellular proliferation of ECs and other cell types (Cummings et al. 1997, Kehrer 2000, Galle et al. 2006). The oxidation of proteins by ROS can generate a range of stable as well as reactive products. Among the reactive products are the protein hydroperoxides that can generate additional radicals, particularly in interactions with transition-metal ions. Although most oxidized proteins are are rapidly removed, some may contribute to the damage associated with ageing and chronic diseases (Dean et al. 1997, Kehrer 2000, Stocker and Keaney 2004).

2.3.2. Lipid damage

Lipids have a critical structural and functional role in membranes. The double bounds found in polyunsaturated fatty acids are prone to free radical attack. The abstraction of a hydrogen atom from one of these double bounds results in a new radical lipid species that can readily interact with molecular O_2. The resulting lipid peroxyl radical can abstract a hydrogen atom from another fatty

acid yielding another radical and lipid hydroperoxide (LOOH) establishing a chain reaction. The LOOHs formed are unstable and can decompose into various species including malondialdehyde (MDA) or it can be reduced to the more stable alcohol form. As these reactions progress, ionic channels may be affected, membrane transport proteins or enzymes may be inactivated or the lipid bilayer itself may become more permeable thereby disrupting ion homeostasis. In addition, some of the oxidized fatty acid species such as the isoprostanes or hydroperoxides, have biologic activity and an ability to affect signaling pathways (Kehrer 2000, Stocker and Keaney 2004).

2.4. LDL metabolism, oxidation and formation of autoantibodies against oxLDL

2.4.1. LDL metabolism

Serum cholesterol is transported in the circulation by several lipoproteins which are specialized in transporting dietary and endogenously produced lipids. The dietary lipids are transported by chylomicrons and the endogenous lipid transport is carried out by very low density lipoproteins (VLDL), LDL and high density lipoproteins (HDL). Triglyceride (TG) rich VLDL particles are synthesized by the liver and contain apolipoprotein B (apoB) and apolipoprotein E (apoE) (Glass and Witztum 2001). After TG removal in the peripheral tissues, such as adipose tissue and muscles, a portion of the remaining VLDL remnants progressively changes into lipoproteins with intermediate density and finally to cholesterol-rich LDL. VLDL and intermediate density lipoprotein (IDL) have a short half-life and are removed from the circulation within hours, whereas the LDL particles have a rather long life and circulate in the blood for about two days before they are cleared (Esterbauer et al. 1992).

The human principal cholesterol carrier LDL consists of a hydrophobic core containing TGs and cholesterol esters in a hydrophilic shell of phospholipids, free cholesterol and ligands for lipoprotein receptors, the apolipoproteines, predominantly apoB (Smith et al. 1978, Osterud and Bjorklid 2003, Spiteller 2005). LDL binds to a specific LDL receptor (LDLR) which is expressed in ECs, monocytes, macrophages and SMCs in atherosclerotic lesions (Hiltunen and Ylä-Herttuala 1998). By the elevated LDL serum levels and the following cholesterol loading, the LDLR expression is downregulated (Jeon and Blacklow 2005).

Modification of LDL lipids and apoB increases its effects and enhances the inflammatory process in atherosclerosis (Steinberg 1997). LDL can be oxidized in the subendothelial space and depending on the degree of oxidation, minimally modified LDL and fully oxidized oxLDL are

formed (Chisolm et al. 1999). In contrast to native LDL, minimally modified LDL is bound not only to LDLR but also by a number of SRs whereas oxLDL is attached only by SRs. LDLR and SRs are expressed in macrophages and some are found in platelets and SMCs (Hiltunen and Ylä-Herttuala 1998). Modified LDL participates in the development of atherosclerosis by increasing the monocyte recruitment to the vessel wall and by foam cell formation. It induces the adhesion molecules and chemokines in ECs and has direct effects on monocytes and promotes (Gleissner et al. 2007).

2.4.2. Oxidative modification of LDL in atherosclerosis

Definition of oxLDL. The term oxLDL was traditionally used to describe the LDL modified by exposure to copper ions which catalyzed lipid peroxidation. Nowadays, the term oxLDL has been extended to additionally describe several chemical, biological and immunological entities such as the measurement of conjugated dienes, susceptibility of LDL to oxidation and autoantibodies against various epitopes of oxLDL (Fraley and Tsimikas 2006). Oxidation of LDL may involve fragmentation of its constituent molecules, including cholesterol, fatty acids, antioxidants and apoB. Therefore, oxLDL does not describe only a single particle but also a spectrum of oxidized particles in different stages (Ahotupa et al. 1998). Immunological methods for the determination of oxLDL are based on the use of antibodies generated against oxidatively damaged LDL. However, the specificity of these assays may be impaired by several possible antigenic sites. Antibodies prepared to identify oxidized LDL may also recognize epitopes on proteins other than LDL (O'Brien et al. 1996, Ahotupa et al. 1998).

Susceptibility of LDL to oxidation. There are several intrinsic properties of LDL that can affect its susceptibility to oxidation, such as the antioxidant content, fatty acid composition (Reaven et al. 1993) and LDL particle size (Chait et al. 1993). There are contradictory studies on the HDL ability to protect LDL from oxidation and it has been suggested that HDL particles may be more susceptible to oxidation than LDL (Solakivi et al. 2005).

The antioxidant status of LDL and plasma are important determinants of the susceptibility of LDL to peroxidation (Stocker and Keaney 2004). In addition, the LDL size and density also influence the extent of oxidation and small dense LDL is more susceptible to oxidation than large-buoyant LDL (de Graaf et al. 1991, Berliner and Heinecke 1996). LDL particles carry lipophilic antioxidants, mostly α-tocopherol, but also minor amounts of γ-tocopherol, carotenoids, oxycarotenoids and ubiquinol-10 (Esterbauer et al. 1992, Hevonoja et al. 2000). Vitamin E (α-tocopherol), the major antioxidant of LDL, may influence LDL oxidation. However, increased

dietary supplementation of vitamin E does not correlate with the susceptibility to LDL oxidation. In fact, it may even promote lipid peroxidation (Berliner and Heinecke 1996, Stocker 1999). Vitamin C (ascorbate) is in turn a water soluble antioxidant which prevents lipid peroxidation in the presence of iron overload (Chen et al. 2000, Mashima et al. 2001). The susceptibility of LDL oxidation varies among individuals and for example, in patients with non-insulin dependent DM LDL is more susceptible to oxidation than in non-diabetic subjects (de Graaf et al. 1991, Berliner and Heinecke 1996).

LDL oxidation. The oxidative process in the arterial wall is a complex reaction involving several cell types including monocyte-macrophages, granulocytes, lymphocytes, ECs and SMCs. In the plasma circulation, LDL is protected from oxidation by the presence of antioxidants, but in the arterial wall the LDL particle is a more vulnerable subject of oxidation. Typically, the oxidation takes place in a microenvironment where the number of antioxidants is low as in the vessel wall and only to a minor extent in the blood (Rosenfeld 1998, Osterud and Bjorklid 2003, Stocker and Keaney 2004). However, the exact mechanisms of this process are not yet fully understood (Gaut and Heinecke 2001).

The LDL particles undergo a series of modifications such as non-enzymatic glycation, enzymatic degradation and aggregation, which generates a wide spectrum of oxidation-specific neo-epitopes (Hörkkö et al. 2000, Binder et al. 2002). Oxidation involves the lipid moiety of LDL in a chain reaction mechanism. In the initial phase, free radicals preferentially attack highly oxidizable polyunsaturated fatty acids. Polyunsaturated fatty acids present in LDL phospholipids are oxidatively converted to LOOHs, which are subsequently cleaved forming aldehydes (Girotti 1998). When polyunstaturated fatty acids undergo peroxidation, a variety of higly reactive breakdown products is formed, such as MDA which in turn can form covalent adducts with the lysine residues of the apolipoproteins (Hörkkö et al. 2000, Binder et al. 2002)**.** Aldehydes are able to covalently modify apoB-100 which leads to the negative overall net charge of the LDL particle, which is then more recognizable to macrophage SRs (Osterud and Bjorklid 2003). The LDL oxidation also leads to a significant loss of cholesterol as it is converted into a range of oxysterols (Jessup and Kritharides 2000). The modified LDL particles and oxidized lipids are pro-inflammatory and trigger both humoral and cellular immune response (Hörkkö et al. 2000, Binder et al. 2002).

Scavenger receptors (SRs). Oxidation-altered apoB of oxidized LDL is recognized by the macrophage SR, which is responsible for foam cell formation. Recognition of oxLDL is related to the derivatization of lysine residues or fragmentation of apoB which leads to a net negative charge (Stocker and Keaney 2004). The SRs of monocyte-derived macrophages can recognize a wide

range of negatively charged macromolecules, oxLDL, damaged or apoptotic cells, and pathogenic microorganisms. In physiological conditions, SRs serve to scavenge or clean up cellular debris and other related materials as a part of the host defence (Yamada et al. 1998). OxLDL is known to be taken up via SRs in a manner which is independent of the cholesterol-dependent LDLR downregulation. The unlimited accumulation of cholesterol in the macrophages eventually leads to the formation of foam cells, a cell type already involved in early atherosclerosis (Henriksen et al. 1981, Noguchi et al. 1993).

2.4.3. Proatherogenic activities of oxidized LDL (oxLDL)

OxLDL has several proatherogenic effects such as the inhibition of eNOS, promotion of vasoconstriction and adhesion, cytokine stimulation and stimulation of platelet aggregation (Stocker and Keaney 2004, Madamanchi et al. 2005, Singh and Jialal 2006). OxLDL has also been shown to upregulate vascular endothelial growth factor (VEGF) expression in macrophages and ECs through activation of peroxisome proliferator-activated receptor-γ (PPAR-γ) and stimulate TF and PAI-1 synthesis (Stocker and Keaney 2004, Singh and Jialal 2006). OxLDL have been shown to powerfully inactivate NO (Chin et al. 1992) and decrease its production in experimental studies (Liao et al. 1995).

It has been demonstrated that the vascular endothelial function is inversely associated with oxidized LDL already in childhood (Järvisalo et al. 2004) and coronary reactivity in young healthy men (Raitakari et al. 1997). OxLDL measured directly from plasma has been reported to be independently associated with subclinical carotid artery atherosclerosis in middle-aged men (Metso et al. 2004).

Table 2. Proatherogenic activities of oxLDL. Modified from Stocker R and Keaney JF (2004): Role of oxidative modifications in atherosclerosis. Physiol Rev 84:1381-1478.

Potential proatherogenic activities of OxLDL.
OxLDL supports macrophage foam cell formation.
OxLDL-derived products are chemotactic for monocytes, T-cells and tissue macrophages.
OxLDL-derived products are cytotoxic and can induce apoptosis.
OxLDL is mitogenic for SMCs and macrophages.
OxLDL alters inflammatory gene expression in vascular cells.
OxLDL can increase the expression of macrophage SRs.
OxLDL is immunogenic and promotes autoantibody formation and activated T-cells.
OxLDL may undergo aggregation, which independently leads to enhanced uptake.
OxLDL induces TF expression and platelet aggregation.
Products of oxLDL impair NO bioactivity.
OxLDL binds C-reactive protein activating the complement pathway.

Abbreviations: NO; nitric oxide, oxLDL; oxidized low density lipoprotein, SMC; smooth muscle cell, SR; scavenger receptor, TF; tissue factor

2.4.4. Autoantibodies against oxLDL

Cellular immunity and oxLDL. In general, antibodies provide protection against exogenous pathogens and endogenous altered molecules to maintain homeostasis by neutralization and clearance. Antibodies can also induce other components of the immune system, such as complement pathways and effector functions of other immune cells (Binder et al. 2002). OxLDL is immunogenic and a wide range of epitopes within the apoB component of oxLDL is known to

provoke an immune response (Fredrikson et al. 2003, Stocker and Keaney 2004). The presence of the antigen-presenting plaque macrophages and T-cells allow the local cellular immune responses to oxLDL. The occurrence of oxLDL-specific T-cells is supported by the development of IgG antibodies specific for oxLDL (Hörkkö et al. 2000). T-cells from human atherosclerotic plaques recognize oxLDL suggesting that the inflammatory infiltrate in the atherosclerotic plaque is involved in a T-cell-dependent, autoimmune response to oxLDL (Stemme et al. 1995).

OxLDL autoantibodies. Immune responses against oxidized forms of LDL play a critical role in the activation and regulation of the inflammatory process that characterizes all stages of atherosclerosis. Human plasma contains immunoreactivity towards epitopes generated from oxLDL. In humans oxidized LDL is targeted by both IgM and IgG autoantibodies. These immunoglobulins are present in atherosclerotic lesions (Ylä-Herttuala et al. 1994, Hörkkö et al. 2000, Binder et al. 2002, Shoenfeld et al. 2004). For the measurement of these immunoresponses, two models of oxLDL are widely used: MDA modified LDL (MDA-LDL) which is generated by the derivatization of LDL with MDA yielding mainly MDA-lysine epitopes and, secondly, $CuSO_4$-oxidized LDL, which has many different oxidation-specific epitopes (Binder et al. 2002).

OxLDL-ab in the pathogenesis of atherosclerosis. Several studies have shown that circulating levels of oxLDL-ab can be used to distinguish between patients with and without clinically evident atherosclerosis (Shoenfeld et al. 2004). Baseline titer of autoantibodies against MDA-LDL has been shown to predict the progression of IMT (Salonen et al. 1992) and elevated oxLDL-ab concentrations may predict the development of CVD (Puurunen et al. 1994, Wu et al. 1997). High oxLDL-ab titers have been associated with the impairment of coronary reactivity in young adults (Laaksonen et al. 2002), angiographically verified CAD (Lehtimäki et al. 1999), angina pectoris and serum MMP-9 (Kalela et al. 2002). Elevated oxLDL-abs are also associated with hypertension arterialis (HTA), peripheral artery disease and endothelial dysfunction (Bergmark et al. 1995, Maggi et al. 1995, Fang et al. 2002). ApoE allele ε 2 is associated with decreased levels of oxLDL-abs in both patients with CAD and healthy controls (Metso et al. 2003). OxLDL-abs are also prevalent in diseases other than atherosclerosis, namely autoimmune diseases and DM (Bellomo et al. 1995, Orchard et al. 1999, Shoenfeld et al. 2004). Interestingly, the level of oxLDL-abs is not necessarily associated with vascular complications in type 2 DM patients (Uusitupa et al. 1996).

In general, however, the antibodies neutralize pathogens and immunogens and in theory, the humoral immunity may reduce the incidence of atherosclerosis (Zhou et al. 2001, Shoenfeld et al. 2004). Human oxLDL-abs may play an important role in the regulation of oxLDL levels as the oxLDL concentrations in the plasma have been shown to be inversely correlated to oxLDL-abs

(Shoji et al. 2000). The antibody titer against MDA-LDL has been reported to inversely correlate with the risk of severe CAD (Rontu et al. 2005). In addition, the inverse relationship between oxLDL-ab titer and carotid IMT in healthy subjects has been published (Fukumoto et al. 2000). However, there are also several studies where no association was found between oxLDL-abs and CVD (Uusitupa et al. 1996, van de Vijver et al. 1996, Shoenfeld et al. 2004).

3. Myeloperoxidase (MPO)

Activated phagocytes produce highly reactive oxidants during inflammatory response against invading microorganisms and tumor cells (Klebanoff 1980, Klebanoff 1999). MPO is a heme containing lysosomal enzyme of the activated neutrophils, monocytes and tissue macrophages (Winterbourn et al. 2000). MPO is a critical component of the oxidative activity of the neutrophils as its activity functions against several microorganisms, from viruses to fungi as well as against mammalian proteins and cells (Klebanoff 1999, Winterbourn et al. 2000). Besides leukocytes, MPO has been found in the microglia, granule-containing neurons and pyramidal neurons of hippocampus in the brain (Nagra et al. 1997, Green et al. 2004) and in the Kupffer cells in the liver (Brown et al. 2001).

3.1. MPO in atherosclerosis

Several studies in humans and data available from the animal studies suggest that MPO may have a crucial role in the development of atherosclerosis (Nicholls and Hazen 2005). Peroxidatively active MPO as well as its protein and oxidation products like 3-chlorotyrosine and L-tyrosine oxidation remnants have been detected in human atherosclerotic lesions (Daugherty et al. 1994, Hazen and Heinecke 1997, Heller et al. 2000). MPO is expressed both intra- and extracellularly predominately in macrophage-rich transitional lesions. In addition, extracellular MPO has been detected in the lipid-rich domains of transitional and complicated lesions and in the cholesterol clefts of advanced lesions (Daugherty et al. 1994). MPO-containing macrophages are also present in those atherosclerotic lesions which provoke acute coronary syndromes (ACSs) (Sugiyama et al. 2001).

Animal studies of atherosclerosis have yielded conflicting results. In the study on LDLR-deficient MPO-knockout mice the extent of atherosclerosis was increased (Brennan et al. 2001). In rat model, however, HOCl induced pathological neointimal growth (Yang et al. 2006). However,

the murine leukocytes carry 10- to 20-fold less MPO than the corresponding human leukocytes, and therefore the murine model may fail to predict the role of MPO in human atherosclerosis (Nauseef 2001, Nicholls and Hazen 2005).

Apart from the studies reporting the polymerase chain reaction (PCR) amplification of MPO transcripts from tissue monocytes or macrophages there is no evidence of MPO protein synthesis in non-malignant cells other than myeloid precursor cells (Hansson et al. 2006). It is also possible that MPO present in tissue macrophages may be taken up from the tissue and not synthesized in the cell itself (Sugiyama et al. 2001, Klebanoff 2005). In fact, blood-derived MPO can also bind and infiltrate into the vascular wall directly (Baldus et al. 2001, Zhang et al. 2003, Yang et al. 2006). An alternative hypothesis is that tissue macrophages situated in the atheromatous plaque reinitiate transcription of the MPO gene. Because the foam cells possess no azurophilic granules, the synthesized proMPO in could enter the foam cells by secretory pathway and be released into the extracellular space (Nauseef 2001, Malle et al. 2007).

3.2. Storage, biosynthesis and structure of MPO

3.2.1. Storage

The MPO synthesis is initiated in the promyelocyte stage of neutrophil development and terminated at the beginning of the myelocyte stage, at which time the MPO containing azurophil granules are distributed to daughter cells where they enter the specific granules (Kinkade et al. 1983, Klebanoff 2005). Human monocytes also contain these granules, although in a smaller amount. The MPO containing granules are usually lost while monocytes mature into macrophages (Nichols and Bainton 1973).

3.2.2. Biosynthesis and proteolytic maturation

Mature MPO has a molecular mass of approximately 150kDa and consists of a pair of heavy-light protomers, whose heavy subunits are linked by a disulfide bond (Olsen and Little 1984). The post-translational processing of the 80-kDa primary translation product, including the heme insertion and lysosomal targeting, are essential for the maturation of the enzymatically active lysosomal MPO (Nauseef 2004, Hansson et al. 2006). The active site of MPO is buried deep in the center of the protein (Zeng and Fenna 1992) and these two hemes are covalently bound to the heavy subunit

(Hansson et al. 2006). The peroxidase activity of MPO depends on a normal heme group, which interacts with the protein structure with three covalent bonds and eight hydrogen bonds (Furtmüller et al. 2006). Structural features unique to the heme group of MPO make it the only member of the human peroxidases capable of oxidizing chloride ion (Cl^-) and thus generating HOCl, at physiological pH (Marquez and Dunford 1994, Furtmüller et al. 2000).

The primary 80 kDa translation product preproMPO is processed in the endoplasmic reticulum as a single-chain precursor and undergoes a complex series of post-translational modifications prior to packaging into azurophilic granules (Pinnix et al. 1994). PreproMPO undergoes cotranslational N-glycosylation resulting in 90 kDa apoproMPO, and heme incorporation to generate enzymatically active proMPO that is exported into the Golgi compartment. After exiting the Golgi, the propeptide is removed before final proteolytic processing in azurophilic granules (Hansson et al. 2006). Some proMPO escapes granule targeting and becomes constitutively secreted to the extracellular environment as a monomer (Hansson et al. 2006). According to current knowledge, the secreted proMPO remains as a monomer and its physiological function is unknown. MPO species isolated from human plasma include both precursor and mature forms of MPO (Nauseef 1986, Olsen et al. 1986, Nauseef 1987). In has been theorized that the enzymatically active MPO could primarily function in the oxidative cell killing and inactive MPO functions as an immunoregulative molecule through the induction of numerous cytokines (Lefkowitz and Lefkowitz 2001).

The processed MPO protein is a glycosylated, predominantly α-helical cationic 146 kDa dimer with a single disulfide bridge between symmetry-related halves (73 kDa), each containing light chain of 14.5 kDa and heavy chain of 58.5 kDa (Hansson et al. 2006). This dimeric MPO is found in neutrophils and monocytes (Hansson et al. 2006, Malle et al. 2007) and its granule MPO comprises 1% of the monocyte cell mass but is lost as monocytes differentiate to tissue macrophages (Nauseef et al. 1988).

3.3. Physiological actions of MPO

MPO catalyzes several modifications including tyrosyl radical formation, chlorination, tyrosine peroxide generation and oxidation of serum lipoproteins (Daugherty et al. 1994, Savenkova et al. 1994, Domigan et al. 1995, Hazen et al. 1996a). MPO is a transcytosable protein which can bind to and infiltrate into the vascular wall directly and enter the vascular cells. It can remain in the vasculature for several days (Baldus et al. 2001, Eiserich et al. 2002, Zhang et al. 2003). As a

strongly cationic enzyme, it is easily attached to negatively charged biological membranes, especially those found in the sites of the inflammatory processes (Johansson et al. 1997). These include glycosaminoglycans of the extracellular matrix as well as a number of proteins and lipoproteins LDL (Daphna et al. 1998, Carr et al. 2000, Baldus et al. 2001). The active site of MPO is located in a hydrophobic, pocket-like structure, which restricts the accessibility of substrates (Furtmüller et al. 2006). In vitro studies suggest that water soluble antioxidants, like ascorbate, inhibit the oxidative reactions of the MPO rather than lipid soluble ones as vitamin E (Savenkova et al. 1994).

