RIIKKA RONTU

Polymorphisms of the Antioxidative Enzyme Paraoxonase-1 in the Development of Atherosclerosis

Clinical and Autopsy Studies

■

ACADEMIC DISSERTATION

To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the small auditorium of Building B,
Medical School of the University of Tampere,
Medisiinarinkatu 3, Tampere, on March 14th, 2003, at 12 o'clock.

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To my husband Mikko

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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications referred in the text by the representative Roman numerals I-VI.

I Malin R, Laaksonen R, Knuuti J, Janatuinen T, Vesalainen R, Nuutila P, Lehtimäki T. Paraoxonase genotype modifies the effect of pravastatin on high-density lipoprotein cholesterol. Pharmacogenetics 2001;11:625-633.

II Malin R, Laine S, Rantalaiho V, Wirta O, Pasternack A, Jokela H, Alho H, Koivula T, Lehtimäki T. Lipid peroxidation is increased in paraoxonase L55 homozygotes compared with M-allele carriers. Free Rad Res 2001;34:477-484.

III Malin R, Knuuti J, Janatuinen T, Laaksonen R, Vesalainen R, Nuutila P, Jokela H, Laakso J, Jaakkola O, Solakivi T, Lehtimäki T. Paraoxonase gene polymorphisms and coronary reactivity in young healthy men. J Mol Med 2001;79:449-456.

IV Malin R, Loimaala A, Nenonen A, Mercuri M, Vuori I, Pasanen M, Oja P, Bond G, Koivula T, Lehtimäki T. Relationship between high-density lipoprotein paraoxonase gene M/L55 polymorphism and carotid atherosclerosis differs in smoking and non-smoking men. Metabolism 2001;50:1095-1101.

V Malin R, Järvinen O, Sisto T, Koivula T, Lehtimäki T. Paraoxonase producing PON1 gene M/L55 polymorphism is related to autopsy-verified artery-wall atherosclerosis. Atherosclerosis 2001;157:301-307.

VI Rontu R. Karhunen PJ, Ilveskoski E, Mikkelsson J, Kajander O, Perola M, Penttilä A, Koivisto A-M, Lehtimäki T. Smoking-dependent association between paraoxonase M/L55 genotype and coronary and aortic atherosclerosis in males: an autopsy study. (submitted for publication).

ABBREVIATIONS

AN(CO)VA	analysis of (co)variance
apo	apolipoprotein
BMI	body-mass index
CA	celiac artery
CAAD	carotid artery atherosclerotic disease
CAD	coronary artery disease
CE(s)	cholesteryl ester(s)
CFR	coronary flow reserve
CHD	coronary heart disease
CI	confidence interval
DNA	deoxyribonucleic acid
EC(s)	endothelial cell(s)
ELISA	enzyme-linked immunosorbent assay
GFR	glomerular filtration rate
HDL	high-density lipoprotein
HPLC	high-performance liquid chromatography
HSDS	Helsinki Sudden Death Study
IAP	International Atherosclerosis Project
IEL	internal elastic lamina
IMA	inferior mesenteric artery
IMT	intima-media thickness
8-iso-$PGF_{2\alpha}$	8-iso-prostaglandin $F_{2\alpha}$
LAD	left anterior descending coronary artery
LCAT	lecithin-cholesterol acyl transferase
LCX	left circumflex coronary artery
LDL	low-density lipoprotein
Lp(a)	lipoprotein (a)
LSD test	Least Significant Difference post-hoc test
(lyso-)PC	(lyso-)phosphatidylcholine
MI	myocardial infarction

M/L55	methionine for leucine polymorphism at position 55
MMax IMT	mean maximum intima-media thickness
mm-LDL	minimally modified LDL
mRNA	messenger ribonucleic acid
OP(s)	organophosphate(s)
ox-LDL	oxidized LDL
PAF-AH	platelet-activating factor acetylhydrolase
PET	positron emission tomography
PON1	paraoxonase-1 protein
PON1	paraoxonase-1 gene
RANOVA	analysis of variance for repeated measures
RCA	right coronary artery
RPP	rate-pressure product
R/Q192	arginine for glutamine polymorphism at position 192
SD	standard deviation
SMA	superior mesenteric artery
SMC(s)	smooth muscle cell(s)
VLDL	very low-density lipoprotein

INTRODUCTION

Atherosclerosis is a disease of large and medium-sized arteries and a primary cause of coronary heart disease (CHD) and myocardial and cerebral infarction. In Westernized societies it gives rise to about 50% of all deaths (Ross 1993). The probability of developing atherosclerosis correlates strongly with several "traditional" risk factors including age, sex, hypertension, hypercholesterolemia, diabetes, obesity, smoking, lack of physical activity and stress (Criqui 1986). However, the tendency to develop atherosclerosis runs in families and numerous studies have provided evidence that there is also a significant genetic component related to the risk of atherosclerosis (Marenberg et al. 1994). The heritability of atherosclerosis (the fraction of disease explained by genetics) within a population has been high in most studies, frequently exceeding 50% (Goldbourt and Neufeld 1986). The genes involved with atherosclerosis in human can be identified by genome scans in family-based studies (Soro et al. 2002), if there is no previous knowledge of gene products, or by the candidate gene approach, if there is a possibility to utilize information from prior biochemical or physiological studies. Today, gene expression studies of coronary artery specimens are also becoming a widely used technique for the identification of genetic factors which may play a role in atherogenesis (Hiltunen et al. 2002). Understanding the genetics of human atherosclerosis is, however, problematic, since atherosclerosis results in complex clinical phenotypes, the endpoints of atherosclerosis usually occur later in life, and there is considerable heterogeneity of genetic and environmental factors.

A primary initiating event in atherosclerosis is the accumulation of low-density lipoprotein (LDL) in the intima of the arterial wall (Faggiotto et al. 1984). Once entered into the intima, LDL undergoes modifications, including oxidation, by any of the major cell types found in arterial wall (Witztum and Steinberg 1991). Accumulation of oxidatively modified LDL (ox-LDL) in the intima contributes significantly to the early steps in atherosclerosis by increasing monocyte adherence and penetration to the lesion (Berliner et al. 1995). In the intima, monocytes differentiate into macrophages that can internalize significant amounts of ox-LDL and turn into lipid-laden foam cells. These events can lead to the formation of clinically silent fatty streak lesions, which, if

developed further, are the precursors of more complex lesions that can eventually lead to coronary narrowing and clinical manifestations (Fuster et al. 1992).

High-density lipoprotein (HDL) protects strongly against atherosclerosis. This is partly explained by the role of HDL in the removal of excess cholesterol from peripheral tissues. In addition, HDL inhibits lipoprotein oxidation (Mackness and Durrington 1995). The antioxidative properties of HDL are partly due to paraoxonase-1 (PON1), an esterase enzyme carried on HDL that can degrade certain biologically active oxidized phospholipids (Hegele 1999). The activity and concentration of PON1 are under genetic control (Humbert et al. 1993): Coding region methionine for leucine polymorphism at position 55 (M/L55 polymorphism) affects mainly on concentration of PON1 but also its activity (Garin et al. 1997). Arginine for glutamine substitution at position 192 (R/Q192 polymorphism), in turn, modulates the activity of the enzyme but not its concentration (Davies et al. 1996). The M55 and Q192 enzyme variants could protect lipids more effectively from *in vitro* peroxidation than the L55 and R192 enzyme variants. In line with this observation some studies, but not all, have suggested that the L55 and R192 alleles may predict CHD (Mackness et al. 1998a). However, the impact of these coding region polymorphisms on different atherosclerotic stages and factors that could modify the functions of PON1 are still unclear.

In this thesis, the effect of PON1 genotypes on serum lipids and apolipoproteins during pravastatin treatment, and the role of PON1 in oxidation of lipids were elucidated in two clinical series. One of these studies utilized positron emission tomography (PET) to examine the association between PON1 polymorphisms and coronary function and reactivity. In the third clinical series, the relation between PON1 genotypes and sonographically measured carotid artery intima-media thickness (IMT) was studied. The association between the PON1 genotypes and early and advanced atherosclerotic lesions in coronary arteries, aorta and mesenteric arteries was examined in two autopsy series. Since the assessment of the impact of genetic factors is not meaningful without consideration of environmental factors, the effect of smoking on the function of PON1 was also investigated.

REVIEW OF THE LITERATURE

1. Atherosclerosis

1.1. Structure of arteries

Intima. Arteries have three main structural layers of which the innermost layer is called the intima. The intima includes the endothelial surface and the subendothelial part underlying it. At the luminal surface, endothelial cells (ECs) of healthy muscular and elastic arteries form a continuos single-cell layer. The subendothelial part of the arterial intima is composed of two layers: The inner layer, subjacent to endothelium, is called the proteoglycan layer, which contains nonfibrous connective tissue (Wight and Ross 1975). In this layer, some macrophages and the synthesizing type of smooth muscle cells (SMCs) are present mainly as single, isolated cells. The thicker layer adjacent to the media is called the musculoelastic layer, since it contains more SMCs, elastic fibers and collagen than the upper layer (Stary 1987).

Media. The media lies under the internal elastic lamina (IEL) and consist of the synthesizing type of SMCs, which produce collagen, and of diagonally oriented contractile type of SMCs, which are involved in vasodilatation and vasoconstriction of the artery (Ross and Glomset 1976). The external elastic lamina bounds the media, forming an elastic border with the third arterial layer called the adventitia.

Adventitia. The adventitia contains fibroblasts intermixed with SMCs, mast cells, proteoglycan and loose collagen fibrils (Ross and Glomset 1976, Laine et al. 2000). Vasa vasorum and nerve endings localize in this outermost layer of the arterial wall.

Adaptive intimal thickening. Thick segments of intima are present in the large arteries of healthy subjects of all ages. This change is called adaptive intimal thickening and it is due to physiological adaptation of the arterial wall to changes in flow and wall tension (Caro et al. 1969). Thick segments may be focal (eccentric) or they may be more extensive (diffuse). Eccentric intimal thickening (also called intimal cushion or pad) is a relatively abrupt increase in the thickness of the intima associated with branches and orifices of the arteries. In humans, eccentric intimal thickening can

occasionally be observed already from the first week of life (Pesonen 1974, Stary 1987). In turn, diffuse intimal thickening is a spread-out and often circumferential pattern of adaptive intimal thickening. Adaptive increases in intimal thickness do not obstruct the vascular lumen and should not be considered abnormal although the turnover of ECs (Wright 1968) and SMCs (Stary and McMillan 1970) and the concentration of LDL (Spring and Hoff 1989) and other plasma components is increased.

Atherosclerosis-prone locations. Some adaptive intimal thickening, particularly the eccentric pattern, coincide with the locations where the first advanced atherosclerotic lesions tend to develop (Zarins et al. 1983, Stary 1989). These regions are called atherosclerosis-prone locations. In theory, it is possible that when lipoproteins exceed a certain critical level, the mechanical forces may enhance lipoprotein deposition in these areas leading to the development of an atherosclerotic lesion.

1.2. Definition and classification of atherosclerosis

Atherosclerosis is characterized by the accumulation of lipids and fibrous elements in the large and medium-sized arteries leading to a pathological thickening of the vessel wall and to arterial luminal obstruction. In atherosclerosis, the intima of the muscular and elastic arteries is affected, most commonly of the coronary arteries, aorta, iliac, femoral and cerebral arteries (Ross and Glomset 1973).

Macroscopical, visual examinations of the degree of atherosclerosis originated in the 1960's from the first large-scale autopsy survey by the International Atherosclerosis Project (IAP) that used validated methods and a detailed standard operating protocol to evaluate and quantify arterial lesions (Guzman et al. 1968). Atherosclerotic lesions were stained with Sudan IV and graded visually as fatty streaks, fibrous plaques and complicated lesions. The risk factor information was collected retrospectively from clinical records or autopsy protocols. 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. The classification was based on microscopical 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) (Table 1).

Table 1. Classification of atherosclerotic lesions with corresponding findings in carotid ultrasonography

Histological classification (Stary et al. 1994, Stary et al. 1995)	Macroscopic classification by the International Atherosclerosis Project (IAP) (Guzman et al. 1968)	Appearance in carotid B-mode ultrasonography (Salonen and Salonen 1993)
Intimal thickening	Usually not visible	Intima-media thickening
Early lesions		
Type I	Initial lesions, usually not visible	Intima-media thickening
Type II (a, b)	Fatty streak, usually visible with Sudan stain	Intima-media thickening
Intermediate lesions	Preatheroma	Intima-media thickening/
Type III	Fatty streak or fibrous plaque	soft plaque
Advanced lesions		
Type IV	Fibrous plaque (atheroma)	Soft plaque*
Type Va (V)	Fibrous plaque (fibroatheroma)	Soft plaque*
Type Vb (VII)	Calcified lesion	Hard plaque
Type Vc (VIII)	Fibrous plaque	Soft plaque*
Type VI (a, b, c)	Complicated lesion (surface disruption, hematoma or hemorrhage, thrombosis)	Soft plaque*

*If no mineralization.

Type I (initial lesion). Type I and II lesions are generally found in infants and children although they occur in adults also. The histological change is minimal and consists of isolated groups of single macrophages distended with lipid droplets (Stary 1994). There is also an increased number of macrophages without lipid droplets. Moreover, SMCs lack lipid inclusions (Stary 1987).

Type II (fatty streak). Fatty streaks stain red with Sudan III and Sudan IV and may also be visible as yellow-colored streaks or spots (Stary et al. 1994). They are

composed of more lipid-laden foam cells, including some SMCs, than initial lesions (Stary 1990). Isolated lymphocytes and mast cells may be found, but both are less numerous than macrophages which tend to form adjacent layers and accumulate in the lower part of the proteoglycan layer (Katsuda et al. 1992). At this point, most of the electron microscopically visible lipid is within cells.

In the case of average levels of atherogenic lipoproteins, a subgroup of fatty streaks, type IIa lesions, will probably proceed to advanced lesions and are thus called progression-prone lesions. A larger subgroup of type II lesions that does not progress, or progresses only slowly, is called progression-resistant or type IIb lesions. Type IIa lesions contain more lipid, macrophages, mast cells and probably more lymphocytes than type IIb lesions (Stary 1994).

Type III (preatheroma). Type III lesions, or "intermediate lesions", may evolve soon after puberty and they constitute a link between IIa lesions and the first type of advanced lesion, type IV. Preatheromas have microscopically visible accumulations of particles of lipids and cell debris. The lipid pools replace intracellular matrix and drive SMCs apart. However, proliferation of SMCs or a massive accumulation of extracellular lipid has not yet developed (Stary et al. 1994).

Type IV (atheroma). This lesion type is relatively frequent from the third decade of life and is characterized by an aggregate of extracellular lipid often called a lipid core. Extracellular particles and droplets have damaged and disorganized the intima by displacing structural SMCs, and SMCs have changed their morphology. The layer above the lipid core represents preexisting adaptive thickening that includes macrophages, foam cells, SMCs, lymphocytes, plasma cells, mast cells and capillaries, while the periphery (shoulder) of the atheroma contains more proteoglycan matrix and foam cells and is more susceptible to fissuring. At this stage, the arterial lumen is not much reduced and changes may not be visible by angiography (Stary et al. 1995).

Type V (fibroatheroma). Type V lesions begin to develop from the fourth decade of life. A fibroatheroma is formed when layers of newly proliferated SMCs and thick layers of collagen are added and there is a change in the nature of the region between the lipid core and the EC layer. This area is named the "fibrous cap" and it increases the thickness of the arterial wall. Type Va lesions (fibroatheroma) may also be multilayered with several lipid cores separated by layers of fibrous connective tissue

(Glagov et al. 1988). The numerals Vb and Vc are used to denote morphological variants that may follow or precede type VI lesions. Classification into type Vb (VII, calcified lesion) is appropriate when mineralization of the lesion dominates the picture (Stary 1992). Type Vc lesions (VIII, fibrotic lesion) consist entirely or almost entirely of dense collagen and the lipid component is minimal or absent (Stary 1994).

Type VI (complicated lesion). Type VI lesions are divided into VIa (fissure), VIb (hematoma or hemorrhage) and VIc (thrombus) according to the dominant change (Stary et al. 1995). Shearing fissures of the lesion surface is thought to be the underlying cause of massive hemorrhage into the lesion, thrombotic deposits and lesion expansion that can lead to symptomatic disease (Davies and Thomas 1985) and unstable angina (Fuster et al. 1992). The type VI lesion is not necessarily greater in thickness than type V and if the lesion is completely filled with collagen, it is again relabeled type V.

1.3. Development of early atherosclerotic lesions

The early events in atherogenesis involve primarily altered endothelial function, recruitment and accumulation of leukocytes and LDL particles into the intima, and modifications of the LDL particles. As a consequence, these events lead to the formation of foam cells and to the development of fatty streak lesions (Geer 1965).