Chlorination by HOCl. MPO is the only human enzyme known to generate HOCl (Harrison and Schultz 1976). It is suggested that MPO promotes toxicity mainly by the production of HOCl and the chlorinating species (Winterbourn et al. 2000). HOCl is a potent oxidizing agent which is capable of oxidizing a variety of biological molecules such as carbohydrates, nucleic acids, peptide linkages, amino acids and lipids (Hazen et al. 1999a, Klebanoff 1999). HOCl is known to oxidize at a significant rate sulfhydryl and thioether groups of proteins (Winterbourn 1985) and it chlorinates the amino groups to chloramines (Thomas et al. 1982). HOCl converts L-tyrosine to 3-chlorotyrosine and cholesterol to chlorinated compounds (Hazen et al. 1996a, Hazen et al. 1996b).

Tyrosine radicals. MPO also acts as a classic peroxidase and about 5% of the hydrogen peroxide consumed by the enzyme creates tyrosyl radicals (Marquez and Dunford 1995). The conversion of L- tyrosine to tyrosyl radical may contribute to several oxidizing events such as LDL lipid peroxidation (Savenkova et al. 1994). Tyrosyl radical is able to promote protein and lipid oxidation independently of the tocopherol radical of vitamin E (Buettner 1993, Heinecke 1998). Both 3-nitro and 3-chlorotyrosine are found in elevated levels in human atherosclerotic plaques (Hazen and Heinecke 1997, Leeuwenburgh et al. 1997a).

Reactive nitrogen species. MPO may be considered an important NO oxidase in the vasculature as it can oxidize nitrite (NO_2^-) to reactive species. MPO uses NO_2^-, a decomposition product of NO, to generate chlorinating and nitrating intermediates (van der Vliet et al. 1997, Eiserich et al. 1998). It has been reported that the MPO system is known to nitrate tyrosine and tyrosyl residues (Eiserich et al. 1996, Eiserich et al. 1998).

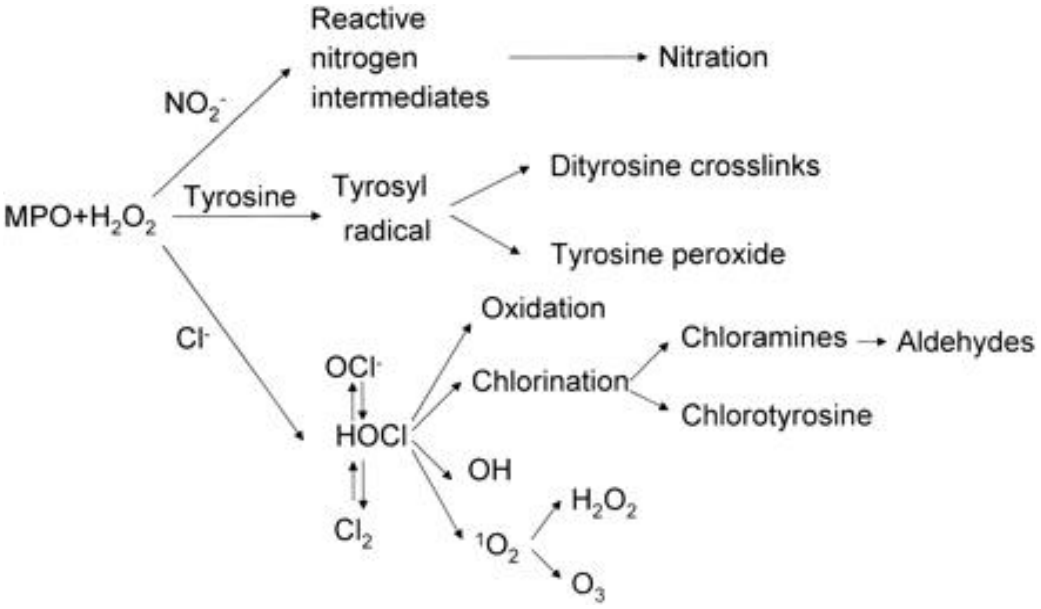

Figure 4. MPO catalyzed oxidative modifications.

MPO reacts with H_2O_2 that oxidizes chloride (Cl^-) to create HOCl which is able to oxidate a wide range of substrates. MPO is the only pathway for generating reactive chlorinating species at physiological levels of Cl^- in humans. HOCl reacts with amines to produce chloramines. In the absence of physiological Cl^- concentration MPO also acts as a classic peroxidase and tyrosine and nitrate serve as the likely physiological substrates for MPO. Tyrosine is reduced in a one-electron reaction to produce tyrosyl radical which promote protein ctosslinks via dityrosine formation. MPO can oxidize nitrite to produce nitrogen dioxide which can create nitrated lipids. Both radical species are able to induce lipid peroxidation. See text for details. Modified from Klebanoff SJ (2005): Myeloperoxidase: friend and foe. J Leukoc Biol 77:598-625.

Abbreviations: Cl^-; chloride ion, H_2O_2; hydrogen peroxide, HOCl; hypochlorous acid, MPO; myeloperoxidase.

3.3.1. MPO as a part of host defence sytem and bacterial killing

The bactericidal activity of MPO has been suggested to be dependent mainly on the production of HOCl (Hampton et al. 1998, Winterbourn et al. 2000). It has been established that HOCl is produced in the phagosomes corresponding to approximately 12% of the overall consumption of neutrophil oxygen metabolism (Hazen et al. 1996b, Hammer et al. 2001). Despite the potential for nitrite oxidation, this reaction is not facilitated because the conditions in the phagosome inhibit this oxidative reaction by MPO (Jiang and Hurst 1997).

The cellular uptake of MPO is accompanied by modulation of the activation state of monocyte-macrophages leading to the release of MPO generated radicals (Lefkowitz et al. 1992).

When monocyte-macrophages are exposed to the MPO released by neutrophils, they exhibit enhancement of the respiratory burst and increased phagocytosis (Lefkowitz et al. 1996). The MPO-deficient neutrophils have impaired bactericidal activity, although alternative oxidative mechanisms are thought to compensate the defect (Winterbourn et al. 2000). According to present knowledge, only the killing of Staphylococcus aureus is largely MPO dependent (Hampton et al. 1996).

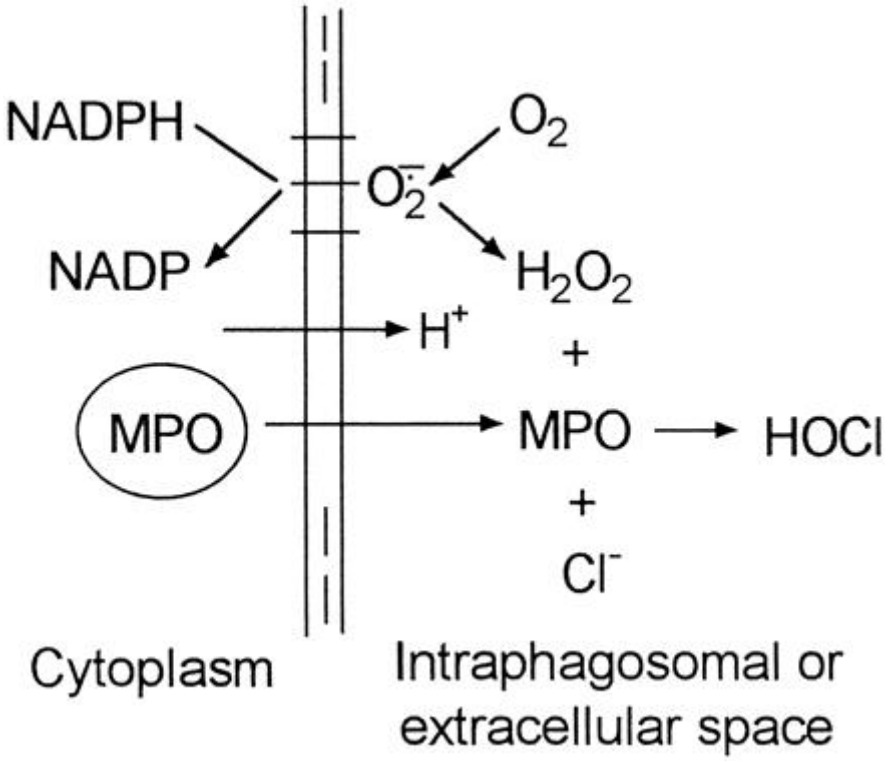

Figure 5. NAD(P)H oxidase -derived H_2O_2 as a substrate for MPO.

Alongside the release of MPO into the phagosome or extracellular space the NAD(P)H oxidase is activated to generate the H_2O_2 for MPO to mediate HOCl generation. See text for details. Modified from Klebanoff SJ (2005): Myeloperoxidase: friend and foe. J Leukoc Biol 77:598-625.

Abbreviations: Cl^-; chloride ion, H_2O_2; hydrogen peroxide, HOCl; hypochlorous acid, MPO; myeloperoxidase, NAD(P)H; nicotinamide adenine dinucleotide (phosphate).

3.3.2. MPO in inflammatory processes

In addition to acute inflammatory response, MPO-derived oxidants are also present in several inflammatory diseases such as ischemia-reperfusion injury, respiratory distress syndrome, glomerulonephritis, arthritis and gastric cancer (Couser 1993, Daher et al. 1997, Winterbourn et al. 2000, Matthijsen et al. 2007, Steenport et al. 2007, Steinbeck et al. 2007). The MPO system and HOCl have been established to activate the tumor suppressor protein p53 and activate the nuclear factor κB (Schoonbroodt et al. 1997, Vile et al. 1998). In addition, antibodies against MPO have been associated with several inflammatory diseases (Kallenberg 1998).

3.3.3. Catalytic mechanisms and substrates of MPO

During phagocytosis of the invading microorganisms the NAD(P)H-dependent oxidase is activated in the plasma membrane of the stimulated neutrophil. The activation produces O_2^- and H_2O_2 from the molecular oxygen (O_2) (Griendling et al. 2000). In the same process, MPO is released into the phagolysosome. In the presence of H_2O_2 and halide anion, which in human physiological state is mainly Cl^-, MPO catalyzes the generation of HOCl to kill the ingested microorganisms (Klebanoff 1980, Klebanoff 2005). MPO may modulate the inflammatory actions of PMNLs by inactivating secreted granule contents and contributing to the termination of the influx of PMNLs in the inflammatory locus (Nauseef 1988). MPO-deficient PMNLs exhibit a stronger and prolonged respiratory burst (Rosen and Klebanoff 1976).

3.4. Biomarkers for and the activity assays of MPO

Enzyme immunoassay (EIA) determined serum MPO concentrations represent circulating levels of MPO released from the neurophils (Hoy et al. 2001). In most studies, the measurement of MPO gene expression has been evaluated using the peroxidase activity of the blood leukocytes. Quantification of MPO is often reported as the measurement of MPO enzymatic activity in neutrophils by the mean peroxidase activity index (MPXI) calculated on an automated hematological analyser. However, the measurements may be biased as MPO is not the only peroxidase of the circulating granulocytes as the eosinophil peroxidase (EPO) may contaminate the measurements and can markedly affect the total peroxidase activity of the sample (Nauseef et al. 1998).

Chlorohydrins are formed by the addition of HOCl to double bonds which are present in cholesterol or various unsaturated ester and ether-phospholipid species, but their usefulness as biomarkers is limited (Malle et al. 2006a, Malle et al. 2007). MPO expressing macrophages are able to chlorinate uracil and the marker of DNA damage, 5-chlorouracil, has been detected in human atherosclerotic lesions (Takeshita et al. 2006). Another specific MPO-associated biomarker is 3-chlorotyrosine, which has been identified in human atherosclerotic lesions and lipoproteins extracted from lesions (Hazen and Heinecke 1997, Malle et al. 2007). Immunohistochemistry with specific monoclonal antibodies generated against HOCl-modified epitopes enables the identification of chlorinated biomarkers in atherosclerosis (Malle et al. 1995, Hazell et al. 1996, Malle et al. 2000). Fractionation of human plaque homogenate by centrifugation and subsequent immunoblot analysis of the LDL fraction is able to detect the MPO-modified apoB-100 (Hazell et al. 1996).

The chlorination activity of MPO can be measured by different assays. The chlorination of monochloride by HOCl results in a decrease in absorbance (Kettle and Winterbourn 1988). The assay has a tendency to underestimate the chlorinating activity of MPO but is useful for detecting HOCl, as shown by its complete inhibition by methionine (Kettle and Winterbourn 1988, Malle et al. 2007). The chlorination of nitrogen compounds with HOCl results in several different chloramines and the formation of taurin chloramines can be followed sensitively by spectrophotometric measurements (Thomas et al. 1986, Dypbukt et al. 2005). Also, loss of ascorbate has been used in assaying the chlorination activity of MPO, although ascorbate can act directly as a peroxidase substrate which may override the chlorination activity (Chesney et al. 1991, Malle et al. 2007).

The oxidation of tyrosine to dityrosine by peroxidation reactions of MPO can be followed spectrofluorimetrically (Marquez and Dunford 1995). The loss of H_2O_2 catalyzed by MPO can be monitored using an H_2O_2 electrode which in the presence of Cl^- as the only substrate to MPO allows a direct assessment of the MPO chlorination activity (Kettle and Winterbourn 1994, Malle et al. 2007).

3.5. MPO deficiency and clinical symptoms

Inherited deficiency of MPO is relatively common in Caucasian population with a prevalence of 1 in 2000 to 4000 individuals. Several studies have identified the most common genotypes involved in this condition (Nauseef 1990, Nauseef 1998, Nauseef et al. 1998). According to epidemiological studies hereditary MPO deficiency may be associated with increased susceptibility to candidiasis

by Candica Albicans and incidence of malignancies (Lehrer and Cline 1969, Lanza et al. 1987, Lanza 1998). According to some epidemiological studies, individuals with inherited MPO deficiency have less CAD than normal population (Kutter et al. 2000). Alternative bactericidal mechanisms are functionally dominant in human neutrophils, which presumably effectively compensate the immune responses in the absence of MPO (Lehrer and Cline 1969).

3.6. MPO activity related factors

Age. MPO serum levels tend to increase with age and the MPO release from PMNLs increases (Mohacsi et al. 1996, Hoy et al. 2001). However, reduced neutrophil function and enzyme release after middle-age has also been reported (Suzuki et al. 1983).

Smoking. Smoking affects the leukocyte count, but independent leukocyte activation has also been reported (van Eeden and Hogg 2000). Nicotine is known to enhance O_2^- anion generation in human neutrophils and findings also suggest that smoking increases the MPO activity (Bain et al. 1992, Pitzer et al. 1996, Hoy et al. 2001). Levels of MPO are elevated in smokers compared with non-smokers and the difference is also evident when former smokers are compared to never-smokers. The enhanced MPO production in smokers may be associated with the development and progression of CAD (Lavi et al. 2007, Rudolph et al. 2008).

Diabetes. MPO activity has been reported to be decreased in subjects with type 1 DM with a significant correlation between HbA1 levels and MPO activity (Sato et al. 1992). Also, in subjects with type 2 DM the MPO activity in leukocytes is reported to be significantly reduced (Uchimura et al. 1999). However, in diabetic rats the MPO activity is markedly increased (Zhang et al. 2004).

Estrogen. The intake of oral contraceptives may increase the levels of circulating MPO and in women on HRT, the intracellular activity and the amount of released MPO is increased (Bekesi et al. 2001a, Bekesi et al. 2001b, Hoy et al. 2001). Also, women treated with high estrogen levels have increased plasma MPO concentrations and, in vitro, the presence of estrogen leads to the release of MPO from PMNLs (Jansson 1991, Santanam et al. 1998). The release of MPO was lowered in coronary artery bypass graft surgery patients who were given 17β-estradiol before surgery (Wei et al. 2001).

Statins. Statins, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase strongly inhibit MPO mRNA expression in human and murine monocyte-macrophages. Reduction of MPO mRNA levels by 20- to 200-fold was observed in vivo in leukocytes from statin-fed mice, correlating with reductions in MPO protein and enzyme activity (Kumar and Reynolds 2005).

4. MPO and its role in the development of atherosclerosis

4.1. MPO as a catalyst for LDL oxidation in atherosclerosis

Products of MPO activity, including HOCl, tyrosyl radicals and NO can contribute to oxidative damage to host lipids and proteins predisposing to atherogenesis (Podrez et al. 2000, Tsimikas 2006). MPO- and HOCl-modified LDL are highly expressed in animal and human atherosclerotic vessels but not in normal control vessels (Hazen and Heinecke 1997, Malle et al. 2000, Malle et al. 2001). Modified LDL is found both in vascular cells and extracellular spaces and a potent chemotactic target for leukocytes (Malle et al. 2000).

MPO generated chlorinating oxidants. The 3-chlorotyrosine content of LDL and proteins derived from human atherosclerotic aorta are significantly increased in atherosclerotic intima when compared to normal vessel (Hazen and Heinecke 1997). Exposure of LDL to HOCl promotes lipoprotein aggregation (Hazell et al. 1994) and conversion into a high uptake particle for macrophages (Hazell and Stocker 1993).

Tyrosyl radical generated by MPO. Dityrosine is found enriched in LDL derived from human atherosclerotic lesions (Leeuwenburgh et al. 1997b). MPO-generated tyrosyl radical promotes the initiation of lipid peroxidation (Savenkova et al. 1994) and modification of HDL (Francis et al. 1993).

MPO-generated reactive nitrogen species and LDL nitration. MPO-generated reactive nitrogen species promote apoB-100 protein nitration and the initiation of LDL lipid peroxidation (Hazen et al. 1999b, Podrez et al. 1999). LDL modified by reactive nitrogen species generated by MPO-H_2O_2-NO_2^- system is converted into a form nitrotyrosine containing LDL that is avidly taken up by macrophages (Podrez et al. 1999).

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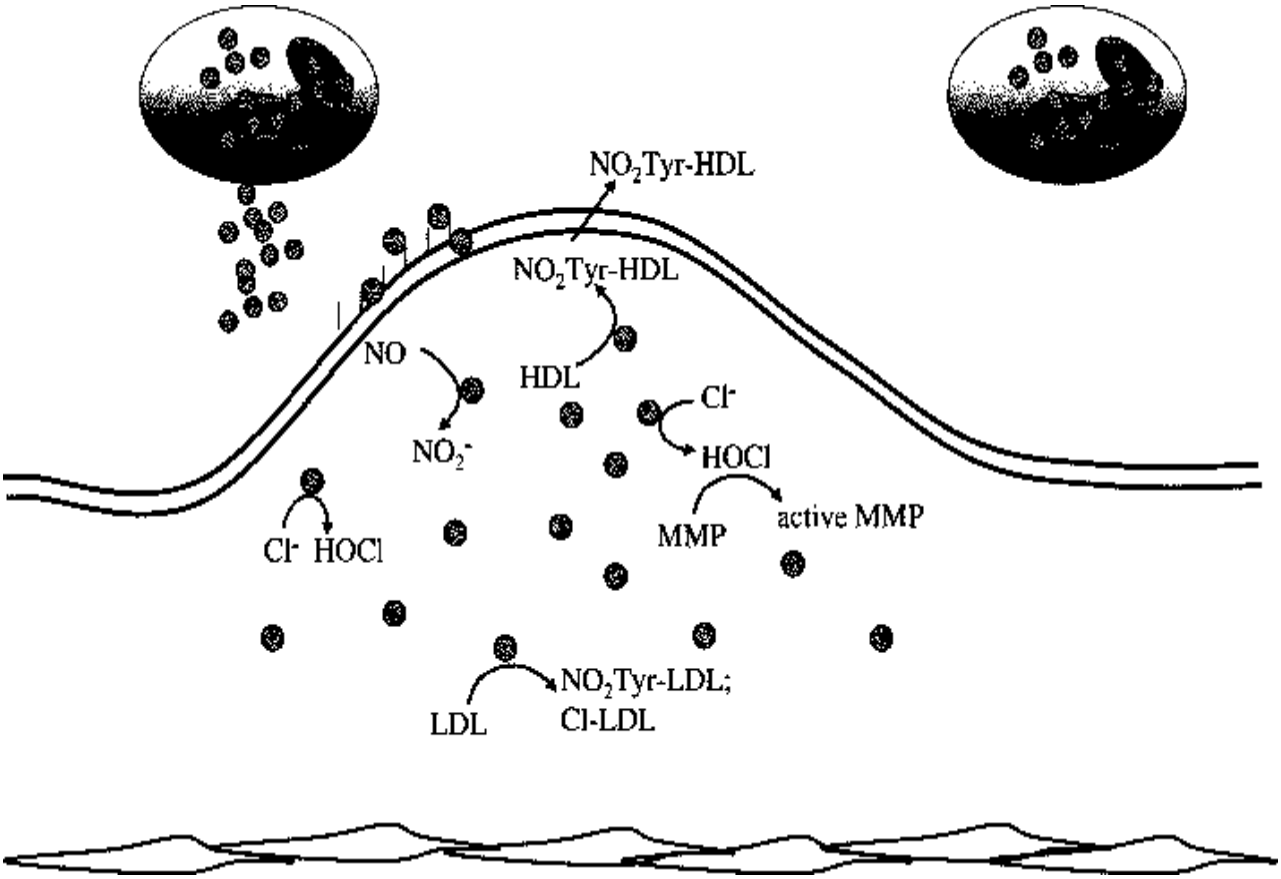

Figure 6. Proatherogenic properties of MPO exposed to the vessel wall.