1.3.1. Endothelial function in normal and pathological conditions

The luminal surface of a normal healthy artery is covered with a monolayer of ECs that have several important physiological functions (Jaffe 1987). The endothelium serves as a selective permeability barrier between intra- and extravascular compartments by not only limiting the efflux of large molecules into the intima but also by exchanging substances between the plasma and the subendothelial space (Ross 1993). ECs provide a nontrombogenic link for the cardiovascular system by releasing inhibitors of platelet aggregation (Sage et al. 1981, Fogelman et al. 1988). In normal physiological conditions, ECs secrete growth factors and cytokines that inhibit SMC migration and proliferation (Consigny 1995). Moreover, ECs take part in maintaining vascular tone by releasing small molecules, such as nitric oxide, that modulate vasodilatation and

vasoconstriction (Vanhoutte 1987). ECs are also able to change their gene expression in response to mechanical forces generated by pulsatile blood flow (Davies and Tripathi 1993).

The earliest event in fatty streak formation in the intima probably results from endothelial injury or dysfunction under pathological conditions (Virchow 1856 in Ross 1993, Ross 1986). Factors that can promote such injury include several cardiovascular risk factors (Libby 1987, Vane et al. 1990) such as hypercholesterolemia, and especially an increased concentration of ox-LDL, free radicals, increased blood pressure or shear stress, viral or bacterial infections, smoking, local cytokines and proteases, homocysteine, diabetes and hypoxia. As a response to injury, endothelium-dependent vasodilatation is impaired and ECs are activated to produce growth promoters and cytokines (Luscher and Noll 1994). Injury increases the permeability of ECs and enhances the penetration of LDL and other large particles into the subendothelium (Goldstein and Brown 1977). Also, as a result of injury, the balance is shifted toward thrombosis which increases the adherence of platelets and monocytes to the endothelium (Ross and Glomset 1976).

1.3.2. LDL retention and oxidation and antioxidants

Retention. The concentration of LDL in the intima may increase because of increased permeability of ECs and also because of elevated plasma LDL levels (Steinberg and Witztum 1990). The retention of LDL in the vessel wall seems to involve interactions between the LDL constituent apolipoprotein (apo) B-100 and negatively charged matrix proteoglycans (Boren et al. 1998). This increases the probability that LDL will undergo a series of changes in several ways: First, LDL particles can aggregate and form larger lipid droplets (Khoo et al. 1988, Öörni et al. 2000). Second, complexes of proteoglycan with LDL can be formed (Schwenke and Carew 1989) and, third, there is formation of immune complexes of modified LDL with autoantibodies (Klimov et al. 1988). Finally, LDL can be modified by oxidation (Witztum and Steinberg 1991).

Oxidation. LDL can be modified by oxidiation when it passes through the endothelium and the oxidation process can continue because those antioxidant components present in whole plasma or in extracellular fluid do no entirely protect LDL

from oxidation in the microenvironment of the intima (Henriksen et al. 1981). Studies on cell culture have identified many cell types capable of oxidizing LDL including ECs, SMCs, monocytes, macrophages, fibroblasts and neutrophils. A key event, however, is macrophage-mediated oxidation (Aviram and Fuhrman 1998). Several enzyme systems of these cells can play a role in the oxidation, including NADPH oxidase, 15-lipoxygenase (Kuhn et al. 1994), myeloperoxidase (Leeuwenburgh et al. 1997) and the mitochondrial electron transport system. Macrophages also generate proteases and lipases that can degrade protein and lipid moieties of LDL (Aviram and Fuhrman 1998). The interaction of LDL with macrophages under oxidative stress activates cellular oxygenases, which can then produce reactive oxygen species capable of oxidizing lipoprotein particles (Parthasarathy et al. 1986). Also, exposure of LDL to iron, copper or other oxidants can lead to the generation of metabolites of lipid peroxidation (Witztum and Steinberg 1991). Such modifications can first give rise to "minimally modified" LDL (mm-LDL) where the lipid part of the particle is affected but the apolipoprotein part is still quite intact (Berliner et al. 1995, Hajjar and Haberland 1997). Mm-LDL bears a cargo of biologically active products of lipid peroxidation, most important of which are the oxidized fatty acids of phospholipid fraction (Watson et al. 1997). The polyunsaturated fatty acids in cholesteryl esters (CEs), phospholipids and triglycerides are all subjected to free radical initiated oxidation and can participate in chain reactions that amplify the extent of the damage. The polyunsaturated fatty acids are also broken down to yield a broad array of smaller fragments, including aldehydes and ketones that can be conjugated to other lipids or to apo B (Esterbauer et al. 1987). If oxidation and degradation continues, the protein portion of LDL is also modified (Berlett and Stadtman 1997) leading to a loss of recognition by the LDL receptor, and to the formation of extensively modified i.e., "highly oxidized" LDL that is cytotoxic to artery wall cells (Hessler et al. 1979). Both the lipid and the protein part can thus be oxidized and, obviously, there is a continuous spectrum of degrees of oxidation and a great deal of molecular heterogeneity in what we call "oxidized LDL".

Antioxidants. Antioxidants which are bound to LDL (vitamin E, carotenoids and flavonoids), or those present in the plasma environment (ascorbate and flavonoids) can inhibit LDL oxidation mainly by scavenging free radicals (Aviram 1999b). Antioxidants, which accumulate in arterial cells, including macrophages, can also block

cell-mediated oxidation of LDL (Halliwell 1996). In addition, cellular enzymatic antioxidants, such as the glutathione system and reduced glutathione, superoxide dismutase and catalase, play an important protective role against cell-mediated oxidation of LDL (Aviram 1996).

Since there could be a negative correlation between coronary artery disease (CAD) and antioxidant status, considerable interest has focused on vitamin E, ascorbate, β-carotene and the polyphenols of red wine as inhibitors of oxidation (Steinberg and Chait 1998). It appears, however, that a rather high threshold of antioxidant effect must be reached before any antiatherosclerotic effect is evident *in vivo* (Sasahara et al. 1994). In agreement with this is the clinical claim that even 5-year clinical study may be insufficient to show the protective effects of some antioxidants (Steinberg 1997, Heart Protection Study Collaborative Group 2002). This could be related to the complex nature of some antioxidants, such as vitamin E. Although vitamin E has attracted most attention as an anti-atherosclerotic factor, it can also exert a pro-oxidant activity (Bowry et al. 1992).

1.3.3. Foam cell formation

The oxidation of LDL is important for the formation of fatty streaks for several reasons (Ross 1993). Modified LDL itself attracts circulating monocytes (Quinn et al. 1987). It attracts monocytes also by stimulating the release of monocyte chemotactic factor-1 and monocyte colony stimulating factor-1 from ECs (Cushing et al. 1990). In addition to monocytes, T lymphocytes tend to accumulate in early human atherosclerotic lesions because ECs express leukocyte adhesion molecules, such as intracellular adhesion molecule-1, vascular cell adhesion molecule-1 and selectins (Berliner et al. 1990). Especially mm-LDL stimulates the overlying endothelium to produce these pro-inflammatory molecules and is thus immunogenic (Salonen et al. 1992). Modified LDL inhibits also the motility of tissue macrophages from the lesion and enables cells to take up large amounts of lipids (Henriksen et al. 1982). Moreover, ox-LDL is cytotoxic (Hessler et al. 1983), it inhibits vasodilatation that is normally induced by nitric oxide (Kugiyama et al. 1990) and it is mitogenic for macrophages and SMCs (Yui et al. 1993).

Once recruited into the intima, monocytes undergo a series of phenotypic alterations and turn into macrophages (Fogelman et al. 1981) which can internalize large amounts of ox-LDL and become lipid-laden foam cells. The rapid uptake of ox-LDL particles by macrophages is mediated by a group of scavenger receptors that recognizes a wide array of ligands (Goldstein et al. 1979, Pearson et al. 1995). Expression of scavenger receptors is not down-regulated by cholesterol, and therefore macrophages can internalize substantial quantities of CE (Witztum and Steinberg 1991). This is precisely opposite to the normal delivery of cholesterol to vascular cells, which is mainly mediated by the ubiquitous LDL receptor (Sudhof et al. 1985) and apo E receptor (Brown and Goldstein 1983). Regulation of these receptors occurs primarily at the transcriptional level and is controlled by the levels of free cholesterol in the cell (Brown and Goldstein 1986). Fatty streaks composed primarily of macrophages are likely to be reversible and the development of fatty streaks further to more advanced types of lesions depends on the total risk factor load of the individual. The fatty streak is thus crucial for the development of clinical atherosclerosis - the fatty streak is silent itself, but it is the precursor of more complex lesions which can cause stenosis and limited blood flow (Quinn et al. 1988).

1.4. Development of advanced atherosclerotic lesions

The conversion of the fatty streak into a fibrotic lesion involves accumulation of more SMCs (Stary et al. 1995) which finally become the dominant cell type (Burk and Ross 1979). The fibrotic lesion is characterized by a fibrotic cap composed of SMCs recruited from both the subendothelium and the media and by extracellular matrix that accumulates in the atheroma as a result of SMC secretion. In the proliferation and migration of SMCs, the generation of growth factors and chemotactic factors plays an important role. One of these factors is platelet-derived growth factor, a chemoattractant released from adherent platelets at sites of endothelial loss and from activated ECs, macrophages and SMCs (Ross 1999). Platelet-derived growth factor acts in conjunction with other mitogens to stimulate SMCs to convert from a contractile to a noncontractile, synthetic cell type (Campbell et al. 1988). After this phenotypic change, SMCs can migrate from the media to the intima and undergo mitosis. The accumulation of SMCs

is fundamental to the entire process because the lipid deposits occur either within or outside the SMCs in association with the secretory products of the SMCs (Ross and Glomset 1973).

In advanced lesions, the deeper layers of the plaque become ultimately necrotic, presumably because of the buildup of toxic extracellular lipids and radicals, ischemia and cell death (Ross 1999). Cell death may occur in response to inflammatory cytokines, and T cells may participate in the process by eliminating some SMCs. Since SMCs are the source of the newly synthesized collagen needed to repair and maintain the matrix of the fibrous cap, the lack of these cells may contribute to weakening of the fibrous cap and, hence, increase the propensity of the plaque to rupture (Libby 1995). During the first part of the life span of a lesion, the growth of the plaque is outward and will not lead to luminal stenosis. After the capacity of the artery to remodel outward is exceeded, encroachment on the arterial lumen begins. This will eventually lead to stenosis and impeded blood flow (Yokoya et al. 1999).

The final stage in the development of an atherosclerotic lesion is the conversion of the fibrotic lesion into a complicated type of lesion. A considerable portion of acute ischemic events is caused by plaque complications, such as plaque ulceration, disruption, hemorrhage and, finally, thrombosis (Fuster et al. 1992). Plaque instability and the development of thrombus-mediated acute coronary events depends principally on the composition and vulnerability of a plaque rather than on the severity of stenosis (Ambrose et al. 1986). Vulnerable plaques tend to have thin fibrous caps, large lipid cores and an increased number of inflammatory cells. Rupture usually occurs at the foam cell-rich lesion edges by turbulence or shear stress or by the release of matrix-degrading enzymes and toxic products of lipid peroxidation (Falk 1992). After plaque rupture, platelets aggregate, the coagulation cascade is activated and a thrombus is formed. In addition to thrombosis, coronary vasoconstriction may occur which further worsens the symptoms of the ischemic heart disease (Maseri et al. 1978). Once formed, a thrombus may undergo one of several processes. In most cases, it undergoes dissolution and does not give rise to clinically apparent coronary events. In some cases thrombus may be incorporated into the lesion resulting in lesion enlargement which may or may not give symptoms of unstable angina. Clinical symptoms, including

cerebral infarction, may also result if the thrombus is sloughed off and travels as an embolus to a distal vessel (Fuster et al. 1992).

2. HDL and atherosclerosis

2.1. HDL metabolism

Nascent HDL and its major apoprotein, apo AI, are synthesized in the liver and small intestine. The second most abundant apoprotein of HDL, apo AII, is produced by the liver, as is the case for apo C and apo E, which are transferred to intestinal HDL. Once entered into circulation, HDL turns into small, dense complexes, consisting of about 50% protein and 50% lipid. A lipid core of spherical HDL consists of nonpolar triglycerides and esterified cholesterol surrounded by a surface layer of amphipathic phospholipids and unesterified cholesterol as well as apolipoproteins (Eisenberg 1984). HDL particles are subclassified according to their size and lipid content into HDL_1, HDL_2, HDL_3 and HDL_4. Two of these classes, the larger, more lipid-rich HDL_2 and the smaller, more dense HDL_3 predominate in human plasma (von Eckardstein et al. 1994).

HDL precursors can be formed also by dissociation from large, triglyceride-transporting lipoproteins, namely chylomicrons and very low-density lipoproteins (VLDL) (Musliner et al. 1991). This occurs during lipolysis, when lipoprotein lipase in extrahepatic tissues transfers surface lipids and proteins of chylomicrons and VLDL into the HDL fraction. The dissociating nascent HDL can be incorporated into pre-existing HDL particles to generate mature, lipid-rich and spherical HDL or they can obtain more phospholipids and unesterified cholesterol from cells or apo B-containing lipoproteins by the HDL-associated enzyme, lecithin-cholesterol acyl transferase (LCAT). LCAT is activated by apo AI and it uses phospholipids and cholesterol to generate CEs and lysophospholipid. In the circulation, two HDL-associated transfer proteins facilitate the movement of lipids between HDL and other lipoproteins. Cholesterol ester transfer protein mediates all neutral lipid transfer in human plasma, including the exchange CE of HDL with triglycerides of VLDL and chylomicrons, resulting in CE depletion and triglyceride enrichment of HDL. Phospholipid transfer

protein, in turn, is responsible for the net movement of phospholipids between lipoproteins and it modulates the size changes of HDL (Huuskonen et al. 2001).

Since the liver and steroidogenic organs need CE for the synthesis of lipoproteins, bile acids, vitamin D and steroid hormones, the CE in the HDL particles has to be transported to these sites. First, CE, in addition to phospholipids, can be taken up by the liver through the action of hepatic lipase. When HDL is increased in size and enriched with additional apoproteins, HDL (now labeled HDL_2) is converted to HDL_3 by hepatic lipase. Hepatic lipase has both triglyceride hydrolase and phospholipase activity, resulting in depletion of HDL triglycerides and phospholipids that are taken up by the liver, and a decrease in HDL size. Second, CE can be moved from HDL to apo B-100 containing lipoproteins and then returned to the liver from these particles via the LDL receptor pathway (Tall et al. 2000). Third, catabolism of HDL can be mediated by direct uptake of HDL particles by the liver via scavenger receptor class B type I (Williams et al. 1999). Moreover, apo E receptors, and possibly also other yet unknown HDL receptors, can facilitate the uptake of HDL (von Eckardstein et al. 2001). The liver and possibly the intestine seem to be the final sites of degradation of HDL (Tall and Breslow 1996). Apo AI, after dissociation from HDL, is removed by the kidneys.

2.2. HDL as an antiatherogenic particle

The first large-scale study that suggested the protective effect of HDL against CAD was the Honolulu Heart Program study (Rhoads et al. 1976), although similar suggestions had been presented already in 1950's by the Finnish investigator Esko Nikkilä. Numerous prospective epidemiological investigations have since then shown a significant inverse relationship between HDL cholesterol level and the incidence of CAD (Gordon et al. 1989). The apo AI level that is highly correlated with the HDL cholesterol level predicts the CAD risk equally well (Stampfer et al. 1991). There are several theories that have attempted to explain the inverse relationship between HDL level and atherogenesis:

Reverse cholesterol transport theory. Reverse cholesterol transport means the movement of cholesterol from nonhepatic cells to the liver and steroidogenic organs via the plasma compartment (Genest et al. 1999). It begins when the apolipoproteins or

HDL particles leave the plasma and enter the extravascular space where they serve as acceptors of cellular lipids (von Eckardstein et al. 1994). HDL removes unesterified cholesterol from the cell membranes of macrophages, which stimulates the net hydrolysis of CE in these cells. In general, particles with apo AI are the most effective stimulators of cellular cholesterol efflux (Mahlberg and Rothblat 1992) and apo AI itself can mediate cholesterol efflux from macrophages most likely by forming HDL-like particles. In addition to apo AI, the adenosine triphosphate-binding cassette transporter-1 appears to be essential for this process (Young and Fielding 1999). Once entering into HDL, free cholesterol is converted to CE by LCAT, which thereby provides a driving force for the net movement of cholesterol from cells into HDL. "Mature HDL" travels in the extracellular fluid to the lymphatic circulation from where it finally returns into the blood circulation. According to the hypothesis, reverse cholesterol transport also mediates the removal of cholesterol from atheromas. The level of plasma HDL determines the efficiency of this process.

Atherogenic remnant theory. This theory suggests that HDL is merely a metabolic marker of a defect in the metabolism of triglyceride-rich lipoproteins. According to this theory, a low HDL level might reflect the accumulation of chylomicron or VLDL remnants in plasma. The concentration of chylomicron or VLDL remnants might be increased through overproduction, inefficient lipolytic processing, including inefficient lipolytic transfer of lipids into HDL, or defective receptor-mediated clearance. Remnants would enter the artery wall and contribute atheroma foam cell formation (Tall and Breslow 1996).

Antioxidative theory. HDL can prevent the *in vitro* oxidative modification of LDL caused both by transition metal ions and by cells (Mackness and Durrington 1995). This effect can not fully be explained by chain-breaking antioxidants present in HDL. Instead, there are several other mechanisms by which HDL can inhibit either the formation or the atherogenic properties of ox-LDL. The first of these includes an exchange of lipid peroxidation products between HDL and LDL (Parhasarathy et al. 1990) and conversion of CE hydroperoxides to stable CE hydroxides (Sattler et al. 1995). Second, apo AI and possibly also HDL-associated transferrin and ceruloplasmin can bind oxidation-promoting transition metals (Kunitake et al. 1992). Third, HDL possesses anti-inflammatory properties (Bonnefont-Rousselot et al. 1999) since it

inhibits the cytokine-induced expression of adhesion molecules by ECs and LDL-induced monocyte transmigration. It also in part induces the synthesis of prostacyclin, which is an inhibitor of platelet and leukocyte functions and an efficient vasodilator. Finally, the enzymatic mechanisms present in HDL can inhibit the oxidation of lipids (Mackness and Durrington 1995). Several enzymes including PON1, LCAT, platelet-activating factor acetylhydrolase (PAF-AH) and phospholipase D are present on HDL. Of these enzymes, PON1 and PAF-AH have been reported to detoxify oxidized phospholipids *in vitro* (Watson et al. 1995a, Watson et al. 1995b). LCAT may inhibit LDL oxidation (Vohl et al. 1999) by participating in the hydrolysis of polar pro-inflammatory phospholipids into lysophospholipids (Goyal et al. 1997).

3. Paraoxonase-1 (PON1) protein

3.1. Synthesis and structure

Paraoxonases are widely distributed among various animal species (La Du et al. 1993, La Du 1996, Nevin et al. 1996), except fish, birds and insects (Mackness et al. 1996). In humans, serum paraoxonase activity is present in newborn and premature infants at a level about half of that found of adults but the adult levels are reached quickly, approximately 1 year after birth (Mueller et al. 1983). Comparison of the rabbit and human sequences indicates 85% similarity at both the protein and deoxyribonucleic acid (DNA) levels (Hassett et al. 1991) suggesting an important metabolic role of this protein. In humans, PON1 is expressed mainly in the liver, although found also in kidneys, heart, brain, small intestine and lungs (Primo-Parmo et al. 1996). PON1 accumulates in the artery wall ECs and SMCs during the progression of atherosclerosis, but it is not yet clear whether there is uptake of PON1 by these cells or whether they can synthesize this enzyme (Mackness et al. 1997a). An interesting feature in the synthesis of PON1 is that it retains its signal sequence and only the initiator methionine is cleaved (Furlong et al. 1993). The hydrophobic leader-sequence possibly facilities the interaction of PON1 with the HDL particle. Several authors report that PON1 is associated with an α_2-migrating, apo AI and apo J-containing HDL subpopulation (Kelso et al. 1994), although it is possible that a number of PON1-containing HDL

subspecies, each of which has its own, unique apolipoprotein content, co-exist in the plasma (Laplaud et al. 1998).

Human serum PON1 has been purified (Gan et al. 1991). It has a molecular weight of about 44 kDa and is a highly glycosylated protein, which consists of 355 amino acids (Furlong et al. 1993). The 3D-structure of mammalian PON1 has not yet been characterized and also the active site residues need still to be identified. Human PON1 contains three cysteine residues and it is present in two oxidation states, which differ by the presence or absence of an intramolecular disulfide bond between two of these cysteines. The only free cysteine at position 283 is therefore a strong candidate for the active center nucleophile (Sorenson et al. 1995).

3.2. Activity and concentration

Esterases. Esterases represent a large and heterogeneous group of enzymes, which hydrolyze aliphatic and aromatic ester bonds, but also peptides, amides and halides (Walker and Mackness 1983). Augustinsson separated esterases into three main types, namely arylesterases (including PON1), aliesterases and cholinesterases, based on their hydrolysis of simple substrates (Walker and Mackness 1983). Aldridge, on the other hand, presented a classification based on the interaction between esterases and organophosphates (OPs). According to this classification, A-esterases, i.e., paraoxonases, can hydrolyze organophosphates, B-esterases are inhibited by them and C-esterases (added some time later) do not interact with them (Walker and Mackness 1983).

Many synthetic chemicals, including insecticides (OPs, carbamate and pyrethroid groups), are A-esters. These chemicals are used as pesticides, since A-esterases (arylesterases) are absent from the serum of lower vertebrates. Pesticides are widely used in agriculture as relatively nontoxic sulphur derivatives; cytochrome P_{450}-dependent microsomal mono-oxygenases activate them *in vivo* to their toxic oxygen analogues. Hydrolysis of the OPs by serum and hepatic A-esterases to less toxic metabolites is the most important route for their detoxification (Williams 1987). This has been shown in rodent model system, where injection of PON1 protects against OP

poisoning (Costa et al. 1990, Li et al. 1995). PON1-knockout mice are also susceptible to OP toxicity (Shih et al. 1998).

Measurement of activity. PON1 has two qualitatively different properties, paraoxonase and arylesterase activities, because it hydrolyzes both OPs and aromatic esters (Mackness et al. 1987a). Paraoxonase activity is measured from serum samples by hydrolysis of paraoxon, an active metabolite of the insecticide called parathion, to the nontoxic products, p-nitrophenol and diethyl phosphate (Figure 1) (Aldridge 1953, Erdös and Boggs 1961). Paraoxonase activity is first measured without any added NaCl and then with 1 M NaCl included. The percent stimulation of paraoxonase by 1 M NaCl is used to classify individuals into the non-salt-stimulated A type, and salt-stimulated AB and B types (Eckerson et al. 1983a), since measurement of paraoxonase activity alone does not clearly discriminate between AB and B phenotypes (Flugel and Geldmacher-von Mallinckrodt 1973). The paraoxonase activity has to be measured at pH 7.5 (Eiberg and Mohr 1981), since at higher pH areas the activity will be the sum of the albumin-associated esterase activity (Erdös and Boggs 1961) and the paraoxonase activity (Ortigoza-Ferado et al. 1984).

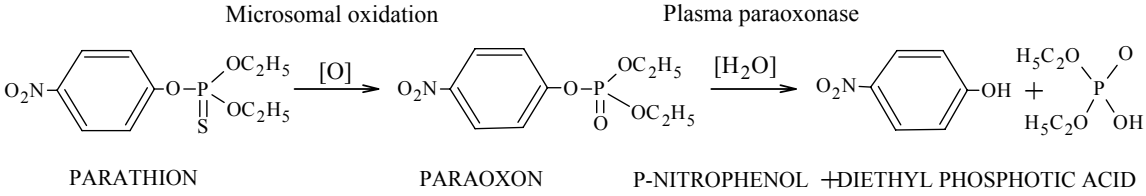

Figure 1. Metabolic activation and detoxification of parathion (modified from Mueller et al. 1983).

The arylesterase activity is usually measured with phenylacetate as a substrate (Mounter and Whittaker 1953, Simpson 1971). The distribution of arylesterase activity in a Caucasian population has a single mode (Simpson 1971) while the paraoxonase activity is bimodally distributed. The distribution of the ratio of salt-stimulated paraoxonase activity to arylesterase activity, however, is clearly trimodal indicating the existence of three paraoxonase phenotypes (Eckerson et al. 1983b).

In addition to the ability of PON1 to hydrolyze paraoxon and phenylacetate, it also hydrolyzes other similar OP compounds, aromatic carboxylic acid esters and

carbamates (La Du 1992). This includes the resulting toxic oxon forms of chlorpyrifos and diazinon, and the nerve agents soman and sarin (Smolen et al. 1991). More recently, the hydrolytic activity of PON1 on 30 lactones, thiolactones and cyclic carbonate esters has been characterized (Billecke et al. 2000, Draganov et al. 2000, Jakubowski 2000), and it has been hypothesized that lactonase activity rather than arylesterase or organophosphatase activity could be a common feature of the PON proteins (Draganov et al. 2000). Despite the fact that humans can detoxify, e.g., diazinon better than paraoxon (Davies et al. 1996), it is common convention to use the term "paraoxonase activity" if the substrate has been paraoxon, and "arylesterase activity" if phenylacetate has been the substrate.

Measurement of concentration. The serum PON1 concentration can be determined by a competitive enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody (Blatter Garin et al. 1994) or by sandwich ELISA using two monoclonal antibodies (Kujiraoka et al. 2000).

3.3. Role in oxidation of lipids

Oxidation of phospholipids in plasma lipoproteins produces a spectrum of phosphatidylcholine (PC) derivatives including hydroperoxides, isoprostanes and core aldehydes (Ahmed et al. 2002) which are inactivated through enzymatic hydrolysis (Watson et al. 1995b). PON1 metabolizes oxidized arachidonic acid derivatives in the Sn-2 position of LDL phospholipids and currently the strongest candidates for the substrates of PON1 are multi-oxidized, long-chain phospholipids (Watson et al. 1995a) and CEs (Aviram et al. 1998c). PON1 and PAF-AH may work in tandem; PAF-AH could be a "second line of defense" that hydrolyzes those lipid peroxides, such as 5-oxyvalerate, that have escaped the action of PON1 (Figure 2). Both enzymes, or only PON1, are probably needed to maintain the antioxidant effects of HDL, since transgenic mice lacking PON1 but with normal murine PAF-AH levels are ineffective in preventing LDL oxidation (Castellani et al. 1997).

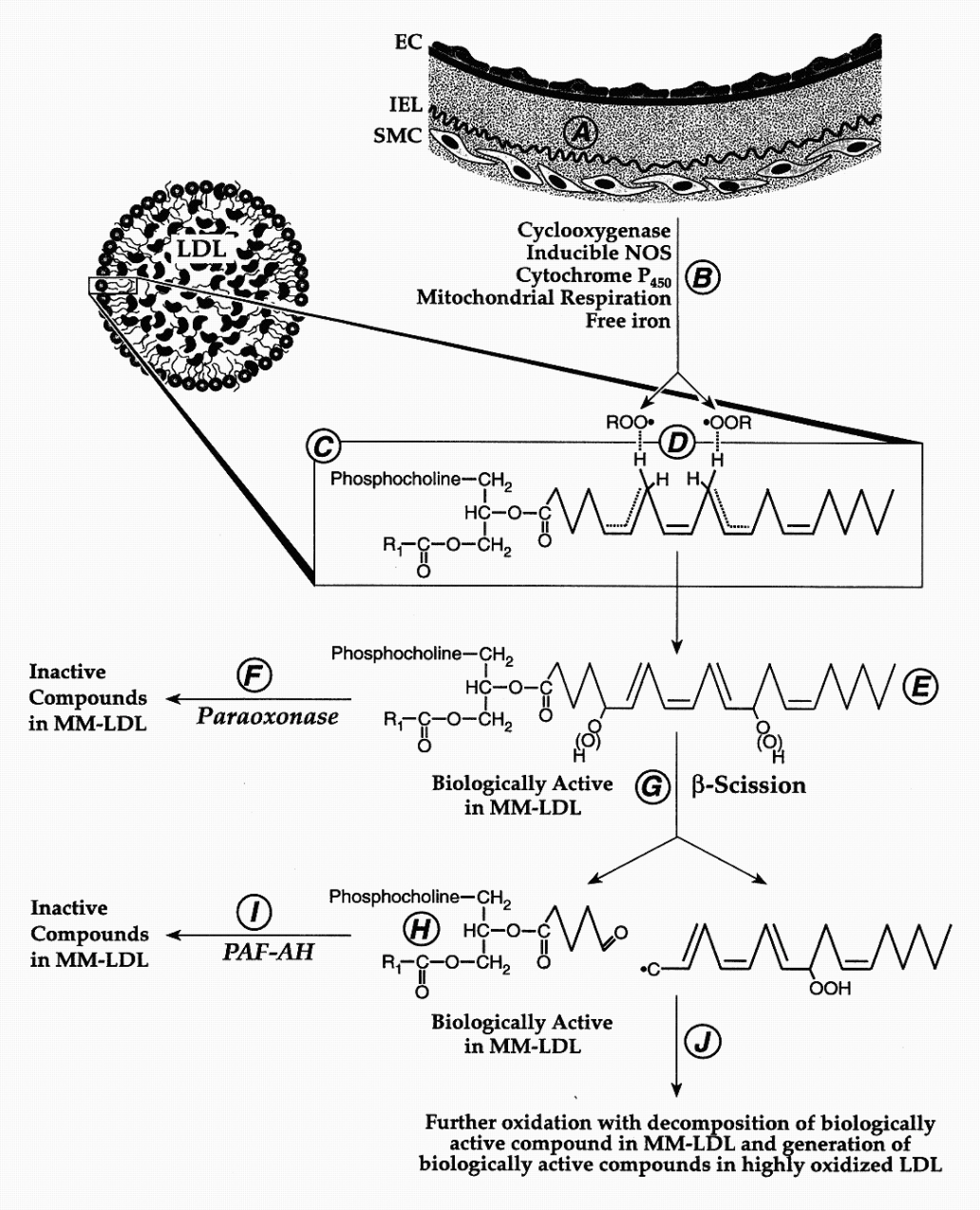

Figure 2. Hypothesis to explain how HDL-bound paraoxonase-1 (PON1) and platelet-activating factor acetylhydrolase (PAF-AH) could destroy biologically active lipids in minimally modified LDL (mm-LDL). (A) Reactive oxygen species may be formed by a variety of mechanisms (B). Oxygen radicals may oxidize phospholipids in LDL (C), e.g., by abstraction of their hydrogen atoms (D). Addition of molecular oxygen may generate multi-oxygenated phospholipids (E), which may be substrates for PON1 (F). In cases where PON1 activity is low or lipid peroxide levels are excessive, oxidized phospholipids may undergo oxidative fragmentation (G) to form particles (H) that may serve substrates for PAF-AH (I). Further oxidative decomposition of lipids in LDL leads to the deposition of highly oxidized LDL (J). ABBREVIATIONS: EC, endothelial cells; IEL, internal elastic lamina; NOS, nitric oxide synthase; SMC, smooth muscle cells (modified from Watson et al. 1995a).

A recent study suggest that also apo AI and PON1 form a coupled system that converts PC fatty acid hydroperoxides to lyso-PC (Ahmed et al. 2002). PON1 is thus perhaps also capable of minimizing the accumulation of PC oxidation products by the hydrolysis of PC isoprostanes and core aldehydes to lyso-PC. PON1 has also been shown to be able to eliminate lipid peroxides and H_2O_2, i.e., to possess peroxidase-like activity, and by this action it could prevent also the initiation of LDL oxidation by oxidants such as copper ion (Aviram et al. 2000b). PON1 can also inhibit LDL-induced migration of monocytes in a vascular cell co-culture system (Castellani et al. 1997), and prevent the oxidation of HDL and preserve its ability to induce cellular cholesterol efflux from macrophages (Aviram et al. 1998c). Taken together, PON1 both prevents the formation of mm-LDL and inactivates LDL-derived oxidized phospholipids once they are formed (Figure 3).

It has been suggested that there are two active sites in the PON1 protein, one required for arylesterase/paraoxonase activities and the other required for the protection against LDL oxidation (Aviram et al. 1998a). As the active center for the hydrolysis of oxidized lipids is probably the cysteine residue at position 283 (Aviram et al. 1999), the amino acids involved in the hydrolysis of OP compounds may include histidine (Doorn et al. 1999) and tryptophan residues (Josse et al. 1999). Calcium is probably required for PON1 arylesterase/paraoxonase activities, but not for the protection by PON1 against LDL oxidation (Aviram et al. 1998a). It is thus becoming more and more evident that paraoxonase/arylesterase activity is not a quantitative measure of the ability of PON1 to protect against LDL oxidation.