MPO catalyzes the generation of HOCl and NO^{-}_{2} by oxidizing Cl^{-} and NO. LDL and HDL can both be modified by HOCL or NO^{-}_{2}; yielding Cl-LDL or NO_2-Tyr-LDL and Cl-HDL or NO_2-Tyr-HDL. MPO modulates MMP activity via HOCl, either directively by activating MMP or by suppressing MMP inhibitors. For details, see text. Reprinted from Lau D and Baldus S (2006): Myeloperoxidase and its contributory role in inflammatory vascular disease. Pharmacology & Therapeutics 111: 16-26, with permission from Elsevier.

Abbreviations used in the figure: Cl^{-}; chloride, Cl-LDL; chlorinated low density lipoprotein, HDL; high density lipoprotein, HOCl; hypochlorous acid, LDL; low density lipoprotein, MMP; matrix metalloproteinase, MPO; myeloperoxidase, NO; nitric oxide, NO_2; nitrogen dioxide, NO_2^{-}; nitrite, NO_2Tyr-HDL/LDL; HDL/LDL containing nitrotyrosine.

4.2. MPO modifies apoA-I creating dysfunctional HDL

There is mounting evidence that anti-atherogenic HDL becomes oxidatively modified by MPO (Nicholls et al. 2005, Malle et al. 2006b). HDL isolated from the blood of the subjects with CVD,

contains elevated levels of chlorinated tyrosine and HDL-associated protein apoA-I, which acts as a selective target for MPO-catalyzed oxidation (Bergt et al. 2004, Zheng et al. 2004). The HDL isolated from the atherosclerotic lesions contains a variety of MPO-derived peptides, including oxidative modification results of reactive chlorinating and nitrating species (Zheng et al. 2004, Zheng et al. 2005). These determinants seem to colocalize with apoA-I and MPO in human atheroma (Marsche et al. 2002, Bergt et al. 2004, Malle et al. 2006b). While the HDL and the apoA-I are chlorinated, the removal of cholesterol from cultured cells by ATP-binding cassette transporter A1 is impaired (Peng et al. 2005, Shao et al. 2005, Zheng et al. 2005). In addition, the modification of HDL by HOCl increases the binding affinity of HDL for MPO. It has been proposed that the binding of MPO with HOCl-HDL protects the MPO from cellular uptake and degradation by ECs (Marsche et al. 2008).

4.3. MPO promotes endothelial dysfunction

The interaction of NO with MPO may serve as a modulator of the peroxidase catalytic activity, influencing the regulation of local inflammatory and infectious events (Abu-Soud and Hazen 2000b). MPO acts as an NO oxidase in the vasculature and affects the anti-inflammatory properties of ECs (Lau and Baldus 2006). Endothelial-derived NO contributes to the relaxation of SMC and the inhibition of muscle cell proliferation, adhesion molecule expression and platelet aggregation (De Caterina et al. 1995, Salvemini et al. 1996, Abu-Soud and Hazen 2000a, Abu-Soud and Hazen 2000b).

MPO-generated oxidants have been reported to inhibit the activity of NOS directly and by chlorinating its crucial substrate, L-arginine (Abu-Soud and Hazen 2000a, Abu-Soud and Hazen 2000b). As a consequence, in in vitro studies the formation of NO by ECs has been diminished and inhibits the acetylcholine-induced relaxation of rat aortic ring segments (Zhang et al. 2001a, Zhang et al. 2001b). There are several possible ways in which the MPO enzyme can affect NO synthesis. In addition to HOCl, MPO-modified HDL and nitrogen species are also able to inhibit the NO synthesis and reduce the availability of the essential NOS cofactor NAD(P)H (Auchère and Capeillère-Blandin 1999, Abu-Soud and Hazen 2000a, Marsche et al. 2004).

In a rodent model of acute endotoxemia, MPO knockout mice exposed to an acute inflammatory stimulus displayed improved vascular function and increased vascular NO bioavailability suggesting that subendothelial MPO is a significant contributor to impaired NO bioavailability in vivo (Eiserich et al. 2002). In the study of symptomatic CAD patients, the forearm perfusion in response to NO-liberating acetylcholine correlated inversely with MPO

plasma levels (Baldus et al. 2004). In acute MI patients undergoing myocardial reperfusion, the plasma samples contained increased levels of MPO, which catalytically consumed NO in the presence of H_2O_2 (Baldus et al. 2004). In addition, while MPO is mobilized from vascular compartments by heparin the forearm perfusion increases (Baldus et al. 2006).

In a study of 298 subjects, the MPO levels were found to predict endothelial dysfunction measured with flow-mediated and nitroglycerin-mediated dilation of the brachial artery. The MPO levels were found have a strong inverse correlation with flow-mediated dilatation to predict endothelial dysfunction, even after multivariable adjustment (Vita et al. 2004). However, in contrast, in a study of 20 patients whose endothelial function was tested during diagnostic coronary angiography, the MPO and nitrotyrosine gradients were similar both in subjects with endothelial dysfunction and controls (Lavi et al. 2008).

4.4. MPO and the development of vulnerable plaque

HOCl-modified proteins are accumulated at ruptured or eroded sites in the coronary atheroma of subjects suffering sudden cardiac death, suggesting a potential mechanistic role for MPO (Sugiyama et al. 2001). Accordingly, both neutrophils and macrophages alongside with MPO- and HOCl-modified proteins are localized in the coronary thrombus (Buffon et al. 2002, Naruko et al. 2002). The neutrophils are activated in ACSs (Biasucci et al. 1996) and an increase in neutrophil MPO activation has been detected in patients with unstable angina pecotris (Buffon et al. 2002). The activation was independent of the site of the stenosis, which may be a marker of a widespread inflammatory process occurring in the coronary vasculature (Buffon et al. 2002).

The in vitro studies demonstrate that HOCl is able to promote the activation of MMP-7 and EC apoptosis which may, in theory, promote the development of the plaque erosion (Fu et al. 2001, Sugiyama et al. 2004). HOCl, generated by the MPO-H_2O_2-Cl^- system inactivates the activity of tissue inhibitors of MMPs (TIMPs) possibly enabling the proteoloytic activity of MMPs during inflammation (Wang et al. 2007). In the ECs exposed to MPO or MPO-expressing macrophages the expression of TF is increased and physiological doses of HOCl also promote the ECs' apoptosis (Sugiyama et al. 2004).

4.5. MPO and CAD in clinical studies

MPO as a prognostic biomarker in acute coronary syndrome (ACS). In a study of 604 patients presenting at the emergency department with chest pain, the initial measurement of plasma MPO

independently predicted early risk of MI, as well as the risk of major adverse cardiac events in the ensuing 30-day and 6-month periods (Brennan et al. 2003). MPO levels, in contrast to troponin T, creatine kinase MB isoform, and CRP levels, identified patients at risk for cardiac events in the absence of myocardial necrosis, highlighting its potential usefulness for risk stratification among patients presenting with chest pain. In the multivariable model adjusted for traditional cardiovascular risk factors, MPO levels were associated with an odds ratio (OR) of 11.9 (95% confidence interval [CI], 5.5–25.5) for the highest versus the lowest quartiles of leukocyte-MPO and an OR of 20.4 (95% CI, 8.9–47.2) for the highest versus lowest quartiles of blood-MPO (Brennan et al. 2003). In the study, plasma levels of MPO tended to be lower in females than in males and MPO levels showed a tendency for to be a stronger predictor of risk for cardiac events in females than in males (Brennan et al. 2003, Hazen 2004).

The significance of the MPO levels as the independent predictive value has also been shown in patients with ACS in a 6 month-follow-up study of 1090 patients. The elevated MPO serum levels powerfully predicted an increased risk for subsequent cardiovascular events even though MPO levels did not correlate with blood levels of troponin T, soluble CD40 ligand or CRP or with ST-segment changes (Baldus et al. 2003). Accordingly, in a cohort study of 193 men with ACS patients were followed prospectively for the development of death and MI, and the baseline MPO levels independently predicted MI at 2 years (Cavusoglu et al. 2007). In a study on 140 patients with acute chest pain and a non-ST elevation in electrocardiography serum MPO level measurements were submitted. MPO proved to be the only independent variable to predict acute MI (Esporcatte et al. 2007).

Angiographically diagnosed CAD. In a case-control study including 158 patients with diagnosed CAD and 175 patients without angiographically significant CAD the leukocyte and blood MPO levels were both significantly higher in patients with CAD than in controls (Zhang R et al. 2001). This case-control study of 874 patients with angiographically proven CAD and 194 subjects with normal coronary angiograms MPO levels were measured before angiography. MPO levels were elevated in patients with CAD and the highest levels of MPO were found in those subjects with progression of CAD from stable CAD to ACS (Ndrepepa et al. 2008).

Myocardial dysfunction. MPO has been demonstrated to contribute to adverse ventricular remodeling after AMI. In a study on 512 acute MI patients plasma MPO concentrations were higher in MI patients than controls and patients with above-median MPO levels in combination with above-median plasma amino-terminal pro-brain natriuretic peptide (NT-proBNP) or below-median left ventricular (LV) ejection fraction had significantly greater mortality than other patients (Mocatta et al. 2007). In a study where 384 post ST segment elevation MI patients were followed

up until death or MI the median MPO was raised in patients suffering death or MI when compared to survivors (Khan et al. 2007). In addition, in a cohort of patients with chronic heart failure, elevated plasma MPO levels were associated with deterioration of the functional class (Tang et al. 2006). In a study on 447 stable outpatients, 113 had impaired LV function resulting from either nonischemic or ischemic cardiomyopathy. MPO plasma levels were significantly higher in patients with impaired LV function than in patients with normal LV function regardless of the presence of CAD (Rudolph et al. 2007a). In MPO knockout mice, a marked reduction in leukocyte infiltration and ventricular dilatation was also demonstrated (Seekamp and Ward 1993).

5. MPO gene

5.1. MPO gene expression

MPO is encoded by a single gene approximately 11kB in size and located on the long arm of chromosome 17q23.1 and composed of 11 introns and 12 exons (Chang et al. 1986, Inazawa et al. 1989, Zaki et al. 1990, Law et al. 1995). Expression of the MPO gene is strictly regulated tissue and development specifically (Lubbert et al. 1991). The MPO expression is restricted to myeloid cells, as, during the granulocyte differentiation in the bone marrow only promyelocytes continue to express and synthesize the MPO enzyme (Borregaard and Cowland 1997, Gullberg et al. 1999). Human MPO messenger RNA (mRNA) is found only during the late myeloblast and promyelocyte stages of myeloid development and the expression decreases sharply as these precursors mature along the granulocyte or monocyte lineages (Sagoh and Yamada 1988, Tobler et al. 1988, Lubbert et al. 1991). When monocytes differentiate to tissue macrophages the MPO gene can be reactivated in subsets of reactive macrophages as in foam cells in atherosclerotic lesions (Sugiyama et al. 2001).

MPO promoter region. The promoter elements that regulate the myeloid-specific expression of the MPO gene are only partially understood and the mechanisms that restrict MPO gene expression are mostly unknown. The molecular analysis of the human MPO promoter region in the promyelocytic cells indicates the presence of a complex array of positive and negative regulatory sites and that some additional elements outside the promoter area are needed to reverse the repression of the human MPO gene in a promyelocyte-specific manner. Several consensus binding sites for transcriptional activators have been found from the promoter area, including SP1 transcription factor (Chumakov et al. 2000).

In murine MPO gene, three functionally active initiation sites for MPO RNA synthesis have been described (Zhao et al. 1997). The analogous three initiation sites for mRNA synthesis have also been found in human cells: P1, P2 and P3 are situated at about bp -925, -310 and +1 of the MPO gene. In contrast to the murine gene, where physiological transcription of intact MPO mRNA may originate at several distinct sites, physiological synthesis of human MPO mRNA appears to be initiated at the promoter P1 site alone (Lin and Austin 2002).

Regulation of MPO gene expression. A variety of transcription factors regulate MPO expression. The MPO gene is regulated by the site-specific acute myelogenous leukemia transcription factor (AML1) and the complete structure of an AML1 binding site is essential for the proximal enhancer site (Nuchprayoon et al. 1994). This site contains an Alu receptor response element (AluRRE), which is recognized by various nuclear receptors including SP1 (Vansant and Reynolds 1995, Piedrafita et al. 1996). The human monocytes exposed to granulocyte-macrophage colony-stimulating factor (GM-CSF) continue to express MPO in vitro as they differentiate into macrophages (Sugiyama et al. 2001). Some nuclear receptors such as PPARγ and ERα are able to regulate MPO gene expression in human macrophages (Piedrafita et al. 1996, Kumar et al. 2004). Moreover, the PPARγ and ERα may compete for binding the Alu receptor response element (AluRRE) in the MPO promoter (Vansant and Reynolds 1995, Piedrafita et al. 1996, Kumar et al. 2004). PPARγ has a binding site in an Alu element preceding the human MPO gene and PPARγ ligands may induce or suppress human MPO gene expression depending on the presence of macrophage colony stimulating factor (M-CSF) or GM-CSF (Kumar et al. 2004).

5.1.1. Mutations of MPO gene in MPO deficiency

Mutations in the MPO gene that alter any of the steps in this biosynthetic pathway may influence the phenotype of MPO deficiency in distinct ways (Nauseef et al. 1998). There is heterogeneity in MPO deficiency at the protein, mRNA and genomic DNA levels (Selsted et al. 1993, Kizaki et al. 1994, Nauseef et al. 1998). Both pre-translational and post-translational defects have been reported (Tobler et al. 1989, Nauseef et al. 1996).

A variety of mutations resulting in MPO deficiency have been reported including seven missense mutations. Four of them have been characterized in detail for their impact on MPO biosynthesis (Nauseef et al. 1994, Romano et al. 1997, DeLeo et al. 1998, Nauseef 2004, Ohashi et al. 2004). The effects of these genotypes on the synthesis of MPO mutant proteins have been assessed using cell lines stably transfected with mutant cDNA. Such studies have suggested

possible structure-function relationships of intracellular progressing and targeting of MPO precursors (Hansson et al. 2006).

Complete hereditary MPO deficiency affects 1 in 2,000 to 4,000 individuals and several mutations causing this disease (Parry et al. 1981). The most common genotype in Europe and the United States is a point mutation C to T in exon 10 causing an arginine replacement with a tryptophan (R569W) in the heavy subunit (Kizaki et al. 1994, Nauseef et al. 1994). A tyrosine replacement with cysteine at codon 173 in exon 10 (Y173C) (DeLeo et al. 1998) and methionine at the light subunit replaced with threonine (M251T) within exon 9 disrupt the light subunit of MPO protein (Romano et al. 1997). In Japanese population glycine replacement with serine at codon 501 (G501S) in the exon 9 region has been also been identified (Ohashi et al. 2004). A marked share of the subjects with complete MPO deficiencies are compound heterozygotes and the phenotype of the patient depends on the relative contribution of each allele to the final product and the interaction between each allelic product during the biosynthesis (Nauseef et al. 1998).

5.2. Polymorphic sites of MPO

According to the Database of Single Nucleotide polymorphism, 123 SNPs have been identified for human MPO. However, most of the polymorphisms described in public databases have not been confirmed. Table 3 presents the detected promoter and coding region SNPs with a known frequency. Five are nonsynonymous amino acid changes within the coding sequence (V53F, M251T, A332V, I642L and I717V) and six are located in the promoter region 2000 bp upstream of the starting site of MPO gene (Hoy et al. 2001, Chevrier et al. 2003).

Table 3. Positions and denominations of MPO promoter and coding region polymorphisms among Caucasian controls. See text for details. Modified from Chevrier I et al. (2003): Myeloperoxidase: new polymorphisms and relation with lung cancer risk. Pharmacogenetics 13:729-739.

Location	Base Change	Mutation allele frequency	Function	Denomination
5´flanking	-1940A/G	0.05	Noncoding	-1940A/G
5´flanking	-1812T/G	0.28	Noncoding	-1812T/G
5´flanking	-638C/A	0.19	Noncoding	-638C/A
5´flanking	-581T/C	0.27	Noncoding	-581T/C
5´flanking	-463G/A	0.24	Noncoding	-463G/A
5´flanking	-129G/A	0.04	Noncoding	-129G/A
Exon 2	2986G/T	0.06	Nonsynonymous	V53F
Exon 6	4311T/C	0.02	Nonsynonymous	M251T
Exon 7	5414C/T	0.02	Nonsynonymous	A332V
Exon 11	11672A/C	0.02	Nonsynonymous	I642L
Exon 12	12684A/G	0.02	Nonsynonymous	I717V

5.2.1. Exonic polymorphisms of MPO

Genetic polymorphisms have been found in exon 2 causing valine replacement with phenylalanine (V53F), exon 6 causing methionine replacement with threonine (M251T), exon 7 causing alanine replacement with valanine (A332V), exon 11 causing isoleusine replacement with leusine (I642L) and exon 12 causing isoleusine replacement with valine (I717V) (Chevrier et al. 2003). Carriers of 53F have been associated with a higher MPO activity (Chevrier et al. 2006). Otherwise, in the two studies considering the exon polymorphisms no evidence of the functionality of these polymorphisms was found (Chevrier et al. 2006, Dolley et al. 2008).

5.2.2. Intronic polymorphisms of MPO

-129G/A. A G/A substitution is located at position -129 upstream from the transcription start site, abolishing an SP1 binding site. The A-allele has been shown to be associated with lower serum MPO concentrations and found to reduce MPO activity in neutrophils (Hoy et al. 2001, Rutgers et al. 2003, Chevrier et al. 2006). The polymorphism has been suggested to account for 2.6% of the variance in the MPO concentration of the population (Hoy et al. 2001).

-463G/A. Of the MPO promoter polymorphisms, the -463G/A is best known and has also been studied in clinical settings. -463G/A (rs2333227) is located within an Alu-encoded hormone response element (AluHRE) consisting of a cluster of four hexamer half sites (Vansant and Reynolds 1995, Piedrafita et al. 1996, Reynolds et al. 1997). This cluster is recognized by various nuclear receptors, and the G-allele creates an SP1 binding site in the first hexamer. The transcriptional activity of the A-allele has been found to be severalfold less in transient transfection assays (Vansant and Reynolds 1995, Piedrafita et al. 1996). In myeloid leukemia cells the GG genotype presents two- to threefold higher expression of MPO messenger RNA and higher levels of MPO than A-allele carriers (Reynolds et al. 1997). Although the presence of an SP1 binding site in the −463G allele has been associated with an increase in MPO expression in vitro, no effect on MPO concentration was detected in vivo (Piedrafita et al. 1996). In one study, the polymorphism has been reported to exhibit gender and age -dependent differences in MPO activity (Rutgers et al. 2003). The A-allele creates a stronger binding site for the ERα which may possibly lead to differential regulation of G- and A-alleles in men and women (Norris et al. 1995, Reynolds et al. 2000, Kumar et al. 2004). Accordingly, ERα ligand 17β-estradiol (E2) has an ability to block the effects of PPARγ, especially on the -463G/A A-allele (Reynolds et al. 2000, Kumar et al. 2004, Reynolds et al. 2006).

The GG genotype is most common, being present in 61% of Northern European populations (Nagra et al. 1997, Reynolds et al. 1997, Reynolds et al. 1999, Cascorbi et al. 2000). There are ethnic variations in allele frequency, with the AA genotype represented in 15% of African-Americans, (London et al. 1997) 3 to 7% of Caucasians, (Nagra et al. 1997, Reynolds et al. 1997, Reynolds et al. 1999, Cascorbi et al. 2000) and 2 to 3% of Japanese-Pacific Islanders (Le Marchand et al. 2000).

-638C/A. The A-allele of the -638C/A polymorphism has been associated with increased MPO activity. However, the SNP does not appear to be located in any known regulatory sequence (Chevrier et al. 2003, Chevrier et al. 2006).

-765T/C and -822C/A are located in the 5´ region of the MPO gene. However, they are not located in any known regulatory sequence but may in theory alter the gene expression (Dolley et al. 2008). The -822C/A has been demonstrated to be associated with increased MPO activity (Chevrier et al. 2006). A study on 680 subjects failed to show any association between these SNPs and the LDL phenotype (Dolley et al. 2008).

-1940A/G. In the studies investigating the polymorphism, no association has been found with MPO activity and -1940A/G polymorphism (Chevrier et al. 2003, Chevrier et al. 2006).

5.3. MPO polymorphism -463G/A and atherosclerotic diseases

Lipid profile. In a cohort of 82 healthy families consisting of both men and women, the A-allele of the polymorphism was associated with higher levels of TGs, total cholesterol, LDL cholesterol and apoB than G-allele homozygotes (Hoy et al. 2001). In a genome-wide scan of 680 subjects, the A-allele was associated with lower plasma total cholesterol, LDL and apoB levels being lowest among AA homozygotes. When analyzed further, the results remained significant only in women. They hypothesized that A-allele carriers had decreased MPO levels, which could attenuate LDL oxidation and, consequently, facilitate the LDL reuptake by the liver (Dolley et al. 2008). In a study on 447 stable outpatients, both men and women, the AA genotype also showed a lower prevalence of hypercholesterolemia (Rudolph et al. 2007a).

Coronary artery disease (CAD). In a cohort of 155 end-stage renal disease patients comprising both men and women, the GG genotype was associated with higher prevalence of CVD (Pecoits-Filho et al. 2003b). In a case-control group which consisted of 229 CAD patients, both men and women, the A-allele was less frequent among cases with CAD than in controls (Nikpoor et al. 2001). In a study on 277 patients of both gender referred for a first diagnostic coronary angiography, the GG homozygotes had an increased risk of developing a cardiovascular event (Asselbergs et al. 2004).

Ventricular dysfunction. In a study on 447 stable outpatients consisting of men and women 113 patients had impaired LV function resulting either from nonischemic or ischemic cardiomyopathy. The -463G/A polymorphism was not associated with the LV dysfunction (Rudolph et al. 2007a).

Stroke. In the study on 450 patients comprising both men and women the allele frequence did not differ between the stroke patients and controls. However, the frequency of A -allele was reported to be higher among those patients whose post stroke recovery was poor (Hoy et al. 2003).

AIMS OF THE STUDY

The association between genetics, lipoprotein oxidation and atherosclerosis is firmly established. MPO has been shown to be able to produce ROS to oxidize LDL in vivo and in vitro. The -463G/A promoter polymorphism (rs2333227) has been associated with CAD and other inflammatory conditions. However, the role of MPO genotypes in different stages of atherosclerosis is still unclear, as are the factors that interact with MPO and modify the functions of MPO polymorphism. The present study used four clinical and one autopsy series to elucidate the relationship between MPO genotypes and indices of lipid oxidation, coronary reactivity, intima-media thickening and autopsy-verified atherosclerotic lesions. Since estrogen have been found to be involved in regulating MPO responses as the A-allele of the MPO polymorphism creates a stronger ERα receptor binding site, the interaction between MPO (rs2333227) genotypes and estrogen replapcement therapy in postmenopausal women was also studied. The specific aims of the study were:

1. To elucidate the association between MPO genotypes and the indices of lipid oxidation both in healthy men and in postmenopausal women receiving HRT (I, IV).

2. To analyze whether MPO genotype is associated with the indices of coronary blood flow as measured by PET in healthy men (I).

3. To assess the interaction of MPO genotypes and type 2 DM in carotid artery IMT in middle-aged men (II).

4. To examine the relationship between the MPO genotypes and autopsy-verified early and advanced atherosclerotic lesions in the abdominal and thoracic aorta (III).

5. To study the interaction of MPO genotypes and disease progression of atherosclerosis in postmenopausal women receiving HRT (V).

SUBJECTS AND METHODS

1. Clinical series

1.1. Positron emission tomography (PET) study (I)

Fifty-one men from the Archipelago Sea Naval Command, Archipelago Coast Guard District, Säkylä Garrison and the Turku Fire Department were invited to participate in the study. The following inclusion criteria were employed: 1) age 25-40 years, 2) total cholesterol level > 5.5 mmol/l, 3) clinically healthy and 4) no continuous drug therapy or antioxidant vitamin use. For the background information, the study subjects were asked about their family history of CAD, alcohol and caffeine consumption, medication, smoking and exercise habits using a validated questionnaire. In Study I, 49 men out of 51 were included in the statistical analyses and two were excluded due to technical problems with the PET measurements. The study was approved by the Ethics Committee of the Turku University Central Hospital and the University of Turku. Each subject gave written informed consent.

1.2. Random sample of Finnish middle-aged men (II)

The subjects for this study were selected from a cohort of 9,058 males aged 50 to 59 years living in the city of Tampere. Three hundred men were randomly invited by letter to participate and 223 (74%) consented while 33 refused and 44 did not answer or could not be reached. The blood pressure of these men was measured and detailed medical histories were collected with a focus on cardiovascular and metabolic diseases, smoking habits and medication. The standard 2-hour oral glucose tolerance test (OGTT) according to WHO 1998 criteria, was used to assess glucose tolerance. All the required data, including MPO genotype were obtained from 198 subjects, which comprised the adjusted study population for the analysis. The Ethics Committee of the UKK Institute approved the study and the participants gave written informed consent.

1.3. Long-term Hormone Replacement Therapy (HRT) Study (IV, V)

In 1993, women attending to a private outpatient clinic in Tampere for annual routine gynecological examinations were invited to participate. For the cross-sectional baseline study in 1993, 120 nonsmoking and nondiabetic postmenopausal women, aged 45-71 years, were enrolled. In 1998, all of these 120 women were invited by letter to participate in the 5-year follow-up study; 87 of 120 (72.5%) consented. They had no clinically evident CVD or HTA and were classified into 3 groups based on the use of HRT. The data from MPO genotype (rs2333227) was available for 87 subjects. The HRT-EVP group (n = 25) used estradiol valerate (EV; 2mg/d) for 11 days, followed by EV continued with progestin (P; levonorgestrel, 0.25mg/d) for 10 days. The HRT-EV group (n = 32) used EV alone, and the control group (n = 30) had never used HRT. At the baseline, the mean duration of EV and EVP treatment was 9.2 ± 3.7 and 10.2 ±2.2 years respectively. The mean time from menopause in the controls was 11.9 ± 4.1 years. The mean ages in the HRT-EVP, HRT-EV and control groups were 60.4 ± 4.8, 59.5 ± 5.5 and 61.5 ± 5.8 years respectively. Ultrasonography was performed at baseline and follow-up to determine intima-media far wall thickness. Study V consisted all of the follow-up atherosclerosis severity score (ASC) data and in Study IV, the baseline values ASC and oxLDL-abs were used. The Ethics Committee of the Tampere University Hospital approved the study. All subjects gave written informed consent.

2. Autopsy series

2.1. The Helsinki Sudden Death Study (HSDS) (III)

The HSDS was launched to study the lifestyle and genetic risk factors predisposing Finnish middle-aged men to sudden death. The HSDS consisted of a series of a total of 300 Caucasian men whose mean age was 53 years (range 33 to 69 years). The series of men was subjected to a medicolegal autopsy at the Department of Forensic Medicine, University of Helsinki between 1991 and 1992 (B-series, n = 300). For the collection of data on CAD risk factors, a relative or a close friend of the deceased were given a detailed questionnaire including a review of past and recent smoking and drinking habits and previous illnesses (Karhunen and Penttilä 1990). In Study III, the data from the autopsies and MPO (rs2333227) genotypes were available in 266 cases. Due to the sudden unexpected death data on CAD risk factors based on the questionnaire was available in 124 of the study cases.

3. Measurements of serum lipids, apolipoproteins and glucose tolerance (I, II, IV, V)

In Study I, the blood test was drawn after the 12-hour-fasting to determine the concentrations of lipids and apolipoproteins, plasma triglyserides and the total and HDL cholesterol. The concentrations were analyzed by a Cobas Integra 700 automatic analyzer using the manufacturer's reagents and calibrators (Hoffmann-La Roche Ltd., Switzerland). LDL cholesterol concentrations were calculated according to Friedewals's formula (Friedewald et al. 1972). ApoB and apoA concentrations were measured by an immunoturbidimetric method using specific controls (Hoffmann-La Roche Ltd., Switzerland) on the same analyzer as the lipids.

In Study II, lipoprotein fractions were assessed from fresh samples by ultracentrifugation where 4 ml of serum was transferred to a 6.5 ml centrifuge tube which was then filled by layering saline on top of the serum. After centrifugation for 16 hours the top layer containing VLDL and the two layers containing HDL and LDL were transferred to separate flasks. The underlying layers were centrifugated for a further 20 hours to distinguish an LDL containing top layer and bottom yellow fraction containing HDL and serum proteins. The three separate VLDL, LDL and HDL fractions were then extracted for determination of TGs and cholesterol (Carlson 1973). Cholesterol was measured from serum and lipoprotein fractions using an enzymatic method (CHOD-PAP), Boehring Mannheim, Germany). TGs were measured by enzymatic hydrolysis (GPO-PAP, Boehringer Mannheim, Germany). ApoB was determined by immunonephelometry (Behring, Behringwerke AG, Germany). To assess the glucose tolerance, the standard 2-hour oral glucose tolerance test (OGTT) with a 75 g glucose load was performed according to the WHO 1998 criteria (Alberti and Zimmet 1998). The blood samples were taken at baseline one and two hours after the glucose load, and the plasma glucose concentrations were measured. Fasting glucose level of 7.0 mmol/l or higher, and/or a 2-h post-challenge glucose level of 11.1 mmol/l or higher were considered to be diagnostic criteria for type 2 DM (Alberti and Zimmet 1998). Glucose analyses were carried out on hemolyzed whole blood samples using the glucose dehydrogenase/mutarotase method (Merck Diagnostica, Germany).

In Studies IV and V, lipid measurements were made at baseline and after 5-year follow-up. Serum total cholesterol and triglyserides were determined by a commercial method (Kodak Echtachem 700XR, Eastman Kodak Co., Rochester, NY). Serum HDL cholesterol and its subfractions (HDL_2, HDL_3) were separated with a dextran-sulfate-magnesium precipation procedure and the cholesterol content was analyzed with a Monarch 2000 Analyzer (Instrumentation Laboratory, Lexington, KY), using the cholesterinoxidase-

peroxidase/antiperoxidase cholesterol reagent (catalog no. 237574, Roche, Mannheim, Germany) and a primary cholesterol standard (catalog no 67265 and 67249, Orion Diagnostics).

4. Measurements of autoantibodies against oxLDL (I, IV)

The levels of autoantibodies against oxLDL were measured by a solid phase enzyme-linked immunosorbent assay (ELISA) as previously described (Lehtimäki et al. 1999). The antigens were prepared from the pooled plasma of ten donors and were divided into two groups: 1) native LDL was protected against oxidation by 0.27 mmol/l EDTA and 20 µmol/l butylated hydroxytoluene in PBS and 2) oxLDL was produced by 24-hour incubation of native LDL with 2 µmol/l $CuSO_4$. Half of the wells on each ELISA plate were coated with native LDL (5 µg/ml). The coated plates were incubated, washed and blocked and the serum samples diluted to 1:15 (I) or 1:20 (IV) were added to the wells. After incubation, peroxidase-conjugated rabbit anti-human IgG antibodies were added and *o*-phenylenediaminen substrate (Sigma, USA) was used to detect the anti-LDL binding of the test samples that was measured as the optical density at 492 nm. The results were expressed as the mean of duplicated samples. The autoantibody titer against oxLDL was calculated both by substraction of the binding to native LDL from the binding to oxLDL and oxLDL to native LDL ratio.

5. Evaluation of myocardial blood flow (MBF) and blood flow reserve by PET (I)

The participants had fasted for 6 hours before the PET studies. At the beginning, two catheters were inserted, one in the antecubital vein of the left arm for the injection of $[^{15}O]H_2O$ and for the infusion of adenosine, the other in the antecubital vein of the right arm for blood sampling. The patients were positioned supine in a 15-slice ECAT 931/08-12 tomograph (Siemens/CTI Inc., USA). After a transmission scan the subjects' nostrils were closed and he inhaled $[^{15}O]CO$ for 2 minutes through a three-way inhalation flap-valve. $[^{15}O]CO$ was allowed to combine with the hemoglobin for 2 minutes before data collection for a static scan was started. During the scan period, three blood samples were drawn at 2-minute intervals and the radioactivity was measured. A 10-minute period was allowed for radioactive decay of $[^{15}O]CO$ before the blood flow measurements were started. Blood flow was measured at baseline and 60 seconds after the beginning of intravenous administration of adenosine. For the blood flow measurement $[^{15}O]H_2O$

was injected intravenously for 2 minutes and dynamic scanning was started for 6 minutes. To calculate the rate-pressure product (RPP), the subject's heart rate and blood pressure were monitored throughout the study.

Large regions of interest were placed on representative transaxial ventricular slices in each study covering the anterior, lateral, septal and whole free wall of the LV (Iida et al. 1995). The regions of interest were drawn on the images obtained at rest and copied input function was obtained at rest and copied to the images obtained after adenosine administration. The arterial input function was obtained from the left ventricular time activity curve using a previously validated method (Iida et al. 1992). Since no regional blood flow differences were found, overall MBF was used for further analyses. The coronary flow reserve (CFR) was defined as the ratio of overall MBF after administration to flow at baseline. The coronary resistance values were calculated both at baseline and during adenosine infusion by dividing the mean arterial blood pressure by the respective flow value. RPP adjusted resting blood flow was calculated by multiplying the subject's basal blood flow by the mean RPP of the study population and dividing the result by subject's RPP. The CFR adjusted for RPP was calculated as the ratio of MBF during adenosine administration to RPP adjusted flow at baseline.

6. Ultrasound measurements of arteries (II, IV, V)

6.1. Intima-media thickness (IMT) (II)

Quantitative carotid ultrasound was done by standardized protocol adapted to the Finnish population (Mercuri 1994, Huang et al. 1999). A high-resolution B-mode ultrasound with a 10 MHz transducer (Biosound Phase 2, Biodynamics Inc USA) was used to examine the left and right carotid arteries. The examinations were recorded on S-VHS videotapes which were read off-line at the ultrasound reading center, Wake Forres University, North Carolina, USA. One certified sonographer and one reader performed all recordings and measurements.

The arteries were identified by Dobbler analysis and imaged from both sides. The protocol involved scanning of the distal 10-mm of the common carotid artery, the bifurcation and the proximal 10-mm of internal carotid artery. The distance between media-adventitia interface and the lumen-intima interface represented the IMT. The maximum IMT of the near and far wall was measured at 12 well-defined arterial segments. The single largest IMT was determined by selecting the largest IMT among the individual maximum IMTs in the 12 standard arterial walls,

i.e., the near and far walls of the common carotid artery, bifurcation and the internal carotid artery at both sides. The mean maximum IMT (MMax IMT, overall mean) was calculated as the mean of 12 maximum IMTs identified at 12 standard sites (Mercuri 1994). Carotid artery atherosclerotic disease (CAAD) was defined as an IMT > 1.7 mm in at least one site.

6.2. Atherosclerosis severity score (ASC) (IV, V)

Ultrasonography at baseline and follow-up were performed with Sonolayer V SSA 100 equipment (Toshiba Corp., Tokyo, Japan). In brief, transverse and longitudinal scans of the extracranial carotid arteries were carried out bilaterally at four different segments of the artery. Only fibrous and calcified lesions were taken into consideration and were defined as plaques when distinct areas of mineralization and/or focal protrusion into the lumen were identified. A far-wall IMT equal to or more than 1.3 mm at any carotid artery segment was defined as an atherosclerotic plaque (Furberg et al. 1989) and the total number of atherosclerotic plaques (NAP) was calculated. All carotid artery examinations were made with a 5.0- MHz convex transducer probe.

Longitudinal ultrasonographs of the abdominal aorta were obtained at 1-cm intervals and transverse scans at 2-cm intervals in the area of three aortic segments. Significant aortic plaques were defined as a far-wall IMT equal to or more than 3.0 mm (Furberg et al. 1989). Longitudinal ultrasonographs of the iliac arteries were performed at two different levels, the common iliac arteries and the external iliac arteries (Study V). All aortic examinations were performed with a 3.75-MHz convex transducer probe.

The replicability of our ultrasonographic protocol for aortic and carotid examination in Study IV and aortic, carotid and iliac examination in Study V, was examined 1 month after the first assessment. Twenty randomly selected subjects were invited to attend a repeat examination. The repeatability of NAP between the first and second examination was 90% for the carotid and iliac artery sites and 100% for the aortic segments. All ultrasonographies were performed in a blinded manner by one experienced ultrasonographer and radiologist.

ASC was constructed by dividing the atherosclerosis into three severity classes: 1 = slight (1.3-2 mm), 2 = moderate (2-3 mm) and 3 = severe (more than 3 mm). The ASC was then calculated as the sum of the severity classes in aorta and carotid (IV) and aorta, carotid and iliac arteries (V). The total NAP was calculated, at baseline only, because 5-year data were not available according to the NAP. Scoring was conducted by one person in a blinded manner without knowledge of HRT or MPO genotype status.

7. Measuring the area of atherosclerotic lesions by morphometry (III)

At autopsy, the thoracic and abdominal aortas were collected for analysis. To measure the area of different types of atherosclerotic lesions, the vessels were dissected free, opened and attached to a cardboard and fixed in buffered formalin. The arteries were radiographed to detect calcified areas and then stained with Sudan IV. The degree of atherosclerotic lesions was evaluated according to standard protocols of the IAP (Guzman et al. 1968) and by the WHO Study Group in Europe (Uemura et al. 1964). The areas of fatty streaks, fibrotic lesions, complicated lesions and calcified plaques were measured with a computer-assisted planimetric technique and by radiography in the case of calcification. The areas of the different types of lesions were expressed in percentage (%).

8. DNA extraction and MPO (rs2333227) genotyping

In Study I, the DNA was isolated from whole blood by Q1Aamp DNA Blood Kit (Qiagen Inc., USA). In Study III, DNA was isolated from pieces of cardiac muscle by a standard phenol-chloroform method. In Studies II, IV and V, DNA was isolated from white blood cells using commercial kit (Qiagen Inc., USA).

MPO -463G/A promoter (rs2333227) genotypes were determined by PCR using restriction enzyme AciI. A region of the MPO gene in the promoter region was amplified using primers designed from those reported by London and colleagues (London et al. 1997). PCR was performed in a 50-µl reaction volume containing MPO forward primer (5´- CGG TAT AGG CAC ACA ATG GTG AG -3´) and MPO reverse primer (5´- GCA ATG GTT CAA GCG ATT CTT C- 3´), each of the four deoxynucleotides, DyNAzyme™ DNA Polymerase and 10 x buffer (Roche Molecular Systems, Inc., Branchburg, New Jersey, USA). The cycling was carried out in a thermal cycler (PTC-225, DNA Engine™ Tetrad MJ Research Inc., Watertown, Massachusetts, USA) at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min with a final cycle at 72°C for 7 min followed by cooling to 8°C. Digestion of the PCR product was carried out in a 25-µl reaction volume with AciI restriction endonuclease and 10 x NEB3 buffer (New England Biolabs, Inc., Beverly, USA). After digestion fragments were separated using agarose gel (2.0 %) electrophoresis and visualized by using ethidiumbromide staining. Genotyping was controlled by analyzing some random samples as duplicates and by including negative (water) controls. Genotyping was always performed without knowledge of the clinical data.

9. Statistical methods

Discontinuous variables were compared with Pearson's χ^2 test. The t-test for independent samples, analysis of variance (ANOVA) or analysis of covariance (ANCOVA) was used to compare continuous variables (I-V). Statistical analyses of the longitudinal data were carried out using analysis of variance for repeated measures (RANOVA) (II, V). In the case of a significant interaction Least Significant Difference (LSD) post-hoc test or Mann-Whitney U-test were utilized to compare the differences between groups. Non-normally distributed data was analyzed after square root or logarithmical transformation, but the results were expressed in crude form. In Studies I, III and V the version 1.0.15 of the PS program was used to calculate the power (1-ß) of the test procedures. In Studies I and III, linear regression analysis was used in the search for the set of variables that best predict CFR (I) and atherosclerotic lesion area in abdominal and thoracic aorta (III).

All statistical analyses were carried out using the Statistica for Windows version 5.1 software package (Statsoft Inc., USA) (I-V) or SPSS version 9.0 (I, III) for Windows (SPSS Inc., USA). Data in the text are presented as mean ± standard deviation (SD) unless otherwise stated. A p-value of less than 0.05 was considered statistically significant.

RESULTS

1. MPO allele (rs2333227) frequencies (I-V)

The distribution of MPO genotypes and allele frequencies in all studies (I-V) are given in Table 3. The genotype distributions in all studies were in agreement with the Hardy-Weinberg equilibrium.

Table 3. Distribution of MPO gene -463G/A (rs2333227) genotypes and allele frequencies in Studies I-V.

		Genotype, n (%)			Allele frequency	
	N	GG	AG	AA	G	A
Study I	49	34 (69%)	13 (27%)	2 (4%)	0.83	0.17
Study II	196	116 (59%)	71 (36%)	9 (5%)	0.77	0.23
DM	37	23 (62%)	13 (35%)	1 (3%)	0.80	0.20
Controls	159	93 (59%)	58 (36%)	8 (5%)	0.77	0.23
Study III	266	180 (67%)	79 (30%)	7 (3%)	0.83	0.17
<53	125	83 (66%)	37 (30%)	5 (4%)	0.81	0.19
≥53	141	97 (69%)	41 (30%)	2 (1%)	0.84	0.16
Study IV, V	87	59 (68%)	26 (30%)	2 (2%)	0.83	0.17
EV	32	22 (69%)	9 (28%)	1 (3%)	0.83	0.17
EVP	25	16 (64%)	8 (32%)	1 (4%)	0.80	0.20
HRT	57	38 (67%)	17 (30%)	2 (3%)	0.82	0.18
Controls	30	21 (70%)	9 (30%)	0 (0%)	0.85	0.15
All	598	389 (65%)	189 (32%)	20 (3%)	0.81	0.19

Abbreviations: DM; diabetes mellitus, EV; estradiol valerate, EVP; estradiol valerate plus sequential progestin, HRT; hormone replacement therapy.

2. The effect of MPO genotypes on oxidation of lipids (I, IV)

Study I. In mildly hypercholesterolemic but otherwise healthy men, when MPO GG homozygotes were compared with A-allele carriers, no significant difference was found between the genotypes in the autoantibody levels against copper-oxidized LDL (Table 4).

Table 4. Autoantibodies against oxidized LDL according to MPO (rs2333227) genotype groups in Study I. Values are expressed as means ±SD.

	MPO GENOTYPE			ANCOVA p-value
	GG (N=34)	AG/AA (N=15)	All (N=49)	
Ox-LDL-ab - native-LDL-ab	0.06 ± 0.05	0.07±0.08	0.06±0.06	0.435
Ox-LDL-ab to native-LDL-ab ratio	1.94 ±0.92	1.99±1.10	1.95±0.97	0.804

Abbreviations: ANCOVA; analysis of covariance, LDL; low density lipoprotein, MPO; myeloperoxidase, OxLDL-ab; autoantibodies against copper oxidized LDL, SD; standard deviation. In ANCOVA age, body mass index, smoking habits and family history of cardiovascular disease were used as covariates.

Study IV. The study examined the relationship between autoantibodies against copper-oxidized LDL and HRT in postmenopausal women. A significant interaction was found between the MPO genotype and HRT treatment in the ratio of autoantibody titer against copper-oxidized LDL to native LDL (two-way ANOVA $p = 0.021$) and with the subtraction of autoantibody titer against copper oxidized LDL and native LDL ($p = 0.046$) (Table 5). In further interaction analysis among the HRT subgroups and controls the oxLDL-ab titer increased in the order of 2.13 in controls, 2.53 in the EV and 3.21 in the EVP group among subjects carrying the GG genotype (two-way ANOVA for trend $p = 0.006$).