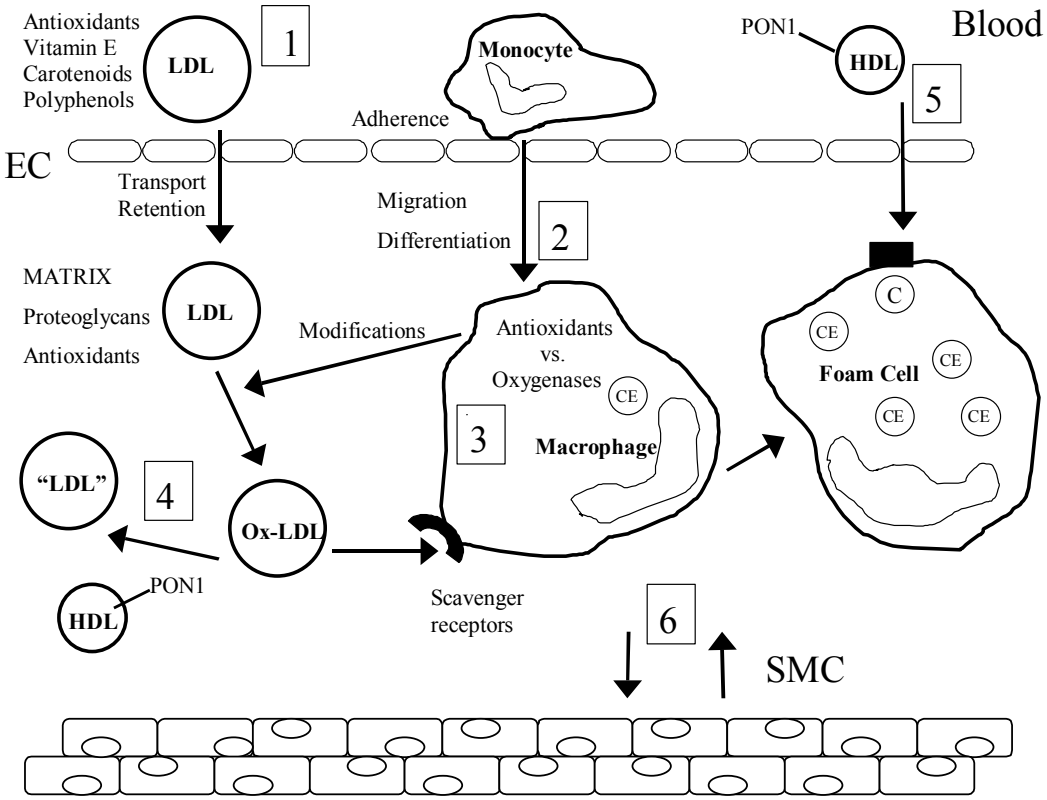

Figure 3. Under oxidative stress, both plasma LDL and monocytes accumulate into the arterial wall and monocytes differentiate into macrophages. LDL binds to extracellular matrix proteoglycans (retention) and can be oxidized, e.g., by macrophages. The extent of LDL oxidation depends on the balance between oxidants and antioxidants and the amount of lipids in the LDL particle (1). In macrophages, the balance between cellular oxygenases and antioxidases determines the extent of LDL oxidation (2). Nutritional antioxidants such as flavonoids are present in LDL particles (1) and in the arterial cells including macrophages (3). The antioxidants in macrophages inhibits cell-mediated oxidation by scavenging of reactive oxygen species and by their effects on cellular oxygenases and antioxidases (3). Finally, if oxidized LDL (ox-LDL) is formed in the arterial wall under excessive oxidative stress, HDL-associated paraoxonase-1 (PON1) can hydrolyze specific lipid peroxides in ox-LDL and convert the atherogenic lipoprotein back to a non-atherogenic ”LDL” (4). Ox-LDL inactivates PON1 and antioxidants can preserve its activity (4). If ox-LDL is taken up to the macrophages, e.g., by scavenger receptors, the macrophages differentiate into foam cells. This process can be reversed by reverse cholesterol transport which is induced by intact HDL (5). PON1 can possibly protect also HDL from oxidation and thus promote cholesterol efflux from foam cells (5). Oxidized lipoproteins, their products and activated cells increase the proliferation, migration and matrix synthesis of smooth muscle cells (SMCs). Oxidized lipoproteins also alter the cytokine and protease synthesis of cells (6). ABBREVIATIONS: CE, cholesteryl ester; C, free cholesterol (modified from Aviram 1999a).

The acute-phase response in animal models and in cardiac surgery in patients reduces the protective effect of HDL and turns it into a pro-inflammatory particle. The appearance of major acute phase reactants and concomitant displacement of HDL-associated PON1, PAF-AH and apo AI appears to play a role in the loss of the protective effect conferred by HDL. Fogelman and coworkers have proposed that HDL is chameleonic (Navab et al. 1996): it changes its associated apoproteins and enzymes in response to environmental changes in the plasma. Moreover, PON1's ability to protect against LDL oxidation is accompanied by inactivation of the enzyme (Aviram et al. 1999) that results from an interaction between the free sulfhydryl group of the enzyme and oxidized lipids. The native phospholipids, however, stabilize PON1 and some antioxidants, like dietary polyphenolic flavonoids from red wine or licorice (Hayek et al. 1997), can preserve PON1 activity, enhance PON1's hydrolytic action on specific oxidized lipids and hence lead to increased PON1 potency against ox-LDL, oxidized HDL (Aviram et al. 1998c) and lipid-peroxidized arterial wall cells (Fuhrman et al. 1997) (Figure 3).

4. *PON1* gene

4.1. PON gene family and structure of PON1

The human genome contains at least three PON genes, designated *PON1, PON2* and *PON3* (Figure 4A). They share ~65% similarity at the amino acid level (Primo-Parmo et al. 1996). All three genes are located on 7q21.3-q22.1 (Primo-Parmo et al. 1996, Mochizuki et al. 1998) and similarities in them strongly suggest that they have arisen by gene duplication. Today, it is known that the coding sequence of human *PON1* is composed of nine exons (Clendenning et al. 1996) and that it has an *Alu* sequence in one intron and a polymorphic CA dinucleotide repeat in the fourth intron (Primo-Parmo et al. 1996) (Figure 4B). Between individuals, there seems to be some variation in the polyadenylation signal of the gene (Hassett et al. 1991). *PON1* contains several polymorphic sites, i.e., two or more variants (alleles) exist at significant frequencies in the population. One specific form of polymorphism is the single nucleotide polymorphism (SNP) defined as a specific difference in one base at a defined location

of an individual's DNA. *PON1* has two SNPs in the coding region of the gene that change the amino acids of the protein: methionine (M) for leucine (L) substitution occurs at position 55 (M/L55) and arginine (R) for glutamine (Q) substitution at position 192 (R/Q192) (Figure 4B). Both of these polymorphisms are easily detectable by restriction endonuclease digestion (Humbert et al. 1993) and can thus be called restriction fragment length polymorphisms (RFLPs). The different allelic forms of PON1 are sometimes called isozymes or allozymes, which underlines the different functional types of the enzyme.

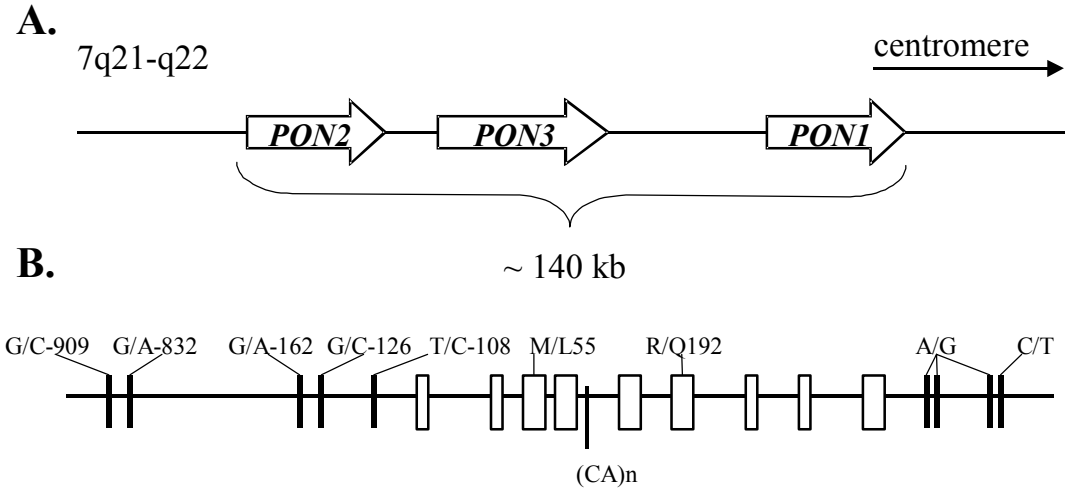

Figure 4. (A) PON gene family and location of *PON1*, *PON2* and *PON3* on human chromosome 7, bands q21-q22. (B) Gene structure of *PON1* (not in scale), where the nine exons are shown as boxes. The figure shows the locations of two coding region polymorphims, five 5' regulatory region polymorphims, four 3' untranslated region polymorphims and the variable-length CA-repeat in the fourth intron (modified from Furlong et al. 2002).

4.2. Coding region R/Q192 and M/L55 polymorphisms

R/Q192 polymorphism. As early as in 1973 it was suggested that the activity of PON1 against paraoxon is genetically determined (Geldmacher-von Mallinckrodt et al. 1973). The existence of three PON1 genotypes, which differed with regard to the activity toward paraoxon, was soon confirmed (Playfer et al. 1976). Isozyme with R at position 192 was later on shown to be responsible for the higher PON1 activity toward paraoxon

(some authors called this a B-type allozyme) and the isozyme with Q at this position for the lower activity (A-type allozyme) (Adkins et al. 1993, Humbert et al. 1993, Primo-Parmo et al. 1996). Since then, a series of studies have showed that within populations of European ancestry there are approximately 50% homozygotes for the low active QQ allozyme, 10% homozygotes for the high active RR allozyme and 40% RQ heterozygotes (Humbert et al. 1993). In some non-Caucasian populations there is a reduced frequency of the low active Q192 allele (Playfer et al. 1976).

M/L55 polymorphism. The second polymorphism in the coding region of the *PON1* is the M/L55 (Humbert et al. 1993). In healthy Finns, like in other Caucasian populations, there are approximately 41% LL homozygotes, 49% ML heterozygotes and 10% MM homozygotes (Salonen et al. 1999). The M/L55 polymorphism affects the activity of PON1 independently of the R/Q192 polymorphism: the allozyme carrying the L55 is a high active against paraoxon whereas the allozyme carrying the M55 is a low active (Garin et al. 1997). Moreover, M/L55 polymorphism modulates serum concentration of the enzyme (Garin et al. 1997), which is related to differences in the expression of the M55 and L55 alleles in the liver (Leviev et al. 1997). The L55 allele is more effectively expressed and, therefore, the carriers of the L55 allele have a higher PON1 concentration in their plasma than the carriers of the M55 allele. It has also been suggested that the M55 isoform is less stable than the L55 isoform (Leviev et al. 2001a). The differences in the PON1 concentration explains partly why there is at least a 40-fold difference in the PON1 activity between individuals, and at least a 13-fold difference within a given genotype class, e.g., in individuals homozygous for the Q192 allele (Furlong et al. 1989). As a consequence of the effects of M/L55 polymorphism both on the concentration and the activity of PON1, the M55 and L55 alleles entail substantial differences in the activity of PON1 not only towards substrates considered discriminatory (paraoxon) but also towards substrates considered non-discriminatory (phenylacetate). In other words, for a "non-polymorphic" substrate such as phenylacetate, simply the amount of the enzyme is important, but for a polymorphic substrate such as paraoxon, both the alleles present and the amount of protein made from each are important.

PON1 haplotypes. The coding region polymorphisms, R/Q192 and M/L55, are in linkage disequilibrium ($p < 0.001$) giving rise to L at position 55 and R at position

192. This favors the simultaneous presence of those alleles associated with "high paraoxonase activity" (Garin et al. 1997). Due to this linkage, PON1 M/L55 and R/Q192 alleles form only three rather than four PON1 haplotypes within the Caucasian population, namely L55/Q192, L55/R192 and M55/Q192, and these haplotypes can be combined in six ways (Cascorbi et al. 1999, Malin et al. 2001).

4.3. Promoter polymorphisms

Five polymorphisms have been identified in the promoter region of the human *PON1* (Leviev and James 2000, Suehiro et al. 2000, Brophy et al. 2001) (Figure 4B). These polymorphisms are frequent in the population and at least three of them, those at positions -108, -162 and -909, have variable impact on promoter activity, with up to 2-fold differences in gene expression (Brophy et al. 2001). The thymidine for cytosine change at position -108 (T/C-108) appears to have a dominant effect on the expression of *PON1* and, therefore, on the concentration of the enzyme. This position is also a possible transcription activation factor binding site. Interestingly, there is strong linkage disequilibrium between the promoter region polymorphisms at positions -108, -162 and -909 and coding region M/L55 polymorphism, although these promoter polymorphisms do not entirely account for the observed association between the M/L55 polymorphism and PON1 concentration (Leviev et al. 2001a).

5. PON1, lipoproteins and atherosclerosis

5.1. PON1 and levels of plasma lipoproteins

HDL concentration as a determinant of PON1 levels. It is poorly known which factors, in addition to PON1 M/L55 polymorphism and promoter polymorphisms, determine the PON1 protein level in plasma. The tight association between PON1 and HDL suggests that HDL and apo AI concentrations could be strong candidates in this respect (Nevin et al. 1996). Low serum PON1 levels occur when HDL concentrations are profoundly low, as in fish-eye disease and Tangier disease (Mackness et al. 1987b, James et al. 1998). The coincident increases or decreases in concentrations of HDL cholesterol and apo AI

and PON1 activity are, however, not a general phenomenon. When serum HDL levels are only moderately decreased, as in type 2 diabetes or familial hypercholesterolemia, the concentration of PON1 is not totally related to changes in HDL (Mackness et al. 1991). The PON1 level is also significantly reduced within two hours after the onset of myocardial infarction (MI) and in CHD patients compared to controls (Ayub et al. 1999, Mackness et al. 2001); this results in decreased PON1 activity. This occurs despite similar HDL concentrations in patients and controls. Moreover, fibric acid derivatives can increase the serum HDL cholesterol concentration by 10% without affecting serum PON1 activity (Durrington et al. 1998).

PON1 as a determinant of serum lipid concentrations. There have been suggestions that PON1 itself could affect serum lipid levels (Mackness et al. 1991, Saha et al. 1991, Blatter et al. 1993, Pavkovic et al. 1993), including apo AI and HDL cholesterol (Boman 1980, La Du and Novais 1989, Abbott et al. 1995). In the studies on the genetic variants of PON1, the R/Q192 genotype has been found to associate with most plasma lipoprotein levels in some isolated populations of non-European origin (Hegele et al. 1995a, Hegele et al. 1995b, Hegele et al. 1997). In these studies, the QQ homozygotes have generally had a more beneficial plasma lipoprotein profile, with lower levels of apo B-related plasma lipoproteins than the carriers of the R192 allele. In contrast, in a sample of European type 2 diabetes patients, the RR and RQ genotype carriers had higher HDL and apo AI concentrations than the QQ genotype carriers but, paradoxically, also increased risk for CAD (Ruiz et al. 1995). Regarding the M/L55 polymorphism, the M55 allele appeared to be associated with higher plasma concentration of total and LDL cholesterol and apo B compared with the L55 allele in two Canadian aboriginal populations (Fanella et al. 2000). These studies imply that PON1 polymorphisms may account for approximately 1% of the variation in total cholesterol and related lipoprotein traits in genetically isolated populations (Hegele et al. 1995a). In more heterogeneous or outbreed populations, the impact of PON1, if any, on lipoprotein levels is inconsistent.

5.2. Activity of PON1 allozymes against LDL oxidation

It was first assumed that subjects with high PON1 activity toward paraoxon (R192 and L55 homozygotes) are better protected also against LDL oxidation and, therefore, against atherosclerosis. This assumption was made despite the fact that the effect of PON1 R/Q192 polymorphism is reversed for the hydrolysis of diazoxon, soman and sarin contrary to hydrolysis of paraoxon (Davies et al. 1996), and that R192 and Q192 isoforms hydrolyze some substrates at approximately the same rate (Furlong et al. 1988, Smolen et al. 1991). This hypothesis was soon demonstrated to be incorrect and the capacity of PON1 allozymes to protect LDL from oxidation was found to be completely opposite to that of paraoxon hydrolysis. Mackness and co-workers showed that HDL containing the PON1 product of the QQ genotype was significantly more efficient at protecting LDL against copper-induced oxidative modification than those incorporating either the enzyme product of the RQ or the RR genotype (Mackness et al. 1997b, Mackness et al. 1998a). This finding was confirmed in another study demonstrating that copper-ion induced LDL oxidation was reduced up to 60% by PON1 with Q192, but only up to 40% by PON1 with R192 (Aviram et al. 1998a), as measured by the production of peroxides and aldehydes *in vitro* after 4-hour incubation. PON1 carrying the Q192 allele was also more efficient at blocking LDL oxidation when added at the initiation of LDL oxidation. Along with LDL oxidation, the activity of R192 PON1 was reduced by 30%, while the activity of Q192 PON1 was only minimally affected. Moreover, HDL isolated from MM homozygotes retained twice as much of the ability to protect LDL after 6-hour incubation as did HDL from either ML heterozygotes or LL homozygotes (Mackness et al. 1997b, Mackness et al. 1998a). When PON1 haplotypes were considered, HDL from those individuals who were homozygous for both the Q192 and M55 alleles was the most efficient at protecting LDL from oxidation. It has also been demonstrated that PON1 can act on oxidized lipids in the atherosclerotic lesion derived from either carotid or coronary artery specimens and that PON1 with Q192 is more potent than PON1 with R192 also in this respect (Aviram et al. 2000b).

5.3. R/Q192 polymorphism and atherosclerosis

The interest in PON1 in the context of atherosclerosis arose in 1986 when it was reported that the activity of PON1 in serum was lower among subjects who had survived on MI than controls (McElveen et al. 1986, Secchiero et al. 1989). The PON1 activity was also lower in several diseases where the susceptibility to develop atherosclerosis is increased (Mackness et al. 1987b, Mackness et al. 1989, Mackness et al. 1991). These findings indicated a protective role of PON1 against atherosclerosis. Later on, PON1-knockout mice were found to be more susceptible to atherosclerosis than their wild-type littermates (Shih et al. 1998).

Coronary artery disease. When the genetic markers of PON1 become available, a study conducted among North American Caucasians showed that the PON1 R192 allele was more often present in angiographically documented patients with CAD than controls (Serrato and Marian 1995). A large case-control study of 434 middle-aged or elderly type 2 diabetes patients with or without history of CAD led to analogous observations and both the R192 allele and RQ and RR genotypes were associated with CAD (Ruiz et al. 1995). Similar results were soon reported regarding different ethnic groups, including Singapore Indians (but not Chinese) (Sanghera et al. 1997) and Japanese (Zama et al. 1997). In case-control studies of 164 Japanese type 2 diabetes patients (Odawara et al. 1997), 200 Asian Indians (Pati and Pati 1998) and 218 Taiwanese Chinese people (Ko et al. 1998), PON1 R192 was a risk factor for CHD. This finding was, however, not reported consistently in all studies, including a Finnish case-control study of 380 well-characterized subjects who had undergone coronary bypass surgery (Antikainen et al. 1996). Studies of 472 Italian CAD patients (Ombres et al. 1998), and of 440 British CAD patients (Rice et al. 1997) were also unable to demonstrate any significant association between the R/Q192 polymorphism and CAD risk.

Since the original observations about ten years ago, several studies have investigated the association between R/Q192 polymorphism and CAD. Durrington and co-workers have conducted a meta-analysis of these studies and found a statistically significant overall association between the R192 allele and the presence of CHD (Durrington et al. 2001) (Figure 5). Most of these studies used coronary angiography

and >50% coronary stenosis or a history of MI as signifying CAD. However, several of these studies that found an association between the R192 allele and CAD concerned only type 2 diabetes patients (Ruiz et al. 1995, Odawara et al. 1997, Pfohl et al. 1999, Aubo et al. 2000, Osei-Hyiaman et al. 2001). Also, the subjects of these studies were middle-aged or elderly and included both sexes except two studies which included only males (Herrmann et al. 1996, Gardemann et al. 2000). It should also be noted that all these studies have been case-control studies and that, as yet, there is only one prospective trial (Turban et al. 2001). In that study, R/Q192 polymorphism was not associated with the progression of coronary atherosclerosis in 356 subjects as determined by serial quantitative coronary angiography. The main inclusion criterion to the study was the presence of 30-75% diameter stenosis in at least one coronary artery.

Myocardial infarction. Several studies have not found an association between R/Q192 polymorphism and MI and that the risk of MI is associated preferentially with the QQ genotype, not the R192 allele (Suehiro et al. 1996, Aubo et al. 2000, Sen-Banerjee et al. 2000, Senti et al. 2000, Senti et al. 2001a, Senti et al. 2001b). A large French study failed to find an association between R/Q192 polymorphism and MI among 642 MI patients and 701 controls (Herrmann et al. 1996). Again, these studies were case-control studies where the patients were survivors of MI. Several studies included also smokers.

Vascular reactivity. In the study of 101 healthy subjects, brachial reactivity after administration of Intralipid® was decreased in RR homozygotes compared with subjects with the QQ or RQ genotype (Paolisso et al. 2001). In another study which enrolled 27 patients with clinical manifestations of CAD who underwent intracoronary administration of serotonin to provoke coronary constriction, the QQ homozygotes had a greater reduction in the lumen diameter than the RQ heterozygotes (Bauters et al. 2000). There were no RR homozygotes in the study.

Intima-media thickness. In the studies on the association between PON1 polymorphisms and carotid atherosclerosis, the atherosclerotic changes have been measured by carotid ultrasonography and recorded as IMT. In general, the results have been negative and no association between R/Q192 polymorphism and carotid IMT has been found in healthy subjects (Schmidt et al. 1998, Dessi et al. 1999, Markus et al. 2001) or in type 2 diabetic subjects (Cao et al. 1998). In familial hypercholesterolemia

patients, the IMT was increased in PON1 wild-type carriers (LL/QQ) but not when both polymorphisms were analyzed separately (Leus et al. 2000).

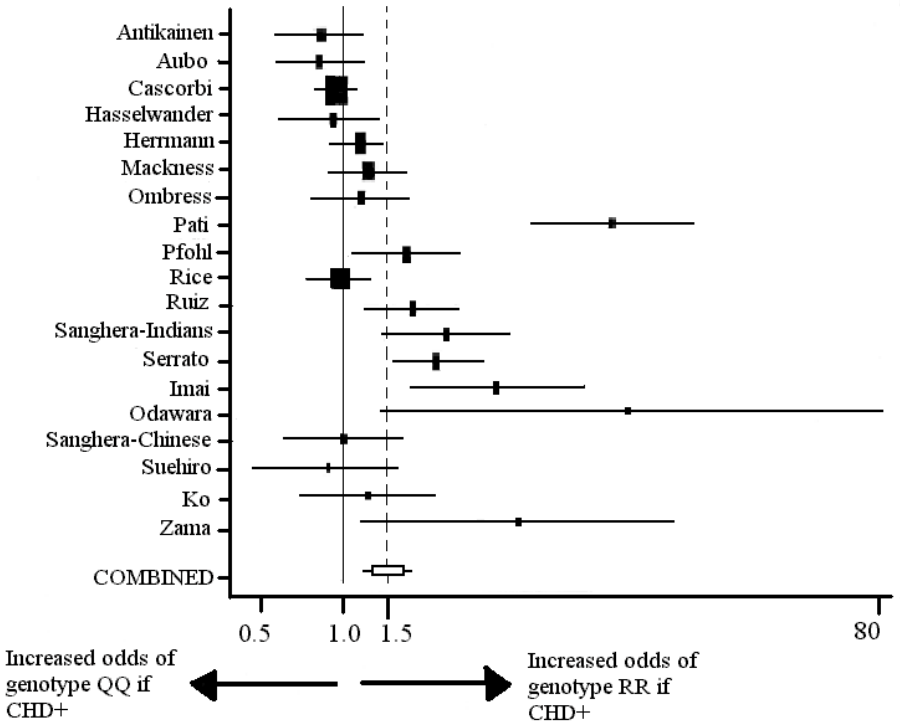

Figure 5. Results of a meta-analysis of case-control studies reporting on the odds ratio of the likelihood of cases of CHD having PON1 R192 allele relative to controls. Confidence intervals that cross unity are not statistically significant. Overall, the odds are statistically significantly increased (open box, combined results). (References: Ruiz et al. 1995, Serrato and Marian 1995, Antikainen et al. 1996, Herrmann et al. 1996, Suehiro et al. 1996, Odawara et al. 1997, Rice et al. 1997, Sanghera et al. 1997, Zama et al. 1997, Ko et al. 1998, Mackness et al. 1998b, Ombres et al. 1998, Pati and Pati 1998, Cascorbi et al. 1999, Hasselwander et al. 1999, Pfohl et al. 1999, Aubo et al. 2000, Imai et al. 2000) (modified from Durrington et al. 2001).

Stroke and cerebrovascular disease. The role of the PON1 R/Q192 genotype with regard to cerebrovascular disease and stroke has not been studied in depth. The PON1 R192 allele frequency was reportedly higher in Japanese ischemic stroke patients than in control subjects (Imai et al. 2000). Type 2 diabetes patients that carried the R192 allele had also an increased risk of cerebrovascular disease (Koch et al. 2001). This result was not confirmed in a study on stroke and carotid stenosis patients and non-diabetic controls (Topic et al. 2001).

Autopsy studies. All of the above mentioned observations are from clinical studies and there are no previous reports on the relation between PON1 R/Q192 polymorphism and autopsy-verified atherosclerosis.

5.4. M/L55 polymorphism and atherosclerosis

Coronary artery disease. All of the studies presented in Table 2, except one (Salonen et al. 1999), considered both PON1 polymorphisms, which gives the opportunity to estimate the effect that results from the combination of the polymorphic sites. In the first study of 408 Caucasian type 2 diabetes patients, homozygosity of the L55 allele was found to be an independent risk factor for CHD (Garin et al. 1997). However, the result was not confirmed in Asian Indian and Chinese populations (Sanghera et al. 1998b), although the combined R192/L55 haplotype was more frequent in Indian CHD patients than in controls. In a large case-control study of PON1 haplotypes (Cascorbi et al. 1999), there was no association between the genotypes and early manifestation of coronary disease, severity of disease, acute coronary syndromes or MI. Several other case-control studies were unable to confirm the result that was first demonstrated in diabetic patients (Zama et al. 1997, Hasselwander et al. 1999, Imai et al. 2000, Leus et al. 2001).

Myocardial infarction. There is only one report of a prospective nested case-control study, which included 2,682 male subjects (Salonen et al. 1999). Among these subjects, MM homozygosity carried a more than threefold risk of a first MI compared with LL homozygosity. This result has not been confirmed in several case-control studies and some studies have presented even contradictory results: A large case-control study of 2,784 males found that the risk of MI was marginally increased in LL homozygotes compared with the M55 allele carriers (Gardemann et al. 2000). Also, the risk of MI was slightly decreased among QQ/MM homozygotes in a recent study (Arca et al. 2002) (Table 2).

Vascular reactivity. The relationship between the PON1 M/L55 genotype and endothelial dysfunction as measured by the coronary function or reactivity has not been investigated previously.

Table 2. Studies on the association between PON1 M/L55 polymorphism and CHD

Study	Design	Cases vs. controls	Sex	Age, years (range or mean ± SD)	CHD phenotype or event	Risk of CHD
Garin et al. 1997	Case-control (type 2 diabetes patients)	168 vs. 240	both	63 ± 10 vs. 59 ± 10	CAD or MI	LL ↑
Zama et al. 1997	Case-control	75 vs. 115	both	63 ± 10 vs. 48 ± 6	CAD	No association
Sanghera et al. 1998a	Case-control, Indians	114 vs. 183	both	55 ± 1 vs. 43 ± 1	CAD or MI	No association
	Case-control, Chinese	119 vs. 181	both	59 ± 1 vs. 46 ± 1	CAD or MI	No association
Hasselwander et al. 1999	Case-control (renal transplant recipients)	103 vs. 388	both	59 ± 10 vs. 43 ± 16	CAD or MI	No association
Cascorbi et al. 1999	Case-control	1,000 vs. 1,000	both	55 - 67	CAD	No association
Salonen et al. 1999	Prospective nested case-control	55 vs. 110	males	42 - 60 at baseline	MI	MM ↑
Imai et al. 2000	Case-control	210 vs. 431	both	63 ± 9 vs. 64 ± 9	CAD	No association
Sen-Banerjee et al. 2000	Case-control	492 vs. 518	both	57 ± 1 vs. 57 ± 1	MI	No association
Gardemann et al. 2000	Case-control	2,249 vs. 535	males	60 ± 10	CAD or MI	LL (↑) (for MI)
Leus et al. 2001	Case-control (FH patients)	83 vs. 114	both	49 ± 9 vs. 48 ± 9	CHD	No association
Arca et al. 2002	Case-control	391 vs. 196	both	61 ± 9 vs. 59 ± 9	CAD or MI	QQ/MM (↓)

ABBREVIATIONS: CAD, angiographically diagnosed disease; MI, myocardial infarction; FH, familial hypercholesterolemia.

NOTE: ↑ increased risk, ↓ decreased risk, (↑/↓) marginally increased/decreased risk.

Intima-media thickness. In a study on randomly selected middle-aged or elderly individuals, the LL genotype was associated with increased IMT values (Schmidt et al. 1998) and, therefore, the LL genotype was considered to be a risk factor for stroke. Another study on familial hypercholesterolemia patients found that subjects homozygous for the wild-type LL/QQ haplotype had the highest mean carotid IMT compared to the other combined genotypes (Leus et al. 2000). When both polymorphic sites were analyzed separately, there was no difference in IMT between the genotype groups. No studies have specifically investigated the role of the PON1 M/L55 genotypes in stroke or cerebrovascular disease.

Autopsy studies. Autoptic material has not been used to study the association between the M/L55 genotype and the area of different types of early or advanced atherosclerotic lesions as measured directly in coronary or other vessel walls.

5.5. Promoter polymorphisms and atherosclerosis

In the study of James et al. (2000b), T/C-108 promoter polymorphism was identified as a possible risk factor for vascular disease in type 2 diabetes patients by virtue of its ability to modulate serum concentration of PON1. The low-expressor TT genotype was associated with a significantly lower serum PON1 level and this genotype was over-represented in type 2 diabetic patients with CHD. The study suggested a complex interaction between the T/C-108 promoter polymorphism and the coding region R/Q192 polymorphism. Consistently with this result, a case-control study of CAD positive and CAD negative subjects identified a decreased risk of CAD in patients aged 60 years or less if they had the high-expressor CC genotype at position -108 (Leviev et al. 2001b). One study has analyzed the genotypes arising from the C/G-909 promoter polymorphism. The GG genotype that associates with high expression of PON1 has also been linked with a reduced risk of MI (Leviev et al. 2002).

6. Environmental and acquired factors versus PON1 concentration and activity

Diet. Animal models have showed that *PON1* expression can be regulated by dietary factors and especially by the amount of fat in the diet (Shih et al. 1996). An atherogenic

diet reduces the PON1 messenger ribonucleic acid (mRNA) levels and PON1 activity in mice that are susceptible to aortic atherosclerosis. This change correlates with a decreased HDL level and stands in contrast to atherosclerosis-resistant mice in which the mRNA levels rise (Shih et al. 1996). The PON1 activity and HDL cholesterol concentration fell also in a study of nontransgenic and transgenic rabbits overexpressing human apo AI that were kept on a pro-atherosclerotic diet (Mackness et al. 2000). A reduction of the HDL cholesterol concentration and PON1 activity and mass was reported also in a study on LDL receptor deficient mice which were fed short-term an atherogenic diet but this time without changes in PON1 mRNA levels (Hedrick et al. 2000).

In humans, the PON1 activity is decreased after fat-rich meals (Sutherland et al. 1999). On the other hand, consumption of pomegranate juice, which is rich in flavonoids, results in a significant elevation in PON1 activity while the HDL cholesterol levels are only minimally affected (Aviram et al. 2000a). Surprisingly, one study demonstrated that a low PON1 activity is associated with a high vegetable intake as calculated from the food records of healthy young Finns (Kleemola et al. 2002). Only one study has so far considered the effect of fat intake on PON1 activity by PON1 R/Q192 genotype (Tomas et al. 2001). A beneficial effect of a high intake of olive oil, which is rich in oleic acid, on HDL and PON1 activity at the population level was reported especially among subjects carrying the R192 allele of PON1.

Alcohol. Moderate alcohol consumption has been associated with a reduction in the risk of CHD and this effect appears to be due to an ethanol-induced increase in HDL cholesterol concentrations (Klatsky et al. 1981, Yuan et al. 1997). The protective effects of alcohol against CHD can also partly be mediated by a decrease in LDL cholesterol, since alcohol-related acetaldehyde can modify LDL chemically and speed up the LDL clearance by macrophage receptors (Castelli et al. 1977). Phenolic substances can, however, also suppress LDL oxidation, which may be related to PON1 since red wine polyphenols can induce serum PON1, at least in apo E-knockout mice (Hayek et al. 1997). In humans, the increases in HDL cholesterol and apo AI concentrations correlate strongly with a coincident increase in PON1 activity during daily moderate consumption of wine, beer or spirits (van der Gaag et al. 1999). The R/Q192 polymorphism may not have an effect on the relative change in serum PON1 activity

according to a study by van der Gaag et al. (1999), but the study included only a small number of subjects, and the observation needs to be corroborated.

Smoking. The activity of PON1 appears to be inhibited by cigarette smoke extract in a dose and time dependent manner (Nishio and Watanabe 1997). Antioxidants do not protect PON1 activity from smoke-induced inhibition, and this suggests that other than free radical compounds in cigarette smoke are responsible for the observed loss of PON1 activity. The activity and concentration of PON1 decreased also in coronary arteriography patients who were current smokers in comparison to non-smokers (James et al. 2000a). There is also a contradictory study according to which cigarette smoke does not affect PON1 (Bielicki et al. 2001). When PON1 R/Q192 and M/L55 polymorphisms are taken into account, the R192 allele is associated with an increased risk of MI but this association is evident only among non-smokers (Sen-Banerjee et al. 2000). Smokers have an increased risk of MI regardless of their PON1 genotype. The R192 allele is a risk factor for CAD in type 2 diabetes patients, and the association is even more pronounced among current and ex-smokers (Pfohl et al. 1999). An association between packs of cigarette smoked and increased risk of MI has reported also in QQ homozygotes (Senti et al. 2000).