Table 5. Autoantibodies against oxidized LDL according to MPO (rs2333227) genotypes and HRT use in Study IV. Values are means ±SD.

	MPO GENOTYPE								
	HRT		CONTROLS		ALL		Two-way ANOVA		
	GG N = 38	AG or AA N = 19	GG N = 21	AG or AA N = 9	GG N =59	AG or AA N = 28	MPO	HRT	Interaction
Ox LDL-ab – native LDL-ab	0.11±0.10	0.10±0.05	0.09±0.07	0.17±0.16	0.10±0.09	0.12±0.11	0.579	0.210	0.046
Ox LDL-ab to native LDL-ab ratio	2.81±1.24	2.51±1.53	2.13±0.67	3.28±2.02	2.57±1.11	2.76±1.71	0.437	0.800	0.021

Abbreviations: HRT; hormone replacement therapy, LDL; low density lipoprotein, MPO; myeloperoxidase, OxLDL-ab; autoantibodies against copper oxidized LDL, SD; standard deviation. Two-way analysis of variance (ANOVA), between MPO genotypes and HRT users and controls was used.

3. MPO genotype and coronary function (I)

The study examined the relationship between the MPO (rs2333227) genotypes and the coronary blood flow and reactivity. In ANCOVA analysis, the age, body mass index (BMI), smoking habits and family history of CVD were used as covariates. The GG genotype carriers had 18.1% lower CFR ($p = 0.019$) and 11.5% lower values of adenosine stimulated flow ($p = 0.049$) than A-allele carriers (Table 6). In linear regression analysis, after adjustment for age, BMI, family history of CVD, smoking habits and MPO genotype, the MPO genotype group and BMI were significant predictors of CFR ($p = 0.019$ and $p = 0.025$ respectively, for the entire model $p = 0.033$, $R^2 = 0.24$). No significant association was found between the MPO genotypes and the RPP-corrected blood flow at rest.

Table 6. Myocardial blood flow according to MPO (rs2333227) genotype groups in Study I. Values are expressed as means ±SD.

	MPO GENOTYPE			
	GG (N=34)	AG/AA (N=15)	All (N=49)	ANCOVA p-value
Blood flow at rest (ml g^{-1} min^{-1})	0.84±0.22	0.81±0.17	0.83±0.21	0.705
Adenosine stimulated flow (ml g^{-1} min^{-1})	3.22±0.79	3.64±0.90	3.35±0.84	0.049
Coronary flow reserve	3.98±1.01	4.60±1.30	4.17±1.13	0.019

Abbreviations: ANCOVA; analysis of covariance, MPO; myeloperoxidase, SD; standard deviation. In ANCOVA analysis, age, body mass index, smoking habits and family history of cardiovascular disease were used as covariates.

4. MPO genotype and carotid artery IMT (II)

The carotid IMT and MPO (rs2333227) genotypes were related according to the presence of type 2 DM. In two-way ANCOVA, with smoking and total cholesterol as covariates, there was a significant MPO genotype-by-study group (non-diabetic vs diabetics) interaction with internal carotid artery IMT ($p = 0.043$) and a borderline significant interaction with overall mean carotid artery IMT ($p = 0.05$) (Figure 7). In similar statistical analyses for other IMT measurements no significant interactions were found.

In non-diabetic subjects, the A-allele carriers had 7.3% higher overall mean IMT values than GG homozygotes ($p = 0.015$ in ANCOVA analysis) whereas the p-values for other measurements were borderline significant. In diabetic subjects, there was no similar genotype-dependent association. When both subjects with type 2 DM and controls were pooled in the same study group, no significant association was found between the MPO genotype and IMT measurements.

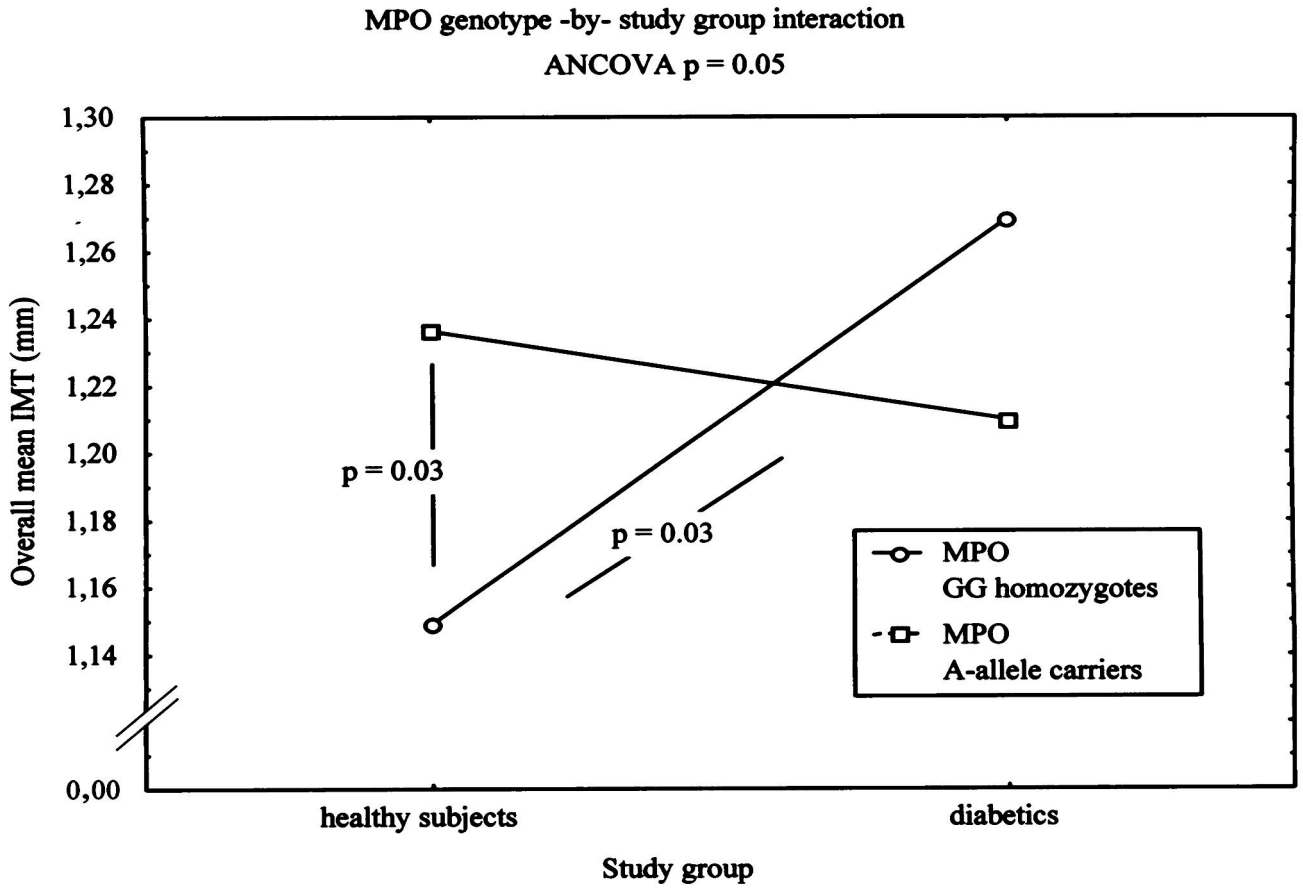

Figure 7. Interaction between MPO (rs2333227) genotype and DM status on the overall mean IMT in Study III. In ANCOVA, smoking and total cholesterol were used as covariates. Least Significant Difference post-hoc test was used to study the difference between diabetics and healthy controls according to MPO alleles.

Abbreviations: ANCOVA; analysis of covariance, DM; diabetes mellitus, IMT; intima-media thickness, MPO; myeloperoxidase.

5. MPO genotype and the areas of aortic atherosclerotic lesions (III)

The aim of this study was to evaluate the age dependent interaction with MPO (rs2333227) genotypes and the severity of atherosclerosis in the abdominal aorta, which is the site where atherosclerotic lesions first develop, and in the thoracic aorta with delayed disease progression. In ANCOVA analysis, age and BMI were used as covariates.

There were significant genotype-by-age interactions for the percent area of both fibrotic ($p = 0.008$) and calcified ($p = 0.015$) lesions at the predilection site of atherosclerosis in the abdominal aorta. In ANCOVA involving all of the available known risk factors, the interaction remained

significant in fibrotic ($p = 0.038$) and in calcified ($p = 0.053$) lesion areas in abdominal aorta. Among the subjects < 53 years old, the A-allele carriers had a 38.6% larger area of fibrotic lesions (LSD $p = 0.017$) (Figure 8A) and 43.8% larger area of calcified lesions (LSD $p = 0.026$) than GG homozygotes. The power of the test measuring the differences between the areas of calcified and fibrotic lesions by MPO was 99% for fibrotic and 91% for calcified lesions. By linear regression analysis including all available atherosclerosis risk factors, BMI, age, HTA and DM, the MPO genotype remained as an independent predictor of fibrotic ($p = 0.04$, for the entire model $p = 0.017$, $R^2 = 0.231$) and calcified ($p = 0.001$, for the entire model $p = 0.001$, $R^2 = 0.324$) lesion areas. The association changed in men ≥ 53 years among whom the A-allele carriers tended (n.s.) to have slightly lower fibrotic lesions.

In the thoracic aorta, there was also a statistically significant ($p = 0.003$, ANCOVA, age and BMI as covariates) interaction between the MPO genotype and age group regarding the fibrotic lesion area in the thoracic aorta. Among men < 53 years the A-allele carriers tended (n.s.) to have larger fibrotic areas which became the opposite among men ≥ 53 years, where the GG homozygotes had on average a 24.5% larger area of fibrotic lesions (LSD $p = 0.012$) than A-allele carriers (Figure 8B). The power of the test to measure the difference between the area of fibrotic lesions according to MPO genotypes was 80%. In linear regression analysis, the MPO genotype did not remain as an independent predictor.

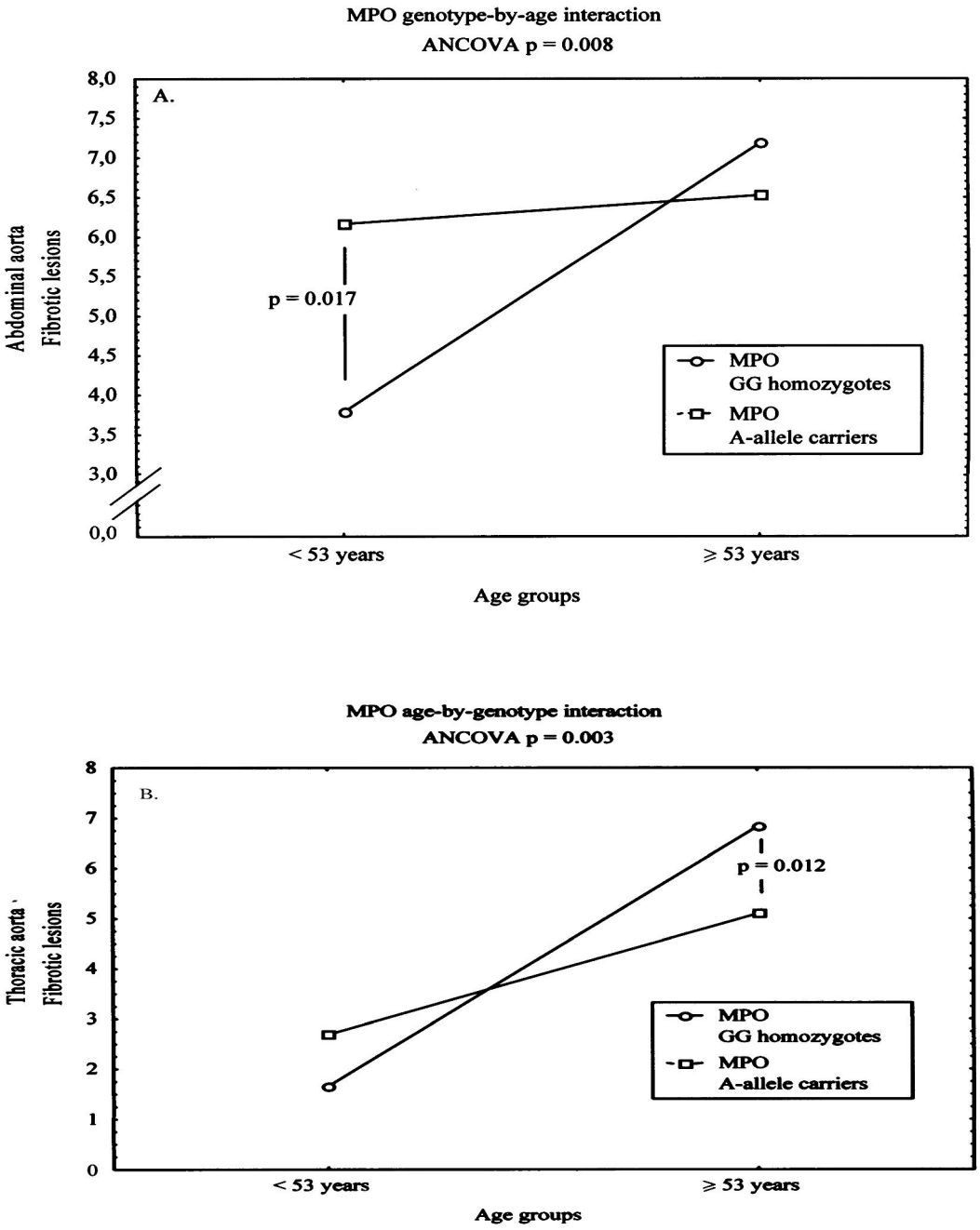

Figure 8. A. MPO genotype-by-age interaction on the area of fibrotic lesions in the abdominal aorta in Study III. Data analysis was based on two-way ANCOVA where age and BMI were used as covariates. The Least Significant Difference post-hoc test was used to study the differences between groups.

B. MPO genotype-by-age interaction on the area of fibrotic lesions in the thoracic aorta in Study III. Data analysis was based on two-way ANCOVA where age and BMI were used as covariates. The Least Significant Difference post-hoc test was used to study the differences between groups.

Abbreviations: ANCOVA; analysis of covariance, BMI; body mass index, MPO; myeloperoxidase.

6. MPO genotype and atherosclerosis development during HRT (IV, V)

Study IV. In this study, the possible association of the MPO genotype and HRT treatment groups in on ASC was examined. In the two-way ANOVA analysis, no significant interaction was found.

Study V. The purpose of the Study IV was to determine whether the MPO genotype modifies the effect of HRT on the development of atherosclerosis. Among GG homozygote subjects, the progression rate of ASC differed significantly between HRT users and controls (treatment group by time point interaction in analysis of variance for repeated measures (RANOVA), p = 0.042) being faster in the control group than in the HRT group (Figure 9).

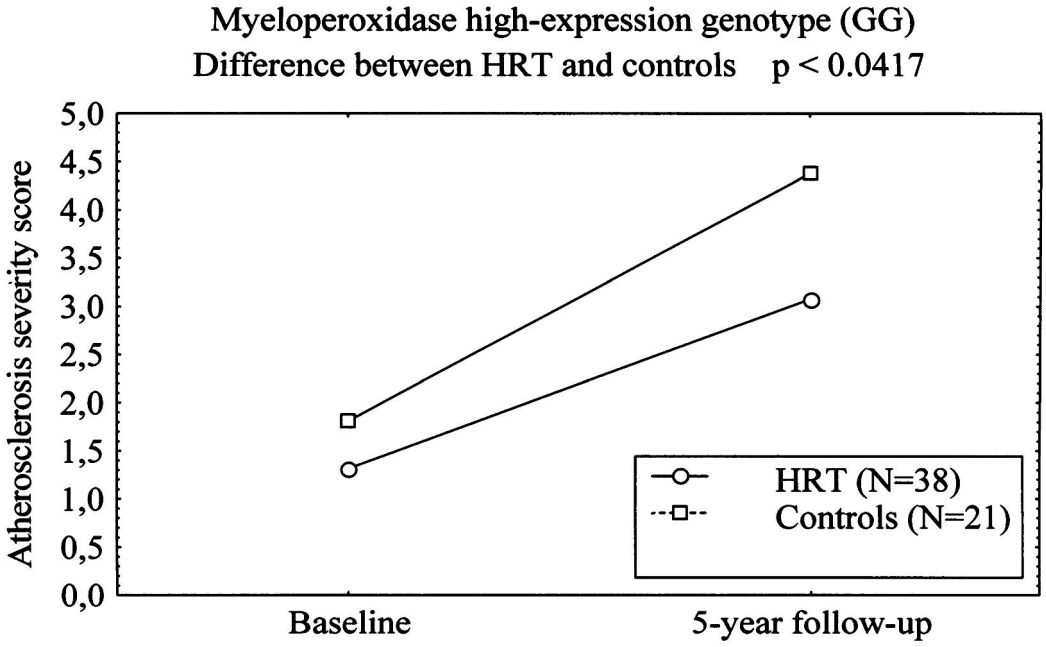

Figure 9. The effect of HRT on the progression of atherosclerosis in Study V, as measured by ASC in postmenopausal women with the GG genotype, compared with the progression in controls with the same MPO genotype and time elapsed from menopause but without HRT. The p-values shown in the figure are from two-way ANOVA for repeated measures.

Abbreviations: ANOVA; analysis of variance, ASC; atherosclerosis score, HRT; hormone replacement therapy, MPO; myeloperoxidase.

At baseline, the GG homozygote carriers on HRT-EV tended to have an average of 32.2% smaller ASC (1.23 vs. 1.80 in controls) and subjects on HRT-EVP had 20.6% smaller ASC (1.44 vs 1.80 in controls) than the controls (for trend p = 0.056, ANCOVA with age and BMI as covariates). After 5-year follow-up, the corresponding differences between the HRT-EV and HRT-

EVP groups and the controls were 31.5% (3.00 vs. 4.38, p = 0.010) and 27.2% (3.19 vs. 4.38, p = 0.040; ANCOVA for trend p = 0.035 with age and BMI as covariates (Figure 10)). The responsiveness to HRT was MPO genotype-specific. Among the A-allele carriers, the progression rate of ASC in users and controls did not differ.

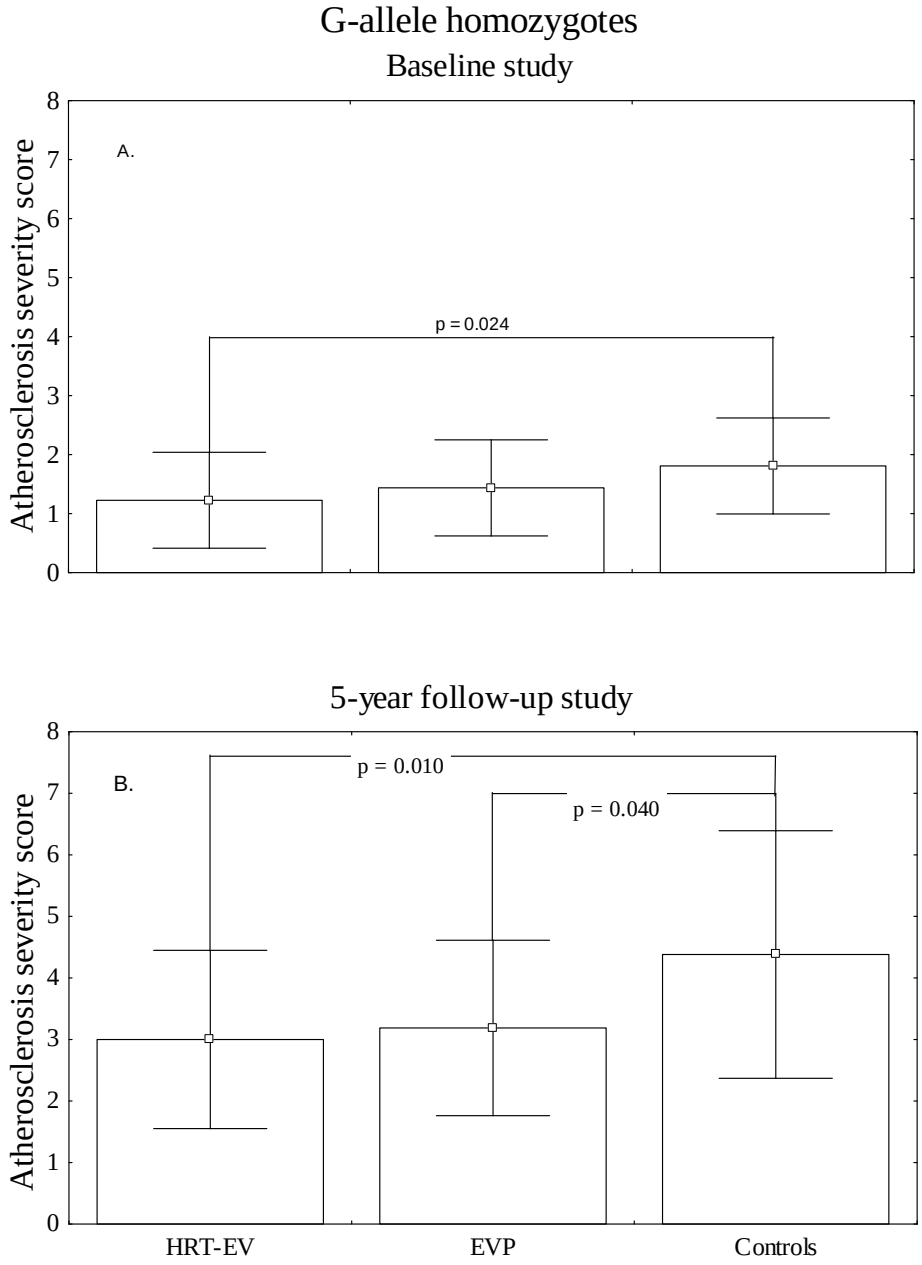

Figure 10. ASC in postmenopausal women with GG genotype by HRT group.