Pharmacotherapy. There is increasing evidence to suggest that statins exert an additional antiatherogenic effect beyond their capacity to lower LDL cholesterol (Vaughan et al. 1996). First, some statins have been shown to raise HDL cholesterol by 4 to 8% (Maron et al. 2000) and, second, statins may possess antioxidative effects by reducing the susceptibility of LDL to oxidation (Aviram et al. 1998b). Simvastatin appears to have antioxidant properties *in vitro* and *in vivo* (Girona et al. 1999). Some of this property may be due to the fact that simvastatin increases serum PON1 activity (Tomas et al. 2000). The effect of statins on PON1 may, however, vary by the statin used. Thus, treatment with atorvastatin does not affect serum PON1 activity in patients with dyslipidemia (Tsimihodimos et al. 2002). An atorvastatin metabolite, in turn, was shown to inhibit HDL oxidation, which was related to preservation of PON1 activity (Aviram et al. 1998b). The ability of simvastatin (Tomas et al. 2000) and fluvastatin (Turban et al. 2001) to increase PON1 activity seems to be independent of PON1 genotype. Interestingly, spironolactone, mevastatin, simvastatin and lovastatin have

been identified as substrates for PON1 (Billecke et al. 2000), and PON1 may thus affect the metabolism of these drugs.

Considering other drugs, PON1 activates the antibacterial agent prulifloxacin to its active form (Tougou et al. 1998) and degrades glucocorticoids (Biggadike et al. 2000). Prulifloxacin is hydrolyzed at a higher rate by the PON1 R192 isoform than by the Q192 isoform. The effect of fibric acid derivates, bezafibrate and gemfibrozil, on PON1 were studied in a randomized, double-blind cross-over study, but neither fibrate influenced plasma PON1 activity in patients with type IIb hyperlipoproteinemia (Durrington et al. 1998). Serum PON1 activity was, however, increased in another study, where type 2 diabetic patients used gemfibrozil three months (Balogh et al. 2001).

AIMS OF THE STUDY

The association between genetics, lipoprotein oxidation and atherosclerosis has been firmly established. PON1 has been shown to be able to destroy certain ox-LDL species *in vitro* and some of its genotypes have been associated with CHD. However, the role of the PON1 genotypes in the different stages of atherosclerosis is still unclear, as are the factors that modify the functions of PON1. The present study used three clinical and two autopsy series to elucidate the relationship between PON1 genotypes and serum lipids and apolipoproteins, indices of lipid oxidation, coronary reactivity, intima-media thickening and autopsy-verified atherosclerotic lesions. The interaction between PON1 genotypes and smoking in relation to the different arterial phenotypes was also investigated. The specific aims of the study were:

1. To study the association between PON1 genotype and serum lipids and apolipoproteins in subjects with and without lipid-lowering medication and to investigate whether the PON1 genotype modulates pravastatin-induced changes in serum lipids (I, IV).

2. To elucidate the association between the PON1 genotypes and the indices of lipid oxidation (II, III).

3. To analyze whether the PON1 genotype is associated with the indices of coronary blood flow as measured with PET (III).

4. To assess the role of the PON1 genotypes in carotid artery intima-media thickening and carotid artery atherosclerotic disease (CAAD) (IV).

5. To examine the relationship between the PON1 genotypes and autopsy-verified early and advanced atherosclerotic lesions in the coronary arteries (VI), the aorta (V, VI) and the mesenteric arteries (V).

6. To study the interaction between smoking and the PON1 genotypes in relation to the stages of atherosclerosis (IV, VI).

SUBJECTS AND METHODS

For more detailed information on study subjects and methods, please refer to the original articles I-VI.

1. Clinical series

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

At study start, 51 men from the Achipelago Sea Naval Command, Achipelago Coast Guard District, Säkylä Garrison and the Turku Fire Department were invited to participate with the following entry criteria: 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. The men were asked about their family history of CAD, alcohol and caffeine consumption, medication, smoking and exercise habits using a validated questionnaire. In study I, 51 men were randomly assigned pravastatin (Pravachol®, 40 mg/day, n = 25) or placebo (n = 26). The plasma lipids and apolipoproteins were measured at baseline and after six months of treatment. In study III, 49 men out of 51 were included into the statistical analysis 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. All subjects gave written informed consent.

1.2. Study of type 2 diabetes patients and controls (II)

The recruitment of patients and control subjects has been discussed in detail previously (Wirta and Pasternack 1995). Originally, the subjects were enrolled during the years 1985-1988 and the study included 150 type 2 diabetes outpatients at the municipal health care center of the city of Tampere and the same number of non-diabetic control subjects, matched for age and gender. The diabetes patients fulfilled the WHO diagnostic criteria for type 2 diabetes and the subjects with other serious disease or otherwise a reduced life expectancy were excluded. The exclusion criteria were the

same for the control subjects and these subjects were tested to have normal glucose tolerance. At the end of nine years of follow-up, 84 diabetic patients and 115 non-diabetic controls were eligible for re-evaluation. At the time of this study, urine samples needed for biochemical measurements were available from 55 diabetics and controls and they comprised the final study population. The subjects included both men (n = 53) and women (n = 57) and they gave written informed consent. The Ethics Committee of the Tampere University Hospital approved the study.

1.3. Random sample of Finnish middle-aged men (IV)

The subjects for this study were selected from a cohort of 9,058 males aged 50 to 59 years who were living in the city of Tampere. Three hundred men were randomly invited by letter to participate and 223 agreed to participate (74%) 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. All required data, including PON1 genotype, were obtained from 199 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.

2. Autopsy series

2.1. Consecutive autopsy series (V)

To evaluate the degree of atherosclerosis in the abdominal aorta and mesenteric arteries, the autopsy material was collected from 81 forensic and 42 medical postmortem examinations performed in the Tampere University Hospital in the summer of 1993. The mean age of subjects was 62 years (range 18 to 93 years) and there were 90 men and 33 women. The history of cardiovascular diseases and classical risk factors for atherosclerosis were collected from the hospital records of each subject. The Ethics Committee of the Tampere University Hospital approved the study.

2.2. The Helsinki Sudden Death Study (VI)

The Helsinki Sudden Death Study (HSDS) was launched to study the lifestyle and genetic risk factors predisposing Finnish middle-aged men to sudden death. The HSDS consisted of two series of a total of 700 Caucasian men whose mean age at their time of death was 53 years (range 33 to 69 years). The first series of men were subjected to a medicolegal autopsy at the department of Forensic Medicine, University of Helsinki between 1981 and 1982 (A-series, n = 400) and second series ten years later, between 1991 and 1992 (B-series, n = 300).

For collection of data on CAD risk factors, a relative or a close friend of the deceased was available for interview in 500 (71%) of the cases. A detailed questionnaire included a review about past and recent smoking and drinking habits and previous illnesses (Karhunen and Penttilä 1990). For coronary arteries, complete data on all risk factors was obtained from 333 cases and for aortas from 134 cases, but these numbers varied slightly according to artery. The Ethics Committee of the Department of Forensic Medicine, University of Helsinki approved the study.

3. Determination of serum lipids and apolipoproteins (I, II, III, IV)

For the determination of the concentrations of lipids and apolipoproteins, blood was drawn after the subjects had fasted for 12 hours. In studies I and III, plasma triglycerides and the total and HDL cholesterol 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 Friedewald's formula (Friedewald et al. 1972). Apo B and apo A concentrations were measured by immunoturbidimetric method using specific controls (Hoffmann-La Roche Ltd., Switzerland) on the same analyzer as lipids.

In study II, serum total cholesterol and triglycerides were measured by the dry slide technique (Ektachem 700 analyzer, Johnson and Johnson Clinical Diagnostics, USA). LDL and VLDL were precipitated with dextran sulphate and $MgCl_2$ and the HDL cholesterol concentration was measured using the same technique. The LDL concentrations were calculated by Friedewald's formula (Friedewald et al. 1972).

In study IV, lipoprotein fractions were assessed from fresh samples by ultracentrifugation (Carlson 1973). Cholesterol was measured from serum and lipoprotein fractions using an enzymatic method (CHOD-PAP, Boehringer Mannheim, Germany). Triglycerides were measured by enzymatic hydrolysis (GPO-PAP, Boehringer Mannheim, Germany). Apo B was determined by immunonephelometry (Behring, Behringwerke AG, Germany) and lipoprotein (a) i.e., Lp(a) by two-site immunoradiometry (Pharmacia, Sweden).

4. Measurements of oxidation

4.1. Urinary 8-iso-PGF$_{2\alpha}$ (II)

The urine collected over 24 hours was mixed, divided into small aliquots and stored frozen at -70°C until analyzed. Thawed urine samples were centrifuged and the supernatant was diluted and used for the determination of 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) by a competitive ELISA according to the manufacturer's instructions (R & D Systems Inc., USA). The glomerular filtration rate (GFR) of each subject was measured by the [^{51}Cr]EDTA plasma clearance technique (Garnett et al. 1967). Urinary 8-iso-$PGF_{2\alpha}$ was expressed as the total amount excreted in 24 hours. The urinary 8-iso-$PGF_{2\alpha}$/GFR ratio was alternatively used in the analysis.

4.2. Autoantibodies against oxidized LDL (III)

The level of autoantibodies against ox-LDL was measured by a solid phase ELISA as previously described (Lehtimäki et al. 1999). 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) ox-LDL was produced by 24-hour incubation of native LDL with 2 μmol/l $CuSO_4$. Half of the wells on each ELISA plate was coated with native LDL (5 μg/ml) and half of the plate with ox-LDL (5 μg/ml). The coated plates were incubated, washed and blocked and the serum samples, which were diluted to 1:15, were added to the wells. After incubation, peroxidase-conjugated rabbit anti-human IgG antibodies were

added and *o*-phenylenediamine 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 duplicate samples. The autoantibody titer against ox-LDL was calculated by subtracting the binding to native LDL from the binding to ox-LDL.

4.3. Susceptibility of LDL to oxidation (III)

The susceptibility of LDL to copper-induced oxidation was measured as previously described (Leinonen et al. 1998). LDL was isolated by single-step ultracentrifucation, and the purity of this preparation was checked by agarose gel electrophoresis with a Sudan-Black lipid staining and by immunonephelometry after desalting the isolated LDL. Isolated LDL was oxidized with 0.167 mM $CuSO_4$ and the production of conjugated dienes was monitored as the change in absorbance at 234 nm. The baseline level of conjugated dienes, the lagtime of LDL oxidation, i.e., the time that LDL can resist the oxidation, the rate of the propagation phase and the maximal concentration of conjugated dienes formed were recorded during the time period of oxidation.

4.4. Plasma antioxidants (II, III)

In study II, the concentration of ascorbic acid was measured from plasma samples obtained from the subjects after an overnight fast by high-performance liquid chromatography (HPLC) with an electrochemical detector (Frei et al. 1988). A modified HPLC method with a LC-amperometric detector (Bioanalytical Systems Inc., USA) was used to measure the concentration of α-tocopherol in the plasma. The protein thiol groups were determined as described previously (Ellman 1959).

In study III, the significant determinants of the lagtime and oxidation rate of LDL, α-tocopherol and ubiquinone were detected from isolated LDL fractions by HPLC with an electrochemical detection with mobile phase that consisted of acetonitrile:methanol:$LiClO_4$:$HClO_4$ (350:350:300:1). This method allowed the simultaneous determination of these two antioxidants.

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

The subjects 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 injection of $[^{15}O]H_2O$ and for 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 transmission scan, the subject's nostrils were closed and he inhaled $[^{15}O]CO$ for 2 min through a three-way inhalation flap-valve. $[^{15}O]CO$ was allowed to combine with the hemoglobin for 2 min before data collection for a static scan was started. During the scan period, three blood samples were drawn at 2-min intervals and the radioactivity was measured. A 10-min 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 sec after the beginning of intravenous administration of adenosine. For the blood flow measurement $[^{15}O]H_2O$ was injected intravenously for two min and dynamic scanning was started for 6 min. 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 left ventricle. The regions of interest were drawn on the images obtained at rest and copied to the images obtained after adenosine administration (Iida et al. 1995). 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 myocardial blood flow was used for further analyses. The coronary flow reserve (CFR) was defined as the ratio of overall myocardial blood flow after adenosine 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 myocardial blood flow during adenosine administration to RPP adjusted flow at baseline.

6. Ultrasonic measurement of carotid artery intima-media thickness (IV)

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 Forrest University, North Carolina, USA. One certified sonographer and one reader performed all recordings and measurements.

The arteries were identified by Doppler 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 the 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). CAAD was defined as an IMT > 1.7 mm in at least one site.

7. Scoring atherosclerosis at autopsy

7.1. Macroscopic classification (V, VI)

The measurements of atherosclerosis in the consecutive autopsy series from the Tampere University Hospital (V) are described in detail previously (Järvinen 1996). The area of atherosclerotic lesions was estimated and expressed in percentages (%). The aortic preparations included initial portions of the celiac (CA), superior mesenteric (SMA) and inferior mesenteric (IMA) arteries. The arteries were graded as 0 if there were no lesions or only fatty streaks and 1 if there were advanced lesions (i.e., fibrous plaques and complicated lesion with ulceration or hemorrhage). The diameter of the

aortic ostium and the narrowest point of the first 3-cm of the artery was measured with calibrated round probes. The normal diameter proximal or distal to a stenotic lesion was measured and an intraluminal diameter reduction of 50% was considered as stenosis. The more distal parts of the SMA and IMA were also studied.

In the HSDS (VI), the areas of atherosclerotic lesions were measured from the left anterior descending coronary artery (LAD), right coronary artery (RCA), left circumflex coronary artery (LCX) and from the thoracic and abdominal aorta (only in the B-series). The arteries were dissected free, opened, attached to a cardboard and fixed, and stained for fat with Sudan IV. The degree of atherosclerotic plaques was defined according to protocols by IAP, Standard Operating protocol 1962 (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 by a computer-assisted planimetric technique and by radiography in the case of calcification. The areas of the different types of lesions were expressed in percentages (%).

7.2. Histological classification (V)

For histological examinations of the mesenteric arteries with light microscopy, 0.5-cm long segments of CA, SMA and IMA were cut at the level of 1 cm distal to their aortic ostium. The segments were fixed in formalin, embedded in paraffin blocks, sectioned and stained with Masson's trichrome. The length of the IEL and the thickness of the intima were measured in millimeters by the MOP 3 image-analysis system (Reichert-Jung, Germany). The intimal thickness was defined as the measure from the lumen to the IEL at the area of greatest intimal thickness (Kay et al. 1976). The number of gaps in the IEL was counted, divided by the circumference of the IEL and expressed as gaps per millimeter.

8. DNA extraction and PON1 genotyping

In the A-series of the HSDS (VI), DNA was extracted from paraffin-embedded samples of cardiac muscle with a method described by Isola et al. (Isola et al. 1994). In the B-

series of the HSDS, DNA was isolated from pieces of cardiac muscle by a standard phenol-chloroform method. In the consecutive autopsy series from the Tampere University Hospital (V), DNA was isolated from paraffin-embedded samples of the mesenteric arteries by QlAamp DNA Tissue Kit (Qiagen Inc., USA). In other cases, DNA was isolated from whole blood by QlAamp DNA Blood Kit (Qiagen Inc., USA).

PON1 M/L55 genotypes were determined by PCR using primers and restriction enzyme *Hsp92*II (Promega, USA) digestion as described earlier (Humbert et al. 1993). After initial denaturation at 96°C for 2.5 min, the DNA was amplified by 40 cycles in the following conditions: denaturation at 96°C for 30 s, annealing at 61°C for 1 min and extension at 72°C for 1 min. PON1 R/Q192 genotypes were determined with the primers described earlier (Humbert et al. 1993) by PCR that involved primary amplification of 30 cycles with annealing at 61°C and secondary amplification of 16 cycles with annealing at 63°C. The PCR-products were digested with *Alw*I enzyme (BioLabs, UK). Digested fragments were separated by electrophoresis and visualized with ethidium bromide staining under UV-light. Genotyping was always performed blind to 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) and analysis of covariance (ANCOVA) were used to compare continuous variables. Statistical analyses of the longitudinal data were carried out using analysis of variance for repeated measures (RANOVA). In the case of a significant main effect or interaction, Scheffe's or Least Significant Difference (LSD) post-hoc tests were utilized to compare the differences between groups. Non-normally distributed data was analyzed after square root or logarithmical transformation. Arlequin ver. 1.1 was used to examine the linkage disequilibrium between the two PON1 polymorphisms.

In the study III, version 1.0.15 of the PS program was used to calculate the power (1-β) of the test procedures. In study I, linear regression analysis was used in the search for the set of variables that best predict apo AI and HDL cholesterol changes during pravastatin treatment. Logistic regression analysis was used in study V to

establish the set of variables that would classify the subjects most adequately by atherosclerosis status and, in study IV, to establish the adjusted odds ratios for CAAD by PON1 genotype groups. In study VI, the results obtained with ANCOVA were confirmed by multinomial logistic regression analysis, which was done by the BMDP Statistical Software version 1990 in the SUN/UNIX mainframe. In other cases, Statistica for Windows version 5.1 software package (Statsoft Inc., USA) or SPSS version 9.0 for Windows (SPSS Inc., USA) was used for statistical analyses. 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. PON1 allele and haplotype frequencies

Taking all studies together, the allele frequency of PON1 L55 varied from 61 to 65% and of M55 from 35 to 39%. The R192 and Q192 alleles occurred in 25 to 26% and in 74 to 75% of cases, respectively. Both genotype distributions followed the Hardy-Weinberg equilibrium and were identical in healthy subjects and in type 2 diabetes patients (study II). There was also a linkage disequilibrium between these two polymorphisms ($p < 0.001$), which favored the simultaneous presence of the R192 and M55 alleles (studies I and III, V). Three PON1 haplotypes, *1 (L55/Q192), *2 (L55/R192) and *3 (M55/Q192), were observed (study I). A haplotype containing both mutations M55 and R192 was not found. Concerning PON1 haplotype combinations, the frequency for the combination without any mutations (*1/*1, wild-type) was 15.7%. PON1 *1/*2 showed a frequency of 23.5%. PON1 *1/*3 occurred in 23.5%, *2/*2 in 5.9%, *2/*3 in 15.7% and *3/*3 in 15.7% of cases.

2. The effect of PON1 genotypes on lipid and apolipoprotein levels (I, IV)

In study IV, there were no statistically significant differences in means with respect to traditional risk factors including lipoprotein concentrations between the M55 allele carriers and L55 homozygotes. In study I, the HDL cholesterol concentration at baseline was higher and apo AI concentration tended to be higher in LL homozygotes than in M55 allele carriers (Table 3). The other lipid or apolipoprotein values did not differ between M/L55 or R/Q192 genotype groups at the beginning of the treatment period. Also, there were no statistically significant differences in the baseline concentrations of lipids between the pravastatin and placebo groups (study I).

Table 3. HDL cholesterol and apolipoprotein AI levels at baseline by PON1 M/L55 genotypes

	M55 allele carriers	LL homozygotes	p-value*
Subjects (n)	29	22	
HDL cholesterol (mmol/l)	1.33 ± 0.27	1.47 ± 0.32	0.049
Apolipoprotein AI (g/l)	1.41 ± 0.22	1.52 ± 0.23	0.094

STATISTICS: *ANCOVA; age and body-mass index as covariates.

The aim of study I was to find out whether PON1 genotypes affect lipid or apolipoprotein responses to pravastatin treatment. In the pravastatin group, the men carrying the QQ genotype had unchanged HDL and apo AI concentrations during pravastatin therapy, while in the R192 allele carriers, the concentrations increased. The time-by-PON1 R/Q192 genotype interaction was of borderline significance regarding the plasma HDL cholesterol concentration ($p = 0.095$) and statistically significant regarding the plasma apo AI concentration (Figure 6A). There was no statistically significant interaction between time and the PON1 M/L55 genotype regarding HDL or apo AI concentration. When the similar analysis was done by haplotype combinations of PON1, the concentrations of HDL and apo AI increased most effectively in the men with the LL/RR or ML/RQ combination (Figure 6B). In the wild-type LL/QQ combination both concentrations decreased and a similar trend was observed in the MM/QQ group.

The linear regression model that included R/Q192 genotype, M/L55 genotype, change in triglyceride concentration during the treatment period and baseline concentrations of apo AI or HDL cholesterol showed that the statistically significant determinants of the apo AI concentration change were R/Q192 genotype, apo AI concentration at baseline and M/L55 genotype. PON1 R/Q192 genotype and M/L55 genotype were, in turn, the most significant predictors of the change in HDL cholesterol level. The model did not yield additional information when other lipid or apolipoprotein values were added to the explanatory variables.

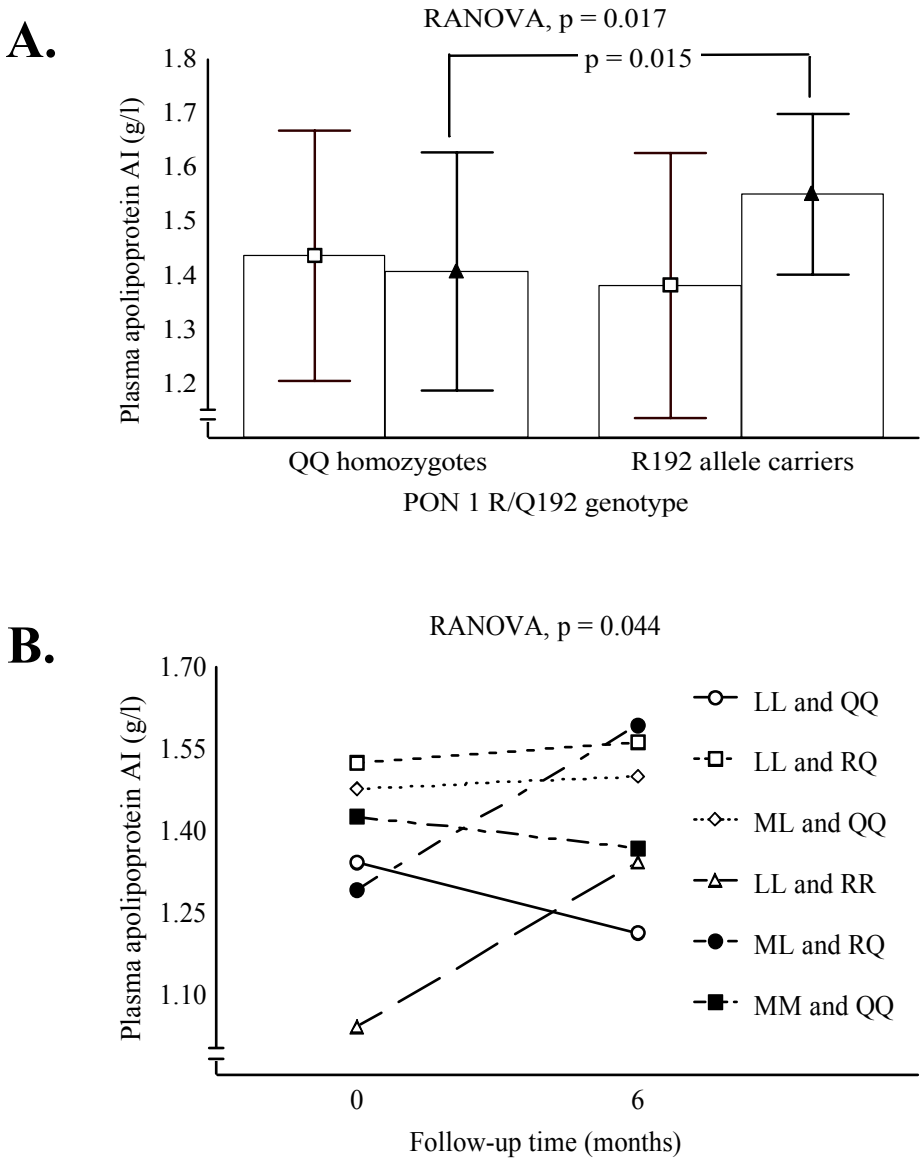

Figure 6. (A) Change in apo AI concentration during pravastatin therapy by PON1 R/Q192 genotypes. □ indicates apo AI concentration at baseline (g/l) and Δ after six months of pravastatin therapy. Least Significant Difference post-hoc test was used to study the differences between genotypes at different time points. Values are mean ± SD. (B) Changes in apo AI concentration during pravastatin therapy by PON1 haplotype combinations.

3. The effect of PON1 genotypes on oxidation of lipids (II, III)

Study II. Considering all study population, non-smokers that were LL homozygotes had statistically significantly higher 24-hour urinary excretion of 8-iso-$PGF_{2\alpha}$ than M55 allele carriers. In the diabetic subjects alone, there was no statistically significant

difference in this excretion marker between PON1 M/L55 genotype groups. In control subjects, the 24-hour excretion of 8-iso-$PGF_{2\alpha}$ was statistically significantly higher among non-smoking LL homozygotes compared to non-smoking M55 allele carriers and this difference persisted after adjustment for GFR (Table 4). When the HDL cholesterol concentration was included into the covariates, the observed associations remained statistically significant.

As expected, the 24-hour excretion of 8-iso-$PGF_{2\alpha}$ and the 8-iso-$PGF_{2\alpha}$/GFR value was increased in non-smoking diabetics in comparison to non-smoking controls as well as in all diabetes patients in comparison to all controls regardless of their smoking status. Smoking seemed to be associated with increased excretion of 8-iso-$PGF_{2\alpha}$ in control subjects but not statistically significantly.

Table 4. Ratio of 24-hour urinary 8-iso-$PGF_{2\alpha}$ to GFR among non-smoking diabetic and control subjects by PON1 M/L55 genotype

	n	LL homozygotes	n	M55 allele carriers
8-iso-$PGF_{2\alpha}$/GFR [ng/(ml/min/1.73 m^2)]				
Diabetic subjects	17	51.3 ± 42.3	31	44.5 ± 36.6
Control subjects*	14	49.3 ± 39.6	33	21.0 ± 13.2

STATISTICS: *p = 0.020 (ANCOVA; gender, hypertension (yes/no), total cholesterol, triglycerides and LDL cholesterol as covariates). Data was transformed logarithmically before analysis.
ABBREVIATIONS: GFR, glomerular filtration rate.

Study III. The PON1 M/L55 or R/Q192 genotype had no effect on LDL susceptibility to oxidation: the lagtime to oxidation and the propagation rate did not differ significantly between subjects with different PON1 genotypes. The maximal diene concentration tended to be increased in the R192 allele carriers compared with the Q192 homozygotes but this trend was not statistically significant. Also, the autoantibody titer against ox-LDL was similar in all PON1 genotype groups. However, the concentration of antioxidant α-tocopherol was increased in L55 homozygotes (p = 0.032) and R192 allele carriers (p = 0.017) compared with the M55 allele carriers and Q192 homozygotes. Also, the concentration of the other antioxidant, ubiquinone, tended to be increased in these subjects.

4. PON1 genotypes and coronary function (III)

The study examined the relationship between PON1 genotypes and coronary blood flow and reactivity. Q192 homozygotes had higher RPP-corrected blood flow at rest compared with R192 homozygotes and RQ heterozygotes (Table 5). The association between R/Q192 genotype and blood flow at rest was more pronounced when the R192 allele carriers were combined into the same group (Q192 homozygotes versus R192 allele carriers, p = 0.011). The MM genotype of PON1 tended to be associated with higher blood flow values at rest since the difference between M/L55 genotypes was of borderline significance (Table 5). Adenosine stimulated blood flow was similar in all PON1 genotype groups. Mainly due to the differences in resting blood flow, the RPP-adjusted CFR was statistically significantly higher in R192 allele carriers than in Q192 homozygotes (Q192 homozygotes versus R192 allele carriers, p = 0.032). When the analysis was done without combining the R192 homozygotes and RQ heterozygotes, the association between the R/Q192 genotype group and RPP-corrected CFR was of borderline significance (Table 5). The difference in coronary resistance between M/L55 or R/Q192 genotype groups was not statistically significant.

Table 5. Variables describing myocardial blood flow by PON1 M/L55 and R/Q192 genotypes

	M/L55 genotype			R/Q192 genotype		
	MM	ML	LL	QQ	RQ	RR
Subjects (n)	6	21	22	26	20	3
Blood flow at rest ($ml \times g^{-1} \times min^{-1}$)[a]	0.95 ± 0.19	0.83 ± 0.25	0.80 ± 0.18*	0.90 ± 0.25	0.76 ± 0.10	0.79 ± 0.32†
Coronary flow reserve[a]	3.44 ± 0.80	4.22 ± 1.32	4.39 ± 1.56	3.90 ± 1.51	4.70 ± 1.12	3.51 ± 1.35‡

STATISTICS: *p = 0.047 over all M/L55 genotypes; †p = 0.042 over all R/Q192 genotypes; ‡p = 0.054 over all R/Q192 genotypes (ANCOVA; age, body-mass index, LDL cholesterol, HDL cholesterol and triglycerides as covariates).

[a]Adjusted by rate-pressure product.

5. PON1 genotype and carotid artery intima-media thickness (IV)

The carotid IMT to PON1 M/L55 genotypes were related according to smoking status. Non-smoking LL homozygotes had 7.8% higher mean common carotid artery IMT than non-smoking M55 allele carriers ($p = 0.012$). The same trend was observed in the carotid bifurcation and in the internal carotid artery but not to a statistically significant extent. Among smokers, the mean IMT did not vary significantly between PON1 M/L55 genotypes in any of the studied arteries. In contrast to non-smokers the smoking M55 allele carriers tended to have a higher mean IMT in the arteries than the smoking LL homozygotes. Therefore, the interaction between M/L55 genotype and smoking was statistically significant in the bifurcation and the internal carotid artery, and of borderline significant in the common carotid artery. Taken together, the non-smoking L55 homozygotes had 8.9% (95% confidence interval (CI), 1.6 to 16.8) higher MMax IMT values than the non-smoking M55 allele carriers but in smokers this association tended to be reversed (Figure 7).

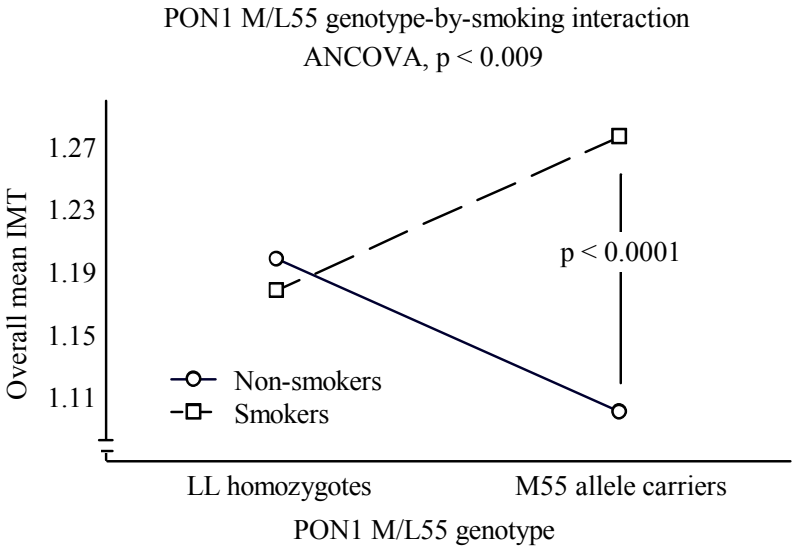

Figure 7. Interaction between PON1 M/L55 genotype and smoking status on the overall mean intima-media thickness (IMT). In ANCOVA, age, body-mass index, systolic blood pressure, LDL cholesterol, HDL cholesterol, apo B, and Lp(a) were used as covariates. Least Significant Difference post-hoc test was used to study the difference between smoking and non-smoking M55 allele carriers.

The interaction between the M/L55 genotype and the smoking status with regard to CAAD was statistically significant also by logistic regression, in which age, body-mass index (BMI), systolic blood pressure, LDL cholesterol, HDL cholesterol, apo B, and Lp(a) were entered into the model as confounding variables. Non-smoking L55 homozygotes had an odds ratio of 4.22 (95% CI 1.06 to 16.8) for CAAD compared with non-smoking M55 allele carriers. Contrary to non-smokers, the odds ratio for CAAD was 2.22 (95% CI 0.82 to 6.01) for the smoking M55 allele carriers when smoking L55 homozygotes was used as a reference group. When non-smoking and smoking M55 allele carriers were compared, the smokers had an odds ratio of 6.45 (95% CI 1.97 to 21.0) for CAAD.

6. PON1 genotypes and early and advanced atherosclerotic lesions (V, VI)

Study V. The aim of this study was to relate PON1 genotypes to the severity of atherosclerosis in the abdominal aorta and common iliac arteries. The R/Q192 genotype, but not the M/L55 genotype, seemed to affect the atherosclerotic lesion area of the abdominal aorta of men and the area tended to be larger in R192 allele carriers compared with QQ homozygotes.

LL homozygous men had more plaques and more complicated lesions in their common iliac arteries than men who carried the M55 allele (Figure 8) but this was not the case in women. The PON1 R/Q192 genotype had an effect on the areas of atherosclerotic plaques in the iliac arteries neither in male nor in female subjects. Logistic regression analysis was able to account 31% of the atherosclerotic status in common iliac arteries and showed that age, PON1 M/L55 genotype and smoking status were the most significant determinants of the atherosclerotic status (i.e., no lesions/only fatty streaks vs. advanced atherosclerotic lesions) in all subjects independently of PON1 R/Q192 genotype, hypertension, diabetes, BMI and sex.

Among men, 21.6% of LL homozygotes had stenotic changes in their SMAs but in M55 allele carriers only 3.8% ($p = 0.008$). In CA and IMA, a similar genotype order emerged, but not to a statistically significant extent. The LL homozygous males had also a statistically significantly thicker intima in the IMA than the M55 allele carriers ($p = 0.035$) and similar trends were seen also in the other mesenteric arteries. The gaps per

millimeter in the IEL did not differ between PON1 M/L55 genotype groups, although the number tended to be increased in LL homozygotes. The PON1 R/Q192 genotype had no effect on the number of stenotic lesions, the intimal thickness nor on the amount of gaps in the IEL.