A. results from the baseline study. B. Results from the cross-sectional study after 5-year follow-up. The p-values for the mean (±SD, whiskers) differences between the HRT groups and controls shown in the figure were obtained from ANCOVA with Least Significance Difference post-hoc test. Results were adjusted for age and BMI.

Abbreviations: ANCOVA; analysis of covariance, ASC; atherosclerosis score, BMI; body mass index, HRT; hormone replacement therapy, SD; standard deviation

DISCUSSION

1. Study subjects

This thesis consists of Studies I-V including one autopsy and four clinical series. They were used to examine the relationship between MPO (rs2333227) genotype and different stages of atherosclerosis. The four series comprised 87 females (IV, V) and 511 males (I-III) who were unrelated to each other.

The Finnish population is considered suitable for genetic studies because of its homogeneity. The relatively small population has a history of genetic isolation, the founder effect and genetic drift, where the inherited risk factors are enriched (Peltonen et al. 1999). The prevalence of CHD has been high in Finland compared to other Western countries although the trend has been declining. The most dominant CHD group has been middle-aged men, whereas nowadays the aged, postmenopausal women comprise the largest CHD group (Tuomilehto et al. 1992, Kattainen et al. 2006).

Subjects in the clinical studies. Subjects in Study I were young and healthy male coastguards and firemen. They had only mildly elevated serum total cholesterol levels and normal PET measurement and therefore it was unlikely that they had any significant stenosis in their coronary arteries. Due to their occupation, however, the subjects may have been healthier and in better physical condition than the population on average.

In Study II, the original study group consisted of 300 randomly selected middle-aged men from ten age-cohorts. Randomized sampling avoided major selection bias, however, 26% of the invited men refused to participate (n = 33) or could not be reached (n = 44). The final population with the data of MPO genotype consisted of 199 subjects. CHD was diagnosed according to medical history or electrocardiographically but not angiographically and therefore, some of the men may have had advanced atherosclerosis.

Studies IV and V are based on the same study population of postmenopausal women who were classified into three groups based on their use of HRT and followed for five years. The original study consisted of 120 women who were invited by letter to participate in the study. The MPO (rs2333227) genotype data was available from 87 subjects (72.5%).

Subjects in the autopsy series. The subjects included in Study III were the victims of sudden death or trauma and therefore had been subjected to a medicolegal autopsy. The conventional cross-sectional or retrospective studies may suffer from the survival bias, which is

avoided in autopsy studies. However, these subjects may present more severe atherosclerosis than in randomly selected studies. The CHD risk factors may also be differently distributed, such as the high alcohol consumption in HSDS subjects. Also, the data available on the traditional CHD risk factors is limited; the series lack any information available from blood samples, such as lipid risk factors. The risk factor information available from the interview has been considered reliable enough to act as confounding factors in statistical analysis. The MPO genotype frequences followed those previously published for Caucasian population, and therefore, the study may be considered a representative sample of Finnish middle-aged men, though it has its limitations. The subjects are males, and therefore the results cannot be generalized to women.

2. Methodological considerations

Candidate gene approach and association studies. Two major catergories of studies are used to investigate the genes that underlie common diseases and traits. These include candidate-gene studies, which use association or resequencing approaches and genome-wide studies, which include both linkage mapping and genome-wide association studies (Hirschhorn and Daly 2005). Association studies are used to identify relevant candidate genes and genotypes involved in polygenic disorders using appropriate controls (Daly and Day 2001). SNPs are single-base differences in the DNA sequence that can be observed between individuals in the population wherein the least frequent allele has prevalence at the minimum of 1%. One way to perform association studies is to select SNPs that are more likely to have functionality, as is the case in promoter variants (Brookes 1999, Daly and Day 2001). In this thesis, the MPO was selected as a candidate gene because of the reported biological significance in atherosclerosis.

PET methodology. PET imaging uses radionuclides that decay with positron emission. A positron has the same mass as an electron but a positive charge. The positron travels a short distance after which it interacts with an electron and the two undergo annihilation. PET imaging consists of the detection of these photons. Imaging by PET with electronic coincidence localization using a ring detector leads to high acquisition efficiency. $[^{15}O]H_2O$ is a freely diffusible tracer with a short physical half-life and therefore the use of $[^{15}O]H_2O$ water is restricted to sites with a cyclotron. However, the ability of water to diffuse freely across plasma membranes makes this tracer a favorite for quantitation of myocardial blood flow (MBF) (Machac 2005). Cardiac PET is the only method allowing the assessment of early atherosclerotic changes in

asymptomatic subjects and has a diagnostic accuracy of ≥ 95% even in asymptomatic subjects (Dayanikli et al. 1994, Pitkänen et al. 1997, Gould 2007).

The earliest finding associated with CAD is abnormal CFR, which is an integrating measurement of endothelial function and SMC relaxation (Dayanikli et al. 1994). The mechanisms potentially causing perfusion abnormalities in asymptomatic persons are reduced CFR due to preclinical, mild, diffuse or segmental coronary atherosclerosis without ischemia or endothelial dysfunction preceding luminal narrowing and cardiac events (Gould et al. 2000, Schachinger et al. 2000, Sdringola et al. 2001). It has been shown that CFR is impaired in young adults with familiar hypercholesterolemia, type 1 DM, familial combined hyperlipidemia and HTA (Pitkänen et al. 1996, Laine et al. 1998, Pitkänen et al. 1998, Pitkänen et al. 1999).

Ultrasound methodology. B-mode ultrasound imaging is a widely used technique to detect the atheroma plaques from relatively large arteries, such as the carotid and femoral arteries (Simon et al. 1995). It has been shown that in middle-aged healthy Finnish men the presence of a carotid plaque multiplies the short-term incidence of acute MI (Salonen and Salonen 1991). The IMT correlates significantly with the main risk factors for atherosclerosis (Davis et al. 2001, Kieltyka et al. 2003) and CAD (Wofford et al. 1991, Burke et al. 1995). B-mode ultrasound imaging overcomes some of the limitations of arteriography, the method is non-invasive and can be used to examine both asymptomatic high-risk subjects and patients repeatedly with a high degree of compliance. To obtain acceptable measurement reproducibility by B-mode ultrasound, it is essential to control for the effects of instrument and operator variability (Mercuri 1994). B-mode ultrasound may, however, underestimate the large or complicated plaques and lack precision in detecting total occlusions. It cannot distinguish fatty streaks from localized intima-media thickening but dense fibrosis and calcified areas are easier to detect because they are more echogenic (Salonen and Salonen 1993). In Studies IV and V, only fibrous and calcified lesions were taken into consideration and defined as plaques.

All ultrasonographies were performed by one experienced sonographer and radiologist, who also scored the severity of atherosclerosis in a blinded manner. The reproducibility of ultrasonographic protocol for significant aortic, carotid and plaques was also examined in the study. In Study II, the recordings were also performed by the same certified sonographer and the images were interpreted and measured at the reading center by one trained reader. The overall mean maximum IMT (MMax IMT) was the mean of 12 maximum IMTs identified at 12 standard sites (Mercuri 1994). These methods ensured optimal validity and reproducibility.

Classification of atherosclerotic lesions at autopsy. Most methods evaluating atherosclerotic lesions in living subjects, such as angiography, only provide information about the

extent and characteristics of arterial lesions that significantly narrow the lumen. Therefore, autopsy studies are needed when the early atherosclerotic lesions are studied. In Study III, different stages of atherosclerosis in thoracic and abdominal aorta were evaluated, from fatty streaks to more advanced lesions. Some limitations should be taken into consideration. The standardized histological classification method was not available at the time of data collection (Stary et al. 1992, Stary et al. 1994, Stary et al. 1995, Stary 2000). Instead the arterial samples were stained red with Sudan IV according to the protocol of the IAP (Guzman et al. 1968). The protocol may fail to show the fatty streaks developing on the site of adaptive intimal thickening and therefore not be visible by staining (Stary et al. 1994).

3. The effect of MPO on oxidation of lipids (I, IV)

In Study I on healthy, relatively young men, the MPO genotype was not associated with oxLDL-abs.

In Study IV, however, the MPO polymorphism (rs2333227) was associated with the oxLDL-abs according to HRT use in postmenopausal women. It has been established in vitro, that estradiols inhibit LDL oxidation but the study outcomes on HRT have been contradictory (Sack et al. 1994, McManus et al. 1996, McManus et al. 1997, Arteaga et al. 1998, Wakatsuki et al. 1998). MPO activity differs according to gender, being higher in women than in men (Kabutomori et al. 1999). These present results seem to support this concept. Estrogen has been reported to enhance MPO activity and to increase the amount of MPO in the plasma (Jansson 1991, Santanam et al. 1998). At the time of menopause, MPO activity is reduced, but may be restored by HRT (Bekesi et al. 1999). The enhanced MPO acitivity may not predict the higher oxidative stress; in neutrophils the MPO is related to simultaneous O_2^- inhibition leading to diminished total production of free radicals in granulocytes, so the net oxidative burden might be even dimished (Bekesi et al. 1999, Bekesi et al. 2001b).

In Study IV, the solid EV administration had no effect on oxLDL ab-titers, whereas among the EVP treated subjects the GG homozygotes showed significantly higher oxLDL-ab levels than A-allele carriers. However, the results were only baseline results, so no follow-up measurements could be made. Is should also be taken into consideration that the autoantibody titer against copper-oxidized LDL is only one of the markers of the complex process of LDL oxidation among several other methodologies developed for the oxidation measurements (Esterbauer et al. 1992) and specific antibodies against HOCl-modified LDL were not used in the present study.

4. MPO genotype and coronary function (I)

In Study I, the A-allele carriers of rs2333227 had higher adenosine stimulated flow and CFR than GG homozygotes whereas the basal blood flow at rest did not differ between the genotypes. About half of the endothelial response to adenosine is endothelium dependent. The exposure of guinea pig hearts to HOCl results in the complete loss of vasodilatation in response to known vasodilatators, such as adenosine (Leipert et al. 1992). MPO appears to be an important modulator of vasomotor function in inflammatory vascular disease during myocardial ischemia (Baldus et al. 2004). MPO can directly modulate the vascular signaling and vasodilatory functions of NO by regulating its bioavailability (Eiserich et al. 2002). Neutrophil adhesion to the vessel wall and the tissue concentration of MPO and HOCl are associated with endothelial dysfunction and reduced NO activity in inflammation and ischemia-reperfusion injury (Leipert et al. 1992, Friese et al. 1996, Granger 1999). In addition, HOCl-modified LDL is known to inhibit the synthesis of NO, which may also lead to endothelial dysfunction (Nuszkowski et al. 2001).

5. MPO genotype and carotid intima-media thickness (II)

The extent of intimal staining for apoB, MPO and HOCl modified proteins correlates with an increase in intima-media ratio of iliac arteries (Hazell et al. 2001). In Study III, the MPO genotype and type 2 DM status were associated in carotid IMT. The A-allele carriers had higher overall mean IMT values among middle-aged men with normal glucose metabolism. In subjects with type 2 DM, no similar effect was found. In Study III, the MPO genotype-dependent effect was absent in diabetic subjects. In type 2 DM patients, the other risk factors for atherosclerosis may have greater significance than mere inflammatory response. It is known that in patients with type 2 DM, MPO activity is significantly reduced, which could to some extent explain our results (Sato et al. 1992, Uchimura et al. 1999). One possible mediator for this could be PPAR-mediated regulation (Kumar et al. 2004).

6. MPO genotype and atherosclerotic lesions in abdominal and thoracic aorta (III)

There was a significant MPO genotype by age interaction on atherosclerotic lesions both in the abdominal aorta, which is known as the site where atherosclerotic lesions first develop, and in the thoracic aorta. In abdominal aorta, the GG genotype carriers < 53 years old had smaller area of

fibrotic and calcified lesions than A-allele carriers. The association changed in men ≥ 53 years among whom the A-allele carriers tended to have slightly smaller area of fibrotic lesions. In thoracic aorta, the genotype and age-dependent trend in fibrotic lesion area seemed to be similar but was statistically significant only among men ≥ 53 years, where the GG genotype carriers had larger area of fibrotic lesions than A-allele carriers.

MPO enzyme is thought to be an active contributor to atherogenesis in all stages of disease progression. However, the MPO-immunoreactive macrophages within atherosclerotic plaques are particularly evident in advanced atherosclerotic lesions (Daugherty et al. 1994, Sugiyama et al. 2001). In aged individuals monocytes exhibit imbalanced production of cytokines and activation (Sadeghi et al. 1999). In neutrophils, the MPO activity differs by gender and age (Kabutomori et al. 1999) and both in older men and women the plasma concentration of MPO is lowered (Bekesi et al. 2001b). The -463G/A polymorphism may be associated with age-dependent differences in MPO activity. A-allele carrying men over 55 years may have higher MPO activity than A-allele carriers under that age (Rutgers et al. 2003). The age range among men in Study III was 33 to 69 years, so the age-dependent differences in MPO activity may to some extent explain our results.

7. MPO genotype and atherosclerosis progression in postmenopausal women receiving HRT (V)

In Study V, the GG homozygotes reflected beneficial effects on atherosclerosis progression during HRT. All study subjects were clinically healthy, non-diabetic postmenopausal women, who were nonsmokers and had normal blood pressure. Although most of the CAD risk factors were equally distributed, the effect of social class cannot be excluded in this study. In general, women who take HRT are more likely to be better educated, have higher incomes and better access to health care services and they tend to be healthier than women without HRT (Matthews et al. 1996).

The effect of HRT on CAD outcomes has been studied extensively in recent years. The effect of HRT on CAD may vary among individuals, possibly due to inherited factors. The effect of genetically determined response is supported by the studies of our own group (Lehtimäki et al. 2002, Koivu et al. 2003). Estrogen has been found to alter MPO activity by influencing MPO gene expression, monocyte number and possibly the release of MPO. In isolated neutrophils, estrogen has been reported to enhance MPO activity (Jansson 1991). In accordance with this, the mean peroxidase activity index (MPXI) is higher in females than in males and fluctuates with serum estrogen levels (Kabutomori et al. 1999). In postmenopausal women, the intracellural MPO

acitivity in neutrophils is lowered whereas during HRT the intracellular MPO activity and MPO release are restored (Bekesi et al. 2001b).

Some findings suggest that the two alleles of MPO -463G/A polymorphism are regulated differently depending on gender (Reynolds et al. 2000). The polymorphism is situated within ER binding site where ERα binds both the G and A promoters, but more effectively to A-allele. Because ERα binds with creater avidity to A-allele than G-allele, the A-allele appears to be more readily blocked to other regulation, for example PPRAγ, by estrogen administration (Kumar et al. 2004). The -463G/A polymorphism is also associated with age-dependent differences in MPO activity particularly in A-allele carriers. Among A-allele carriers, the MPO activity differs by age, being higher in women under middle-age than women over 55 years of age, possibly due to diminishing estrogen levels (Rutgers et al. 2003). However, in Study V the A-allele carriers did not differ in the progression of atherosclerosis in terms of the HRT use.

In the present study, the HRT used was EV and levonorgestrel. Most large trials use a restricted range of preparations, mostly conjugated equine estrogen and MPA (medroxyprogesteroni acetate), which are rarely used by Finnish phycicians. Levonorgestrel and MPA are reported to potentially inhibit the cardioprotective effects of estrogen (Zhu et al. 1999, Zhu et al. 2000). The compounds and the administration route used seem to differ in their impact on CAD risk factors. HRT has modulative effects on several inflammatory markers and some of the modulative effects are also dependent on the administration route used. Interestingly, if the first stage metabolism in the liver is prohibited by using transdermal administration, the MPO levels are reduced (Hermenegildo et al. 2002) and the resistance of LDL to oxidative modification is likewise enhanced (Wakatsuki et al. 1998).

8. Study limitations and future prospects

One of the main study limitations was that the MPO activity was not measured in any of the studies and the impact of MPO polymorphism (rs2333227) on MPO expression and activity in vivo thus remains uncertain. It should also be noted that the MPO polymorphism (rs2333227) may also be linked to some other SNP, which may thus be the major factor behind the results of this thesis.

Studying functionally significant polymorphisms rather than random polymorphisms offers advantages in terms of detecting disease-associated genes. The effects of MPO -463G/A (rs2333227) have been reported in different disease processes but in the beginning of this study, there were only a limited number of studies given the association in atherogenesis. The results

remain somewhat controversial. One explanation might be that the regulation of the MPO gene expression and the protein biosynthesis is complex and mostly unknown. Some studies on the environmental factors influencing MPO protein expression and activity have been published, including gender and age. However, there may be other environmental factors regulating the gene expression possibly confusing the results.

The association of MPO in inflammatory diseases suchs as atherosclerosis has been demonstrated in multiple studies, but whether there is any turning point at which the presence and activity of MPO becomes harmful is not yet clear. Most of the studies on the physiological actions of MPO have been carried out in the neutrophils. In atherosclerosis, the presence of neutrophils seems to be restricted at the time of sudden cardiac events and mostly, the monocyte-macrophages are the source of MPO in atherosclerotic plaque (Rudolph et al. 2007b). In clinical studies, MPO levels have been shown to predict the presence of CAD and the plasma and serum levels of MPO have been shown to predict the risk of major cardiac events. Although at the present moment the routine measurement of MPO blood levels is not used in clinical diagnostics, one could predict that possible future therapeutics for MPO will be targeted at the prevention ACS in high-risk patients.

Future therapeutic implications may include strategies to prevent the proinflammatory actions of MPO in the vessel wall. MPO inhibition could be targeted at different levels such as MPO active site blockade, deflection of MPO from the chlorination cycle or use HOCl scavengers (Malle et al. 2007). To date, no specific MPO inhibitors are available and considering the general heme-centered structure of the protein, they will be difficult to develop (Lau and Baldus 2006). With enhanced statin treatment it could be possible to inhibit MPO expression in an attempt to reduce MPO protein and enzyme activity in risk patients (Kumar and Reynolds 2005). Also, one possible way could be to use the ability of heparin to inhibit the MPO binding to the endothelial wall (Baldus et al. 2001, Baldus et al. 2006). According to current knowledge, the possible hormonal regulation of MPO gene may cause gender-dependent differences in MPO blood levels. In the future, one therapeutic perspective could be the use of HRT in those patients who are most likely to benefit from the treatment. This also includes the male patients who could benefit from the anti-inflammatory effects of estrogen usage in unstable cardiovascular conditions (Wei et al. 2001).

Single disease related SNP alleles may not be sufficient to cause illness, whereas the combined effect of a collection of SNP alleles in several key genes and environmental factors determines the whether a subject suffers from the disease (Brookes 1999). It is essential that the overall effects of a particular combination of linked polymorphisms, haplotypes, is considered

rather than only interpreting the functional effects of a single polymorphic site (Daly and Day 2001, Humphries et al. 2007). In the present study, the MPO -463G/A (rs2333227) genotype served as a genetic marker for atherosclerosis. It would be interesting to include this genotype in haplotype analysis among other known MPO promoter area SNPs.

SUMMARY AND CONCLUSIONS

Earlier studies suggest that the MPO promoter -463G/A polymorphism (rs2333227) is associated with an increased risk of atherosclerosis, but conflicting results have also been published. In this thesis, four clinical studies and one autopsy study were conducted to elucidate the association between the -463G/A genotypes of MPO and coronary reactivity, carotid IMT and development of early and advanced atherosclerotic plaques in thoracic and abdominal aorta. As it was previously known that the -463G/A polymorphism has an ERα binding site the effect of MPO genotype on atherosclerosis progression during long-term HRT was assessed. The main findings and conclusions are as follows.

1. In Study I on young, healthy men there were no significant differences in the autoantibodies against copper-oxidized LDL between the MPO genotypes. However, in Study IV on postmenopausal women there was an interaction between MPO genotype status and the use of HRT in relation to the titers of oxLDL-abs. In women with the GG genotype the oxLDL-ab titer increased along with the HRT use; in the EVP group the GG genotype carriers had the most marked difference when compared to A-allele carriers.

2. MPO G-allele homozygotes had lower CFR and adenosine stimulated flow than A-allele carriers, suggesting that the -463G/A polymorphism may modify coronary reactivity and reflect differences in the early pathogenesis of coronary dysfunction in this study group of healthy young men (Study I).

3. In Study II, the MPO genotype and type 2 DM status were associated in carotid IMT. Among men with normal glucose metabolism, the MPO A-allele carriers aged 50 to 59 years had higher overall mean IMT values than GG homozygotes. In subjects with type 2 DM, no association was observed.

4. In the autopsy study (Study III), the MPO A-allele carrier men < 53 years had larger calcified and fibrotic lesions in the abdominal aorta than men with GG genotype. In the thoracic aorta, there was also a statistically significant interaction between the MPO genotype and age group regarding the fibrotic lesion area. Among men < 53 years the A-allele carriers tended to have larger fibrotic lesion areas (n.s.). The association turned at the

opposite both in thoracic and abdominal (n.s.) aorta among men ≥ 53 years, where the GG homozygotes had on average larger fibrotic lesions than A-allele carriers.

5. In observational study (Study V) of postmenopausal women during long-term HRT, the progression of ASC in subjects with the MPO GG genotype was significantly faster in the control group than in the HRT group, whereas there were no significant differences in ASC progression between the control and HRT groups in A-allele carriers. This result suggests that the beneficial effect of HRT on atherosclerosis progression was restricted to women with the GG genotype.

On the basis of these findings we can conclude that the MPO -463G/A (rs2333227) may serve as an important genetic marker for atherosclerosis in different stages of the disease. Significant associations were seen in all stages of atherosclerosis and in the response to atherosclerosis progression to long-term HRT. The effects of MPO genotype varied according to the age, gender and the state of the disease. Among young men the A-allele was associated with higher CFR values whereas middle-aged A-allele carrier men had higher IMT and the area of atherosclerotic lesions than GG homozygotes. In women, the effect of HRT on atherosclerosis progression and increased oxLDL-abs was restricted to women with the GG genotype. The findings concur with and support the current knowledge of the importance of MPO in the development of atherosclerosis.

ACKNOWLEDGEMENTS

The studies of this thesis were carried out at the Department of Clinical Chemistry, Centre for Laboratory Medicine, Tampere University Hospital, Finland during the period 2000-2009.

First I would like to express my gratitude to my supervisors, Professor Terho Lehtimäki MD, PhD and Professor Pekka J Karhunen MD, PhD, for the opportunity to carry out this doctoral thesis. I have been priviledged to have all the guidance and assistance I have ever needed, even at those moments when I thought I might fail to finish this project. I will always remember all the encouraging and supportive comments I have had.

I am grateful to Professor Mikko Hurme and Professor Seppo Nikkari, the members of my thesis committee, for their feedback and comments.

I express my thanks to Professor Marja-Leena Kortelainen and Docent Ken Lindstedt for their careful review and constructive criticism of the manuscript.

I wish to express my gratitude to all my co-authors – Gene Bond, Prasun Dastidar, Heini Huhtala, Erkki Ilveskoski, Olli Jaakkola, Tuula Janatuinen, Hannu Jokela, Olli A. Kajander, Juhani Knuuti, Timo Koivula, Tarja Kunnas, Reijo Laaksonen, Antti Loimaala, Michele Mercuri, Jussi Mikkelsson, Arja Nenonen, Pirjo Nuutila, Pekka Oja, Antti Penttilä, Markus Perola, Reijo Punnonen, Olli T. Raitakari, Marika Saarela, Jukka T. Salonen, Risto Vesalainen and Ilkka Vuori– for their contribution to this study.

My sincere thanks are due to Docent Erkki Seppälä, Head of the Department of Clinical Chemistry, Centre for Laboratory Medicine and Docent Timo Koivula, the former head of the same department for providing the facilities for carrying out this research.

I am very grateful to Professor Esa Leinonen and Deputy Chief Psychiatrist Riitta Alaja of the Department of Psychiatry for their engouragement and support for finishing this study.

I express my thanks to Virginia Mattila for her careful revision of the language of this thesis.

I have greatly enjoyed my time as a postgraduate student at the Laboratory of Atherosclerosis Genetics thanks to the help and support I have received from the research group. Special thanks go to the current and former group members Riikka Rontu, Nina Airla, YueMei Fan, Meng Fan, Jussi Hernesniemi, Päivi Hämelahti, Salla Höyssä, Mari Levula, Mari Luomala, Nina Mononen, Nina Peltonen, Perttu Pöllänen, Marika Saarela as well as Marita Koli, Irma Valtonen and Aulikki Nuut. They and many other persons have worked in the laboratory and created a unique and inspiring atmosphere which I have been priviledged to enjoy.

Finally, I would like to thank my parents, Matti and Anja Mäkelä, for their help and support during these years.

This study was supported by the Medical Research Fund of Tampere University Hospital, the Research Foundation of Orion Corporation, the Scientific Fund of the City of Tampere, the Finnish Medical Society Duodecim, a scientific grant from the Faculty of Medicine, University of Tampere in addition to travel grants from the Tampere Graduate School in Biomedicine and Biotechnology.

Tampere, February 2009

Riikka Mäkelä

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Original Paper

Journal of Biomedical Science

J Biomed Sci 2004;11:59–64
DOI: 10.1159/000075289

Received: May 2, 2003
Accepted: August 11, 2003

Myeloperoxidase Gene Variation and Coronary Flow Reserve in Young Healthy Men

Riikka Mäkelä[a] Reijo Laaksonen[b,d] Tuula Janatuinen[e] Risto Vesalainen[f] Pirjo Nuutila[e] Olli Jaakkola[a,c] Juhani Knuuti[e] Terho Lehtimäki[a]

[a]Department of Clinical Chemistry, Laboratory of Atherosclerosis Genetics, Center for Laboratory Medicine, Tampere University Hospital and University of Tampere Medical School, [b]Department of Internal Medicine, Tampere University Hospital, and [c]Institute of Medical Technology, Tampere University, Tampere, [d]Department of Clinical Pharmacology, University of Helsinki, Helsinki, and [e]Turku PET Center, University of Turku, and [f]Department of Medicine, Turku University Central Hospital, Turku, Finland

Key Words

Coronary artery blood flow · Endothelial function · Genetics · Myeloperoxidase · Positron emission tomography

Abstract

Chronic inflammation may lead to endothelial dysfunction, which manifests as an impaired coronary reactivity. Impairment in coronary flow reserve (CFR), preceding the clinical symptoms of coronary artery disease, can be measured noninvasively by positron emission tomography. Myeloperoxidase (MPO) is an oxidative enzyme present in phagocytes and atherosclerotic lesions. The MPO gene has a promoter polymorphism (–463G/A) which affects gene transcription. Whether these variants associate with coronary artery function is not known. Myocardial blood flow at rest and during adenosine-induced hyperemia was assessed in 49 healthy young men with normal or slightly elevated serum total cholesterol. These subjects were divided into high (G/G) and low (A/G, A/A) MPO expression groups and effect of MPO genotype on myocardial blood flow was evaluated. We found a significant difference between MPO genotypes in CFR after adjusting for age, body mass index, smoking and family history of cardiovascular disease ($p = 0.019$). Men with G/G genotype had 18.1% lower CFR than subjects with low-expression genotypes (A/G and A/A). This was due to an 11.5% lower adenosine-stimulated flow of the G/G genotype carriers ($p = 0.049$). These findings provide evidence that MPO polymorphism is associated with coronary artery reactivity. However, the number of individuals investigated was low and our observation should be confirmed by a larger number of subjects.

Atherosclerosis is a long-lasting inflammatory process where the activation of inflammatory cells, such as phagocytes, initiates a response to injury processes [45]. Myeloperoxidase (MPO) is an oxidative enzyme found in phagocytes and an essential part of the antimicrobial defense system [22, 47]. MPO is able to form proatherosclerotic particles by its oxidative intermediates [15, 40]. Elevated whole blood and leukocyte MPO levels are associated with the presence of coronary artery disease [50]. Accordingly, persons with MPO deficiency have defects

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1021–7770/04/0111–0059$21.00/0
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Terho Lehtimäki, MD, PhD, Laboratory of Atherosclerosis Genetics
Department of Clinical Chemistry, Center for Laboratory Medicine
Tampere University Hospital, FinnMedi 2, 3rd floor, PO Box 2000
FI–33521 Tampere (Finland)
Tel. +358 3 311 74066, Fax +358 3 311 74168, E-Mail terho.lehtimaki@uta.fi

in leukocyte-mediated initiation of lipid peroxidation in plasma [51] and reduced risk for cardiovascular damage [24]. MPO enzyme is expressed in human atheromas [7] and products of MPO-mediated reactions are present throughout the atherosclerotic disease process [14, 16, 26]. In patients with unstable angina, a widespread activation of neutrophils and MPO is present throughout the coronary vascular bed. This overall inflammatory activation seems to be independent of the location of stenotic sites [3].

MPO is able to produce a wide range of oxidative species, such as hypochlorous acid, tyrosyl radicals, chloramines and reactive nitrogen species, and has therefore a large group of potential biological targets [47]. Presence of hypochlorous acid-modified low-density lipoprotein (LDL) stimulates the production of reactive oxygen metabolites, enzyme secretion and endothelial adhesion molecules in the vessel wall [23]. MPO is able to promote LDL oxidation in vivo [14] whereas oxidized LDL is known to impair endothelial function and coronary reactivity [41]. Furthermore, hypochlorous acid blocks the coronary flow response to known vasodilators such as acetylcholine, bradykinin and adenosine in guinea pig heart [28]. It is interesting, however, that in MPO knockout mice the progress of atherosclerosis is increased [2].

The promoter region of the MPO gene has a single G-to-A base substitution at position –463 inside a SP1 transcription factor consensus sequence [35]. This polymorphism exhibits marked differences in transcriptional activity [35, 42] and leads to high- (G/G) and low-expression (A/A, A/G) genotypes. In a recent study, A/A and A/G genotype had a protective role for coronary artery disease and patients with angiographically proven coronary artery disease had a significantly lower incidence of allele A than the control group [31].

Changes in the peripheral vascular endothelium belong to the earliest signs of developing atherosclerosis and coronary artery disease [8, 39]. Endothelial dysfunction and changes in smooth muscle cell relaxation are manifested as impairment in coronary flow reserve (CFR) [37, 38]. CFR can be measured noninvasively by positron emission tomography, which is today the only method allowing the assessment of early atherosclerotic changes in the coronaries of asymptomatic, healthy subjects [8, 39]. There are no previous studies evaluating the association between MPO genotype and CFR, an indicator of coronary function. To extend our knowledge about the risk factors for coronary dysfunction we investigated the possible relation of MPO gene polymorphism to CFR measured by positron emission tomography in mildly hypercholesterolemic, otherwise healthy men. Moreover, the association between the MPO genotype and the autoantibody titer against LDL, the marker of in vivo oxidation of LDL, was studied.

Materials and Methods

Subjects and Study Design

Fifty-one men were invited to participate in the study. The entry criteria and the background information of the study participants have been previously described [21]. Two men were excluded due to technical problems of the positron emission tomography studies and 49 men were included in the analysis. The mean age of the subjects was 35.0 ± 4.0 years (range 26–40 years) and their mean body mass index was 25.0 ± 2.3. Participants had normal or mildly elevated serum total cholesterol level (average 5.6 ± 0.8 mmol/l), but they were otherwise healthy and none had diabetes. There were 4 smokers in the study population. All subjects had normal electrocardiograms at rest and during adenosine infusion. All flow measurements were considered normal, suggesting that study subjects were free of atherosclerotic lesions detectable with positron emission tomography. The study was approved by the Ethics Committee of the Turku University Central Hospital and the University of Turku. All subjects gave written informed consent.

Positron Emission Tomography Protocol and Calculation of Myocardial Blood Flow

Positron emission tomography studies were performed after a 6-hour fast as previously described [21]. Alcohol, caffeine and smoking were prohibited 12 h before the study. Myocardial blood flow was calculated as previously described [21]. The CFR was defined as the ratio of overall myocardial blood flow after adenosine administration to flow at the baseline.

Genetic and Biochemical Analyses

Blood samples for biochemical analyses were collected after an overnight fast. DNA was isolated from whole blood using a commercial kit (Qiagen, Calif., USA). The DNA fragment of the MPO gene (GenBank accession No. X15377) promoter area was first amplified and then digested with A*ci*I restriction endonuclease (New England Biolabs Inc., Beverly, USA) as previously described [29].

The fasting plasma triglycerides, total and high-density lipoprotein cholesterol concentrations as well as apolipoprotein B and A1 concentrations were analyzed by Cobas Integra 700 automatic analyzer (Hoffmann-La Roche Ltd., Basel, Switzerland). LDL cholesterol concentration was calculated using the formula of Friedewald et al. [11]. Autoantibodies against oxidated LDL were measured as previously described [27].

Statistical Analyses

In view of the small number of low-expression allele A-homozygous subjects (n = 2) we categorized the MPO genotypes into high- (G/G) and low-expression (A/G, A/A) allele groups, as previously done in other studies [5, 31, 44]. Discontinuous variables were compared using χ^2-test. Means of continuous variables between MPO genotypes were compared using one-way analysis of covariance, wherein age, body mass index, smoking habits (+/0) and the family history of cardiovascular disease (+/0) were used as covariates. In

Table 1. Characteristics, lipid and antioxidant values according to MPO genotypes

Variable	MPO genotype		ANCOVA p value
	GG (n = 34) mean ± SD	AG/AA (n = 15) mean ± SD	
Age, years	34.8 ± 3.7	36.3 ± 4.4	0.232
BMI, kg/m^2	25.0 ± 2.4	25.1 ± 2.1	0.878
Systolic blood pressure, mm Hg	126.7 ± 13.3	131.0 ± 11.3	0.187
Diastolic blood pressure, mm Hg	74.1 ± 7.3	76.3 ± 6.9	0.314
Heart rate, beats/min	61.4 ± 10.4	61.7 ± 8.0	0.826
Rate-pressure product mm Hg × beats/min	5,635.4 ± 1,175.3	5,857.7 ± 1,102.1	0.453
Total cholesterol, mmol/l	5.50 ± 0.79	5.57 ± 0.78	0.974
LDL cholesterol, mmol/l	3.58 ± 0.68	3.63 ± 0.63	0.937
HDL cholesterol, mmol/l	1.38 ± 0.31	1.36 ± 0.224	0.807
Triglycerides, mmol/l	1.19 ± 0.68	1.28 ± 0.64	0.698
Apolipoprotein A1, g/l	1.44 ± 0.24	1.45 ± 0.20	0.967
Apolipoprotein B, g/l	1.04 ± 0.17	1.06 ± 0.19	0.962
LDL α-tocopherol, μmol/l	5.36 ± 1.58	5.05 ± 1.86	0.666
LDL ubiquinone, μmol/l	0.32 ± 0.14	0.36 ± 0.24	0.506
Leukocytes, 10^9/mmol	4.94 ± 1.50	5.33 ± 1.07	0.412
Smokers, n[a]	2	3	0.132
Exercise, times/week	2.8 ± 1.5	2.5 ± 1.5	0.515
Family history (+/0)[a]	6/28	6/9	0.094
Use of coffee, cups/day	4.5 ± 2.5	4.1 ± 2.9	0.556

ANCOVA = Analysis of covariance; BMI = body mass index; SD = standard deviation. In ANCOVA, age and BMI were used as covariates for lipids, apolipoproteins, leukocytes and hemodynamic data. [a] p values from χ^2 test.

linear regression model CFR was used as an independent variable and age, body mass index, smoking, family history and MPO genotype group as explanatory variables. Nonnormally distributed data was logarithmically transformed prior to analysis, but the following results are displayed as crude values. A p value of less than 0.05 was considered significant. Values in the text are means ± standard deviation if not otherwise stated. The statistical analyses were carried out by using SPSS 9.0 for Windows 95 (SPSS Inc., Chicago, Ill., USA) and Statistica for Windows version 5.1 software package (Statsoft, Okla., USA). We used the Power and Sample Size (PS) program version 1.0.15 for Windows 95 for power (1–β) calculations.

Results

Descriptive Analysis

The background characteristics did not differ between the genotype groups (table 1). The MPO genotypes G/G, A/G and A/A were found in 34 (69.4%), 13 (26.5%) and 2 (4.1%) of the subjects. The frequencies for G and A alleles were 82.7% and 17.3%, respectively. The observed genotype distribution followed the Hardy-Weinberg equilibrium and was in agreement with that of previously published Caucasian populations [30, 42, 43].

Myocardial Blood Flow and LDL Oxidation

Table 2 shows the values of myocardial blood flow and indices of in vivo LDL oxidation among the subjects by MPO genotype. Subjects with high-expression genotype G/G had 18.1% lower CFR (p = 0.019) and 11.5% lower adenosine-stimulated flow (p = 0.049) than low-expression A/G, A/A genotypes after adjusting for age, body mass index, smoking habits and family history of atherosclerosis. There were no significant differences in blood flow at rest or in the incidence of ex vivo LDL oxidation between the MPO genotype groups. In linear regression analysis, after adjustment of age, body mass index, family history of cardiovascular disease, smoking habits and MPO genotype group, the MPO genotype group and body mass index were significant predictors for CFR (p = 0.019 and p = 0.025, respectively, for the entire model p = 0.033, R^2 = 0.24). MPO was the only significant predictor also in adenosine-stimulated flow (p = 0.046) whereas the whole regression model remained nonsignificant (data not shown).

Table 2. Myocardial blood flow and in vivo oxidation indices according to MPO genotype groups

Variable	MPO genotype						
	GG (n = 34)		AG/AA (n = 15)		All (n = 49)		ANCOVA p value
	mean	SD	mean	SD	mean	SD	
Blood flow at rest, ml g^{-1} min^{-1}	0.84	0.22	0.81	0.17	0.83	0.21	0.705
Adenosine-stimulated flow, ml g^{-1} min^{-1}	3.22	0.79	3.64	0.90	3.35	0.84	0.049*
CFR	3.98	1.01	4.60	1.30	4.17	1.13	0.019*
Ox-LDL to native-LDL difference (n = 33)	0.06	0.05	0.07	0.08	0.06	0.06	0.435
Ox-LDL to native-LDL ratio (n = 33)	1.94	0.92	1.99	1.10	1.95	0.97	0.804

ANCOVA = Analysis of covariance; Ox-LDL = oxidated low-density lipoprotein; SD = standard deviation. * $p < 0.05$ for ANCOVA between classified genotypes. Age, body mass index, smoking habits and family history of cardiovascular disease were used as covariates.

Discussion

This study demonstrates that MPO polymorphism –463G/A is associated with coronary reactivity in young, mildly hypercholesterolemic but otherwise healthy men. The high-expression genotype (G/G carriers) had significantly lower adenosine-stimulated blood flow and CFR than subjects with the low-expression genotypes (A/G and A/A). There are no previous studies in which the impact of MPO gene promoter variation on vascular function would have been assessed. The incidence of low-expression allele A has been associated with reduced morbidity risk to coronary artery disease [31] and now our results link this protective effect to the early manifestation of coronary dysfunction.

The mechanism behind our result remains to be clarified. The CFR, coronary flow response to adenosine or dipyridamole, is not a purely endothelial function indicator, rather it is an integrating measure of endothelial function and vascular smooth muscle relaxation. The endothelial dependency of coronary vasodilation by adenosine has recently been investigated by Buus et al. [4] and they found that about half of the response to adenosine is endothelium-dependent. Impairment of CFR has been shown to be an early manifestation of atherosclerosis and coronary artery disease [1, 6, 8, 39]. It has been observed that CFR is impaired in young adults with classical coronary risk factors, i.e., familial hypercholesterolemia [38], familial combined hyperlipidemia [36], type 1 diabetes mellitus [37] and borderline hypertension [25].

Adhesion of neutrophils and increased tissue concentration of MPO and hypochlorous acid are associated with endothelial dysfunction and reduced nitric oxide activity in inflammation and ischemia-reperfusion injury [12, 13, 28]. The exposure of guinea pig hearts to hypochlorous acid results in the complete loss of vasodilatation in response to known vasodilators, including adenosine, by blocking the endothelial mediation of coronary dilatation [28]. In fact, it has been shown that when the precursor of nitric oxide, *L*-arginine, is modified by hypochlorous acid the synthesis of nitric oxide in endothelial cells of the rat aortic ring segments is inhibited [48, 49]. In addition, the major metabolite of nitric oxide, nitrite, has been reported to serve as a substrate for MPO [9, 17].

To our knowledge, there are two studies concerning MPO –463G/A polymorphism and coronary artery disease. A/A and A/G genotypes have been reported to have a protective role for coronary artery disease in French-Canadian patients with angiographically diagnosed coronary artery disease [31]. In patients with end-stage renal disease, the G/G genotype was associated with a higher prevalence of cardiovascular disease when compared to A/G or A/A genotypes. The G/G genotype carriers also had higher levels of pentosidine, a marker of oxidative protein damage, than A allele carriers, which may indicate a difference in production of free radicals between genotypes [34]. These results are congruent with our results in the early stage of coronary artery disease. A allele has also been associated with increased lipid values and therefore suggested to be a risk factor for cardiovascular disease [20]. However, we did not record any major differences in lipid and lipoprotein levels between MPO genotype groups.

Oxidation of LDL has been associated with the impairment in coronary reactivity [41]. MPO-derived oxidants are known to have a potential role in the LDL oxidation and in the promotion of atherosclerosis [18, 19]. Hypochlorous acid-modified LDL is known to inhibit the syn-

thesis of nitric oxide in endothelial cells, suggesting it to be an important mechanism in the development of endothelial dysfunction [33]. In the current study, autoantibody levels against copper-oxidized LDL were measured in order to indicate the overall atherosclerotic progress [46]. There were no significant differences in autoantibody levels between the MPO genotype groups. Therefore, one could suggest that the mechanism by which MPO polymorphism influences coronary reactivity may not be related to LDL oxidation. However, it should be noted that the autoantibody titer against copper-oxidized LDL is only one marker of the complex process of LDL oxidation among several other methodologies developed for oxidation measurements [10], and specific antibodies against hypochlorous acid-modified LDL were not used in the current study. Thus we cannot rule out the possible association of MPO promoter genotype with these other markers of oxidation describing other phases of lipid oxidation.

There were some limitations in our study. The major limitation was the low number of study subjects. The power of the study protocol remained weak whereas the power of 80% would have been obtained with 85 subjects. Therefore, it would be important to confirm this observation by using other methods suitable for screening a larger number of subjects. In addition, only males were enrolled in the study and therefore our results cannot be extrapolated to female subjects. It is noteworthy that the SP1 transcription factor binding area may function as an estrogen hormone-binding site leading to gender-specific expression of MPO [30, 32, 43]. In the population of 49 men, there were only 2 subjects carrying the MPO A/A genotype.

In conclusion, our results show that MPO gene polymorphism has an effect on coronary artery function, the magnitude of which is similar to traditional risk factors of coronary artery disease. Thus MPO promoter genotype may partly explain differences between individuals in the development of coronary artery disease.

Acknowledgments

This study was supported by grants from the Finnish Foundation of Cardiovascular Research, the Juho Vainio Foundation, the Elli and Elvi Oksanen Fund of the Pirkanmaa Fund under the auspices of Finnish Cultural Foundation, the Medical Research Fund of Tampere University Hospital and the Research Foundation of Orion Corp. The authors thank Ms. Marita Koli and the Turku National PET center for their skilful technical assistance.

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ScienceDirect
Clinical Biochemistry 41 (2008) 532–537
CLINICAL BIOCHEMISTRY

The association of myeloperoxidase promoter polymorphism with carotid atherosclerosis is abolished in patients with type 2 diabetes

Riikka Mäkelä [a,c,*], Antti Loimaala [d], Arja Nenonen [a,c,e,f], Michele Mercuri [g], Ilkka Vuori [e], Heini Huhtala [b], Pekka Oja [e], Gene Bond [g], Timo Koivula [a], Terho Lehtimäki [a,c]

[a] Tampere University Hospital, Centre for Laboratory Medicine, Department of Clinical Chemistry, Tampere, Finland
[b] University of Tampere, Department of Public Health Tampere, Finland
[c] University of Tampere Medical School, Tampere, Finland
[d] Seinäjoki Central Hospital, Department of Clinical Physiology and Nuclear Medicine, Seinäjoki, Finland
[e] UKK Institute for Health Promotion Research, Tampere, Finland
[f] Rheumatism Foundation Hospital, Heinola, Finland
[g] Wake Forest University School of Medicine, Winston-Salem, North Carolina, USA

Received 18 October 2007; accepted 22 January 2008
Available online 6 February 2008

Abstract

Objectives: Type 2 diabetes mellitus (DM) enhances the development of atherosclerosis and reduces the activity of the oxidative myeloperoxidase (MPO) enzyme. MPO gene has a functional promoter polymorphism −463G/A which leads to high- (GG) and low-expression (AG, AA) genotypes.

Design and methods: We studied the association of MPO polymorphism with carotid artery intima-media thickness (IMT) in 198 randomly selected Finnish men of Caucasian origin, 161 non-diabetics and 37 with type 2 DM. Their carotid IMT was measured by high-resolution ultrasonography, and the overall mean IMT value was calculated. MPO genotypes were determined by the PCR-RFLP method.

Results: We found significant MPO genotype-by-study-group (control/DM) interactions with the overall mean IMT and internal carotid IMT ($p=0.05$ and $p=0.04$, respectively). Among non-diabetic subjects, the overall carotid IMT was 7.3% higher in subjects with the low-activity genotype when compared to the high-activity (G/G) group. The results remained significant after adjustment for total cholesterol and smoking ($p=0.015$). No similar genotypic association was found for the subjects with type 2 DM.

Conclusions: This data suggests that in subjects with normal glucose metabolism, MPO gene variation may modify the carotid artery IMT.

Keywords: Myeloperoxidase; Carotid atherosclerosis; Polymorphism; Type 2 diabetes

Introduction

Atherosclerosis is a chronic inflammatory disease process, which is a major cause of morbidity and mortality in the developed countries. According to current knowledge, the accumulation of phagocytic cells in the vessel wall results in enhanced production of reactive oxygen species (ROS) and oxidation of low-density lipoprotein (LDL) cholesterol [1]. Insulin resistance is present in 80% of type 2 diabetes mellitus (DM) patients. Interestingly, insulin resistance is also characterized by a long-term inflammatory process [2] and, accordingly, the development of atherosclerosis is enhanced in subjects with type 2 DM [3,4]. According to prospective and cross-sectional studies, type 2 DM is a major risk factor of coronary artery disease (CAD) [5,6].

High-resolution ultrasonography allows noninvasive and quantitative assessment of atherosclerotic changes in the peripheral vascular wall [7–9]. Carotid atherosclerosis disease can be detected by measuring the intima-media thickness (IMT) by B-mode ultrasonography [10]. IMT is prognostic of ischemic stroke, and it also reflects simultaneous CAD [11]. Traditional atherosclerosis risk factors, such as age, hypertension, smoking,

* Corresponding author. Laboratory of Atherosclerosis Genetics, Department of Clinical Chemistry, Centre for Laboratory Medicine, Tampere University Hospital, FinnMedi 2, 3rd Floor, P.O. Box 2000, 33521 Tampere, Finland. Fax: +358 3 311 74168.
E-mail address: riikka.makela@uta.fi (R. Mäkelä).

0009-9120/$ - see front matter
doi:10.1016/j.clinbiochem.2008.01.017

and DM, are all directly related to an increase in IMT [7,10,12]. In diabetic patients, several studies have shown that their carotid IMT is larger than that of healthy controls [13,14]. In addition to this, genetic factors are known to determine the variation in carotid IMT [15]. However, most of the studies have been performed on clinically healthy subjects—knowledge about the genetic determinants and possible gene–environment interactions therefore remains insufficient.

Myeloperoxidase (MPO) is an oxidative enzyme present in phagocytes, and it is an essential part of the anti-microbial system and inflammatory regulation [16,17]. MPO is expressed in atherosclerotic lesions and is able to modify pro-atherosclerotic lipoprotein particles by its oxidative intermediates [18,19]. Moreover, in humans elevated blood and leukocyte MPO levels are associated with CAD and the incidence of myocardial infarction [20,21], and, interestingly, MPO activity is lowered in DM patients [22]. MPO gene expression is regulated by a single nucleotide polymorphism (SNP) in the promoter region at position −463. The G-to-A base substitution creates high- (G) and low-expression (A) alleles [23,24]. This polymorphism is known to be a determinant of coronary flow reserve in healthy subjects and the progression of atherosclerosis during hormone replacement therapy [25], in addition to being associated with the prevalence of CAD [26,27]. These earlier findings are important because they suggest that specific MPO genotypes may be associated with different outcomes of atherosclerosis, depending on the prevailing CAD risk factors. Furthermore, relevant genetic factors may not be detected at all, unless the target sample is stratified by silent cardiovascular risk factors, e.g., by type 2 DM.

In summary, MPO polymorphism is a potent determinant of atherosclerosis, and DM is known to affect MPO activity and possibly even its gene expression by peroxisome proliferator-activated receptor (PPAR)-mediated regulation [28]. However, there are no previous studies concerning the association or interaction of the presence of type 2 DM with MPO polymorphism in the development of atherosclerosis. The current study was undertaken to investigate this issue and to determine whether this kind of association exists and whether it modifies the extent of the noninvasive marker of atherosclerosis, i.e., carotid IMT, in a random sample of middle-aged men.

Materials and methods

Subjects

Subjects were randomly selected from a total cohort of 9058 men aged 50 to 59 years living in the city of Tampere in southern Finland. Three hundred subjects representing ten age cohorts were invited to enter the study, and 223 agreed to participate, the participation rate being 74%. All required data was obtained for 196 of these participants, and this data constituted the final analysis of clinical characteristics and carotid IMT. The study was approved by the local ethics committee, and all participants gave written informed consent.

Detailed medical histories were collected with particular emphasis on cardiovascular and metabolic diseases, smoking habits, and chronic medication. Weight, height, and resting blood pressure were recorded as described previously [29]. There were 40 smokers and 158 non-smokers, including 71 former smokers, in the study population, and six persons were already treated for DM. None of the subjects had suffered a symptomatic cerebrovascular event.

Oral glucose tolerance test

The standard 2-hour oral glucose tolerance test (OGTT) with a 75 g glucose load, according to WHO 1998 criteria, was used to assess glucose tolerance [30]. The blood samples were taken at baseline and 1 and 2 h after the glucose load, and the plasma glucose concentrations were measured. Fasting glucose level of 7.0 mmol/L or higher, and/or a 2-hour post-challenge glucose level of 11.1 mmol or higher were considered as diagnostic criteria for type 2 DM [30]. Glucose analyses were carried out on hemolyzed whole blood samples using the glucose dehydrogenase/mutarotase method (Merck Diagnostica, Darmstadt, Germany).

Biochemical analyses

Blood samples were drawn after a 12-hour fast. Lipoprotein fractions were assessed from fresh samples after ultracentrifugation [31]. Cholesterol levels were measured from serum and lipoprotein fractions using an enzymatic method (CHOD-PAP, Boehringer Mannheim, Mannheim, Germany). Triglycerides were determined from frozen samples by enzymatic hydrolysis (GPO-PAP, Boehringer Mannheim, Mannheim, Germany). Apolipoprotein B (apoB) was analyzed by immunonephelometry (Behring, Behringwerke AG, Marburg, Germany) and lipoprotein (a) (Lp(a)) by two-site immunoradiometry (Pharmacia, Uppsala, Sweden).

MPO genotyping

DNA was isolated from lymphocytes with the aid of a commercial kit (Qiagen Inc, California, USA). The MPO genotypes were determined by a polymerase chain reaction and restriction endonuclease AciI (New England Biolabs Inc., Beverly, USA) as described previously [32]. Digested fragments were separated by electrophoresis on 2.5% agarose gel and visualized with ultraviolet light after ethidium bromide staining.

Carotid ultrasonography

Quantitative carotid artery ultrasonography was performed according to a standardized protocol [9,29]. A commercially available high-resolution B-mode ultrasound device with a 10 MHz transducer was used (Biosound Phase 2, Indianapolis, USA) to examine the left and right carotid arteries. The examinations were recorded on S-VHS videotapes, and the tapes were then read off-line at the ultrasound reading center, Wake Forest University, North Carolina, USA.

The right and left carotid arteries were scanned from both sides by means of a circumferential scan including the longitudinal views of the lateral, posterior, and anterior angles. The

protocol involved the scanning of the distal 10 mm of the common carotid artery, the bifurcation, and up to 10 mm of the proximal internal carotid artery. The distance between the media-adventitia interface and the lumen-intima interface is the IMT. The maximum IMT of the near and far wall was measured at 12 well-defined arterial segments. The mean maximum IMT (MMax IMT, overall mean) was the mean of 12 maximum IMTs identified at 12 standard sites [9]. The intra-observer variability and measurement reproducibility have been described previously [29], and they compare with other reports on CAAD data [33]. Carotid artery atherosclerosis (CAAD) was defined as an IMT > 1.7 mm in at least one site. The cut-off point (1.7 mm) was calculated in the following manner: overall mean IMT + 2 SD. When this cut-off point was used, the prevalence of CAAD was 21%.

Statistical analysis

Because the number of AA homozygotes was small ($n=9$), the study subjects were categorized into high-expression (G/G) and low-expression (A/G, A/A) allele groups, a categorization previously used in other related studies [34]. Non-normally distributed data was logarithmically transformed prior to analysis, but the results are displayed as crude values. The data were divided into two groups, non-diabetic and diabetic subjects, according to the measurements during OGTT. The MPO genotype and study group (non-diabetic vs. DM) were used as factors in the two-way analysis of covariance (ANCOVA), where the possible confounding effects of smoking and total cholesterol were taken into account by including them in the model as covariates. The least significant difference test was used as a *post hoc* test to study the differences between the genotype groups. For the analysis of carotid IMT between the MPO genotype groups among non-diabetic subjects, one-way ANCOVA was employed with smoking and total cholesterol as covariates. The Student's *t*-test was used for the statistical analysis of descriptive data. Categorized variables were compared with the χ^2 test.

To examine the possible differences in OGTT response curves between MPO genotypes, we used ANOVA for repeated measures (RANOVA), where the MPO genotype was used as a dependent factor and the plasma glucose concentrations measured before (0) and 1 and 2 h after glucose load were included as a repeated (time) factor. Data are expressed as mean and SD. A *p*-value equal to or less than 0.05 was considered statistically significant. Calculations were performed with Statistica for Windows 5.1 (StatSoft Inc., Tulsa, Oklahoma, USA) software on a PC.

Table 1
Clinical characteristics of the study population by myeloperoxidase genotype

	GG	SD/%	AG/AA	SD/%
	$n=116$		$n=80$	
Age (yr)	54.1	3.0	54.3	2.9
BMI (kg/m^2)	26.9	3.7	27.3	3.6
Systolic blood pressure (mmHg)	131	18	131	15
Diastolic blood pressure (mmHg)	83.8	11.3	84.0	8.5
Total cholesterol (mmol/L)	5.50	0.82	5.42	0.96
LDL cholesterol (mmol/L)	3.54	0.76	3.55	0.87
HDL cholesterol (mmol/L)	1.24	0.29	1.21	0.24
VLDL cholesterol (mmol/L)	0.72	0.44	0.64	0.40
Triglycerides (mmol/L)	1.55	0.87	1.51	0.86
ApoB (g/L)	1.30	0.27	1.30	0.32
Blood leukocytes (10^9/L)	5.57	1.50	5.77	1.93
OGTT 0 h (mmol/L)	5.5	1.4	5.5	1.4
OGTT 1 h (mmol/L)	9.5	3.1	9.5	2.8
OGTT 2 h (mmol/L)	7.3	2.9	7.5	3.3
Diabetes mellitus (yes/no)*	23/93	24.7%	14/66	21.2%
CAAD status (yes/no)*	22/94	23.4%	19/61	31.1%
Smoking (yes/no)*	22/94	23.4%	17/63	27.0%
Hypertension (yes/no)*	23/93	24.7%	8/72	11.1%

Statistics: There were no statistically significant differences between genotype groups in the *t*-test or χ^2 test*. Abbreviations used in the table: ApoB, apolipoprotein B; BMI, body mass index; CAAD, carotid artery atherosclerosis; HDL, high-density lipoprotein; LDL, low-density lipoprotein; OGTT, blood glucose on oral glucose tolerance test; VLDL, very low-density lipoprotein.

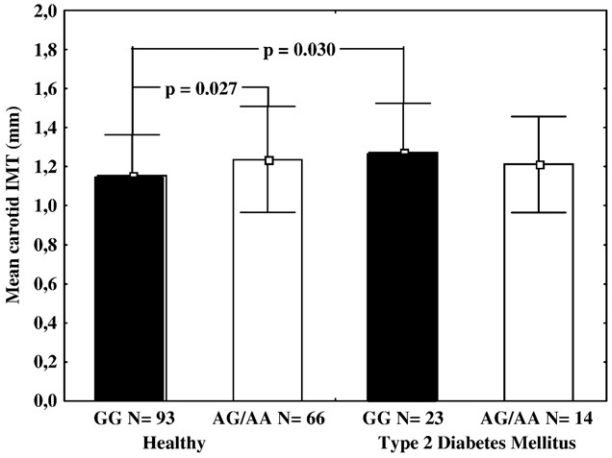

Fig. 1. Mean carotid artery IMT according to study group (non-diabetic/diabetics) and Myeloperoxidase (MPO) genotype status (high-expression homozygotes GG and low-expression A allele carriers AG or AA). The mean IMT values are given as mean ± standard error. *p*-values have been derived with two-way ANCOVA, using the least significant difference (LSD) test as a means of post hoc analysis. Results are adjusted for smoking and total cholesterol.

Results

Descriptive results

The MPO genotype frequencies among the 196 men were as follows: 116 for GG (59.2%), 71 for AG (36.2%), and 9 for AA (4.6%). The allele frequencies were 0.77 and 0.23 for G and A, respectively. The genotype frequencies were in accordance with previous Finnish studies [25], and the genotypes were in Hardy–Weinberg equilibrium. Allele A carriers were combined into one group which was then compared with the GG homozygotes. Table 1 shows the clinical characteristics of all 196 participants. There were no statistically significant differences in the means with respect to traditional risk factors i.e. age, smoking status, BMI, and lipoprotein concentrations—or in CAAD and hypertension status between the A allele carriers and GG homozygotes. There were no statistically significant differences in OGTT response curves between MPO genotypes ($p=0.702$ for MPO genotype-by-time interaction in RANOVA).

Table 2
Mean carotid artery IMT in different segments of the carotid artery by MPO genotype in healthy subjects

Carotid artery IMT (mm)	MPO genotype						One-way ANCOVA *p*-value
	GG	SD	AG/AA	SD	All	SD	
	n=93		*n*=66		*n*=159		
Common carotid artery	1.03	0.21	1.06	0.18	1.04	0.19	0.071
Bifurcation	1.36	0.30	1.46	0.37	1.40	0.34	0.055
Internal carotid artery	1.05	0.33	1.18	0.50	1.10	0.41	0.069
Overall mean IMT	1.15	0.21	1.24	0.27	1.19	0.24	0.015

Statistics: One-way ANCOVA between MPO genotypes and carotid artery IMT. Smoking and total cholesterol were used as covariates. Values are means (±SD). Abbreviations used in the table: ANCOVA, analysis of covariance; IMT, intima-media thickness; MPO, myeloperoxidase.

MPO gene variation and IMT in carotid arteries

In two-way ANCOVA, there was a significant MPO genotype-by-study-group (non-diabetic controls vs. DM) interaction with internal carotid artery IMT ($p=0.043$) and a borderline significant interaction with mean carotid artery IMT ($p=0.05$) (see Fig. 1). In similar statistical analysis for other IMT measurements, the interaction was not significant. Table 2 shows the mean IMTs in the different segments of the carotid artery by MPO genotype group in healthy non-diabetic subjects. The *p*-values for the overall mean carotid IMT were significant ($p=0.015$) and the *p*-values for the other measurements were borderline significant. The subjects carrying the low-expression genotype (AG/AA) had 7.3% higher IMT values than GG homozygotes. The results remained significant after adjustment for total cholesterol and smoking ($p=0.015$, ANCOVA). No similar genotypic association was found in the subjects with type 2 DM, or when the ANCOVA was performed for the whole study group, including subjects with DM.

Discussion

To our knowledge, this is the first study to investigate the interactive effect of MPO promoter polymorphism and presence of type 2 DM on carotid IMT as measured by B-mode ultrasonography. We found a statistically significant interaction between MPO genotype and type 2 DM status in relation to both internal carotid artery IMT and overall mean IMT (Fig. 1). In healthy non-diabetic subjects, the association of MPO genotype with carotid IMT was quite distinct, while a similar association was abolished in subjects with type 2 DM. According to our results, the low-expression allele A carriers had higher overall mean IMT values among men with normal glucose metabolism. In subjects with type 2 DM, there were no significant differences in IMT values between the MPO genotypes.

Insulin resistance increases the risk of metabolic abnormalities such as hypertension and dyslipidemias [35]. Subjects with insulin resistance seem to have a permanent increase in inflammatory markers, such as *c*-reactive protein (CRP), predicting a constant chronic subclinical inflammation state [5]. It has been speculated that CRP levels might predict type 2 DM before the clinical diagnosis of diabetes [36]. In persons with impaired glucose tolerance (IGT), carotid IMT is increased, indicating that even small changes in glucose tolerance may increase the risk of CAD [37]. However, it has been found that in patients with type 2 DM, the increased IMT does not associate with markers of low-grade inflammation [38]. Accordingly, the MPO genotype-dependent difference in IMT was not detected in diabetic patients in the present study. One could speculate that in type 2 DM, other risk factors carry greater significance in artery wall thickening than a mere inflammatory response.

MPO is a potent oxidative contributor to atherosclerosis, with the ability to produce a large group of oxidative compounds [39]. MPO is detected in atherosclerotic lesions, and high blood MPO concentrations correlate with the incidence of CAD [18]. MPO and HOCl-oxidized proteins are also found in segments with an intima-media ratio higher than 1.8, indicating the presence of the enzyme in CAAD [40]. MPO has been detected in the coronary vasculature, and high blood MPO levels predict the risk of acute myocardial infarction [20,41]. In the current study, the genotype-related benefit of the high-expression genotype GG seemed to be lost in diabetic persons. It is known that the oxidative metabolism of leukocytes is significantly reduced in diabetic patients [42], while the glucose transport in polymorphonuclear phagocytes is increased [43]. In addition to the metabolic changes, MPO activity is also decreased by an allosteric enzyme blockade [22,44]. This decrease in the MPO activity of diabetic patients could partly explain our interaction results.

The expression of the whole MPO gene is regulated by several ligands, including the PPARγ ligands and estrogen [28]. The PPARγ agonists, such as rosiglitazone, are used as medical treatment for type 2 DM and are shown to retard the progression of atherosclerosis in both diabetic and non-diabetic patients [45]. The MPO expression is also mediated by the promoter area G-to-A point mutation which causes a multifold decrease in MPO gene expression [23,24]. In addition to our current results, MPO polymorphism has been reported to affect the atherosclerosis process in several previous studies. GG genotype carriers with end-point renal disease have been reported to have both higher levels of oxidation markers and total risk of CAD [27]. Accordingly, the A allele carriers have higher coronary flow reserve and lower prevalence of angiographically verified CAD than GG homozygotes [26,46]. In contrast, GG homozygous postmenopausal women benefit from hormonal replacement therapy in terms of ultrasonographically measured progression of atherosclerosis [25]. A similar protective effect of GG genotype as in our study was observed in an autopsy study where GG carrier men aged under 53 years had lower areas of atherosclerotic lesions in thoracic and abdominal aortas than A allele carriers [47]. According to these recent studies, however, the effect of MPO genotype in atherosclerosis seems to vary in different phenotypes, and further investigation is still required. Our results may also arise from some other single nucleotide polymorphism effect in the MPO promoter area and, therefore, this data should be regarded only as preliminary results.

In conclusion, our study of 196 randomly selected middle-aged men showed that MPO genotype status interacts with the presence of type 2 DM in relation to carotid IMT values. Among the GG genotype carriers, the IMT values were lower

than among A allele carriers, but the effect was abolished in subjects with type 2 DM. In addition to investigating the major effects of certain risk genotypes, the stratification of study groups according to the most important cardiovascular risk factors–e.g., DM in the present study–may be important, as these important results are otherwise not found. However, our results should be considered preliminary, since the number of subjects in the type 2 DM group was low. The clinical importance of MPO genotype with respect to carotid IMT values in subjects with type 2 DM therefore needs to be investigated further in larger studies.

Acknowledgments

This study was supported by grants from the Medical Research Fund of Tampere University Hospital, the Emil Aaltonen Foundation, the Finnish Foundation for Cardiovascular Research, the Finnish Ministry of Education, the Research Foundation of Orion Corporation, and the Finnish Medical Society Duodecim. The authors thank Mr. Matti Pasanen for his statistical expertise and Ms. Marita Koli for her technical assistance.

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