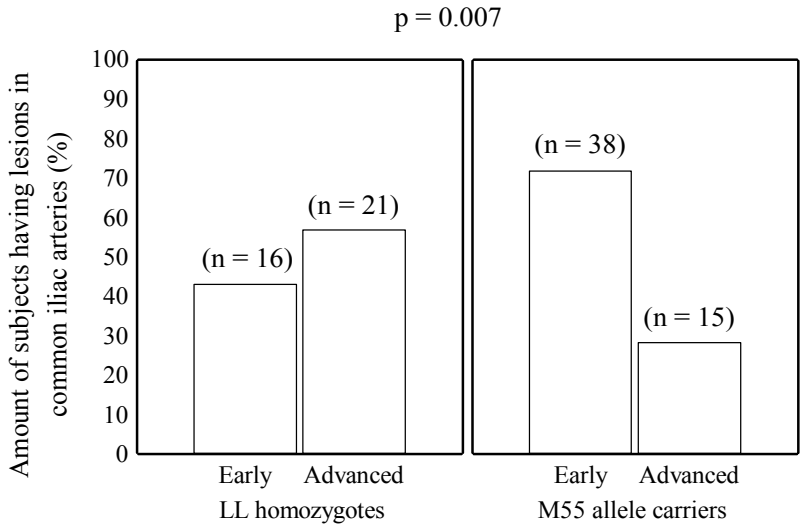

Figure 8. Frequencies of early lesions (i.e., no findings or only fatty streaks) and advanced lesions (i.e., plaques and complicated lesions) in common iliac arteries by PON1 M/L55 genotype in men. The χ^2-test was used to compare genotype distributions.

Study VI. The purpose of this study was to investigate the association between M/L55 genotype and the areas of different types of atherosclerotic lesions in the coronary arteries and aorta. Carriers of the MM genotype had less advanced plaques harboring larger percentual areas of fatty streaks in their LAD ($p = 0.014$) and RCA ($p = 0.004$), as well as in their thoracic ($p < 0.001$) and abdominal aorta ($p = 0.010$) compared to carriers of the LL or ML genotype. A PON genotype-by-smoking interaction was observed on the area of fatty streaks in the LAD ($p = 0.011$) and RCA ($p = 0.005$); the association between fatty streaks and MM genotype emerged only among non-smokers, not in smokers. In the LCX, the interaction was of borderline significance ($p = 0.065$). In subjects under 54 years of age, the PON1 genotype-by-smoking interaction in the LAD and aorta was strengthened, whereas among men older than 54 years the interaction became statistically non-significant. The areas of more advanced

atherosclerotic lesions did not vary significantly between the genotype groups, although MM homozygous non-smokers tended to have smaller fibrotic plaque areas in their RCA and aorta. Figure 9A shows the M/L55 genotype-by-smoking interaction on the area of fatty streak lesions in LAD and Figure 9B the area of fibrotic lesions in smokers and non-smokers by M/L55 genotype.

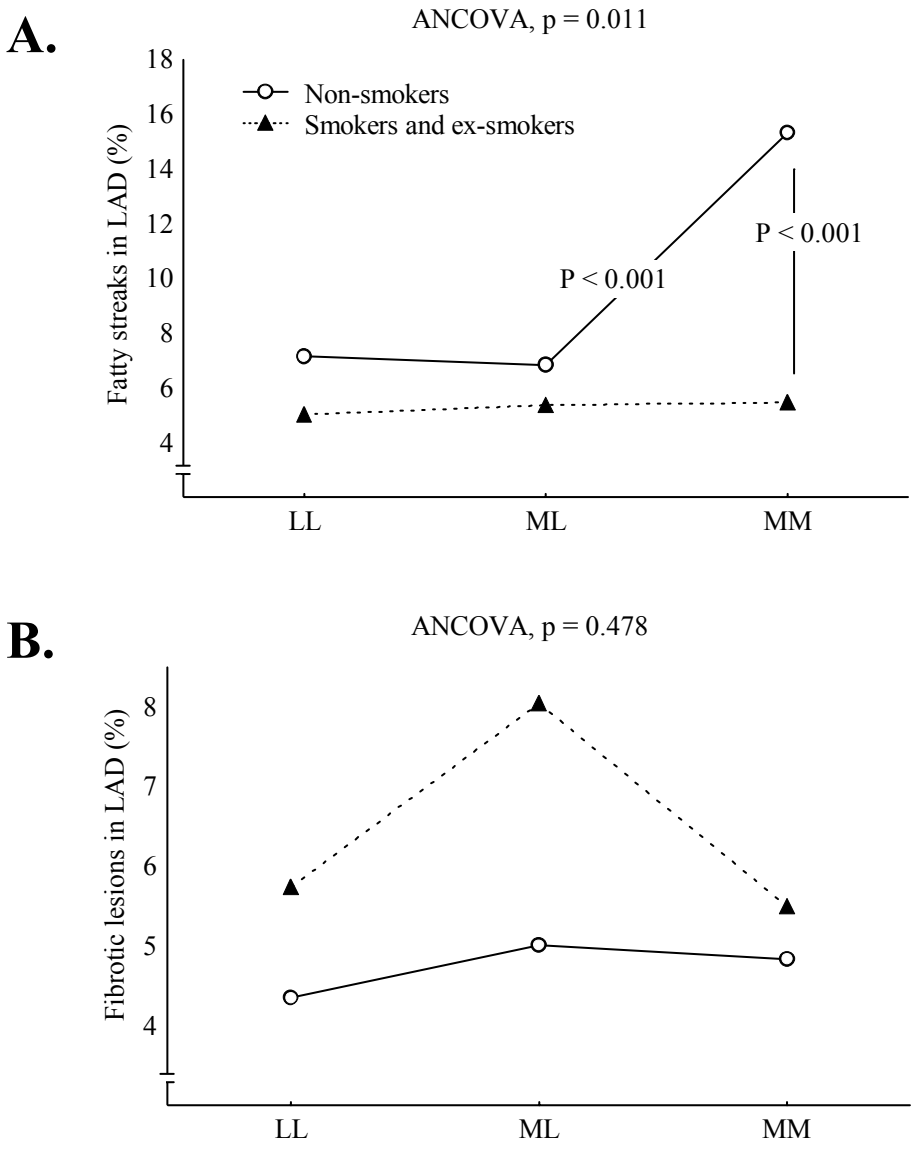

Figure 9. (A) PON1 M/L55 genotype-by-smoking interaction on the area of fatty streaks in the left anterior descending coronary artery (LAD). (B) Area of fibrotic lesions in smokers and non-smokers by M/L55 genotype. In ANCOVA, the M/L55 genotype, smoking and alcohol use were used as factors and age and body-mass index as covariates. In a case of a significant interaction, the Least Significant Difference post-hoc test was used to study the differences between groups.

DISCUSSION

1. Study subjects

In the present study three clinical and two autopsy series provided data to examine the relation between PON1 genotypes and biochemical CHD risk factors and different phases of atherosclerosis. The series consisted of about 1040 Finnish males and females that were unrelated to each other. In general, the Finnish population is particularly suitable for studies of genetic components that contribute to complex diseases such as atherosclerosis: Finns are genetically homogenous due to a founder effect and a history of genetic isolation (Peltonen et al. 1995) and there is a high prevalence of CHD (Vartiainen et al. 1994, Salomaa et al. 1996). In Finland, molecular information can easily be combined with detailed information on subject's diseases. However, because of the isolation and regional differences of the sparsely populated country, it is important to select controls and cases for the case-control studies from the same area.

Subjects in the clinical studies. Subjects in studies I and III were young and healthy male coast guards and firemen. They had only mildly elevated serum total cholesterol levels and normal PET measurements and, therefore, it is highly unlikely that they had significant stenosis in their coronary arteries. Because of their occupation they can be considered to be healthier and in better physical condition than the "average Finnish male" of similar age. It would, therefore, be important to confirm the findings in men and women with detectable atherosclerotic lesions. Also, the relationship between PON1 genotypes and lipid concentration changes during pravastatin treatment in persons with severe hypercholesterolemia remains to be determined. Moreover, due to small number of subjects, it would be interesting to repeat this study with a larger study population although the PET technique is expensive and laborious. The power of the test measuring the differences in the blood flow at rest between PON1 genotype groups was, however, high suggesting that the differences observed are statistically valid.

Study II was a case-control study of type 2 diabetes patients and their age and gender matched control subjects who were followed up for nine years before re-evaluation. At the time of the follow-up, those control subjects that had an elevated fasting blood glucose level were excluded and finally 55 diabetics and 55 controls were

found to be appropriate for the case-control study. The study-design favors the possibility of survival bias among diabetics since during the nine-year follow-up time some of them had died. However, both diabetic and control subjects were well-characterized and, in addition to gender, the analyses were controlled for the confounding factors that were different in diabetic and control subject groups.

In study IV, the original study group consisted of 300 randomly selected middle-aged men from ten age-cohorts. Randomized sampling avoided major selection bias, although some bias is possible since 26% of the invited men refused (n = 33) or could not be reached (n = 44). CHD was diagnosed according to medical history or electrocardiographically but not angiographically. It is thus likely that some of the men had advanced atherosclerotic lesions.

Subjects of the autopsy series. Conventional cross-sectional and retrospective case-control studies can easily lead to survival bias, because only patients with non-fatal events can be studied. This can be avoided in autopsy studies. Autopsy studies can, however, carry selection bias, since it is possible that these subjects have more severe atherosclerosis than subjects selected randomly. In study V, a medical or forensic autopsy was performed, whereas in study VI the men were victims of sudden death or trauma. The CHD risk factors were different in these studies: In study VI, many men were heavy alcohol drinkers (median 60 g/day), and although the results were adjusted for this risk factor, it is relatively difficult to predict the co-timing effects of alcohol and smoking, for example. The PON1 genotype frequencies in these studies were comparable to other studies from Finnish population (Antikainen et al. 1996, Salonen et al. 1999) and, therefore, one may assume that the autopsy series were representative samples of Finnish middle-aged or elderly subjects.

2. Methodological considerations

Candidate gene approach and association studies. Two main approaches have been used for candidate gene studies. Family-based studies involve examination of inheritance patterns for genetic polymorphisms while case-control studies compare genotype frequencies for candidate disease genes in unrelated individuals with and without the disease (Daly and Day 2001). Although family studies are generally to be

preferred, population-based case-control studies with appropriately matched cases and controls usually have to be used when multifactorial diseases such as atherosclerosis are studied. Case-control studies are highly efficient in detecting associations but they require careful study design (Daly and Day 2001). Studied and discovered associations should make biological sense and alleles should affect the gene product in a physiologically meaningful way. In the present study, *PON1* was chosen as a candidate gene because of the differences in the biological function between the PON1 isoforms and assumed association with CHD and lipoprotein oxidation in humans.

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 autopsy studies V and VI different stages of atherosclerosis were evaluated, ranging from fatty streaks to more advanced lesions. These studies were undertaken in an effort to obtain evidence about the risk factors at different time-points of disease development. Although the severity of the disease can be accurately evaluated there may also be some drawbacks. In study VI, the classification of atherosclerotic lesions was done by visual inspection after staining of the arterial samples with Sudan IV according to the protocol of the IAP (Guzman et al. 1968). The first problem with this method is the classification of fatty streaks, since some of the fatty streaks that develop in regions with adaptive intimal thickening are deeper under the endothelium, and may not become visible by staining (Stary et al. 1994). Another problem is that after death, some regions of adaptive intimal thickening may project into the lumen and can be mistaken for as raised lesions. Today, a new standardized histological classification method is available (Stary et al. 1992, Stary et al. 1994, Stary et al. 1995), but this was not the case at the time of data collection to studies V and VI.

Ultrasound methodology. B-mode ultrasound imaging is a widely used technique to study relatively large arteries, such as the carotid and femoral arteries. The ultrasonographic endpoint, combined thickness of media and intima, correlates significantly with the main risk factors for atherosclerosis (Salonen and Salonen 1993). There is also a strong relationship between arterial wall thickness, as defined by B-mode imaging, and CAD (Heiss et al. 1991). 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 (Mercuri 1994). B-mode ultrasound may, however, underestimate large or complicated plaques and lack precision in detecting total occlusions. It cannot distinguish fatty streaks from localized intima-media thickening; dense fibrosis and calcified areas are easier to detect because they are more echogenic (Salonen and Salonen 1993). Ultrasound images are also difficult to interpret in the presence of anatomic variations of the arteries such as coiling (Zwiebel 1986). To obtain acceptable measurement reproducibility by B-mode ultrasound, it is essential to control for the effects of instrument and operator variability. In study IV, all recordings were performed by the same certified sonographer and the images were interpreted and measured at the reading center by one trained reader. This method guaranteed the highest validity and reproducibility. The measurement of MMax IMT, which is an average value of the IMT of 12 standard sites, gives a good picture of the involvement of early atherosclerotic changes in the whole carotid artery tree (Mercuri 1994). The relatively high cut-off limit (1.7 mm) to define CAAD status prevented misclassification of subjects.

PET methodology. PET is a unique method that enables quantification of myocardial blood flow in absolute terms (Gould 1998). The detection is based on two photons created in annihilation reactions between a positron (infused into the circulation) and an electron from tissue. By measuring blood flow at rest and after pharmacological stimulation with dipyridamole or adenosine it is possible to determine CFR. CFR becomes impaired even in apparently healthy young individuals with risk factors for atherosclerosis (Pitkänen et al. 1997). CFR is known to be impaired also in familial hypercholesterolemia (Pitkänen et al. 1996), in type 2 diabetics (Clarkson et al. 1996), in smokers (Campisi et al. 1998) and in hypertensive subjects (Laine et al. 1998).

The advantage of PET is its rather non-invasive nature and in this regard, $[^{15}O]H_2O$ seems to be an ideal tracer for the measurement as it is chemically inert and freely diffusible. Because of the short half-life of the radioactive label, it is possible to repeat the measurement after intervention, e.g., to assess the effects of lipid-lowering medication (Guethlin et al. 1999). PET also affords serial measurements of blood flow at less than 10 minutes intervals. The limitation of study III is that adenosine-induced

vasodilatation, or CFR, is not a pure marker of endothelial function but rather a combined index of endothelial function and smooth muscle relaxation (Dayanikli et al. 1994). Nor is CFR an ideal index to evaluate narrowing in the epicardial coronary arteries, but rather a functional parameter of the whole coronary system (Raitakari 1999).

3. The effect of PON1 on lipid and apolipoprotein levels (I, IV)

According to studies I and IV, PON1 genotypes do not clearly affect lipid or apolipoprotein concentrations in healthy subjects not on lipid-lowering medication. The borderline baseline differences in HDL, apo AI and apo B concentrations between PON1 genotypes in study I were thus most likely fortuitous and due to small group sizes. In study I, both the PON1 R/Q192 and the M/L55 genotypes were the significant predictors of apo AI and HDL cholesterol changes during pravastatin therapy. PON1 haplotype analysis indicated that the R/Q192 genotype is probably a more powerful factor in this respect than the M/L55 genotype. Subjects carrying the R192 allele and having low HDL levels might benefit more from pravastatin therapy since the increase in apo AI and HDL was greatest in them. The strength of the PET study lies in the fact that it used randomized, placebo-controlled and double blind study design.

The number of previous intervention studies on the relationship between PON1 genotypes and lipid concentrations is very limited. In one previous study, no significant differences were found between R/Q192 or M/L55 genotype groups concerning serum concentrations of lipids and lipoproteins at baseline or after simvastatin treatment in a group of 64 familial hypercholesterolemia patients (Tomas et al. 2000). The serum PON1 activity was, however, increased during treatment with simvastatin. In line with this result, another study showed that simvastatin enhances the transcriptional activity of the *PON1* gene promoter (Leviev and James 2000). Promoter polymorphism at position -108 was involved in this action so that the effect was blunted when there was T instead of C at this position. According to this and our findings, we can hypothesize that also pravastatin could change the transcriptional activity of *PON1* and that one or more promoter polymorphisms determines how efficiently the transcriptional activity is changed. The interaction between the coding region polymorphisms and promoter

polymorphisms would then link also the R/Q192 and M/L55 polymorphisms on the statin-affected level of serum PON1. The change in the PON1 concentration and activity could then affect lipid metabolism by one or several hypothetical mechanism(s): First, PON1 may stimulate cellular cholesterol efflux, which is the first step in reverse cholesterol transport (Aviram et al. 1998c). PON1 could also affect the efficiency of lipid transfer between HDL and LDL cholesterol. Second, a low PON1 activity or concentration could allow the accumulation of substrate(s) of PON1 in plasma, which could then activate or inhibit some enzymes in the lipid metabolism pathway with resultant changes in HDL level and lipoprotein composition. Third, PON1 could be involved in the metabolism of statins (Aviram et al. 1998b, Billecke et al. 2000, Tomas et al. 2000).

4. The effect of PON1 on oxidation of lipids (II, III)

F_2-isoprostanes. In study II, PON1 LL homozygotes had a higher excretion of the urinary oxidative stress marker 8-iso-$PGF_{2\alpha}$ than the carriers of the M55 allele and this association persisted after correction for GFR. This finding suggests that PON1 can destroy some lipid peroxides that lead to the formation of 8-iso-$PGF_{2\alpha}$ and that PON1 with the LL genotype is the least effective in this action. This finding is thus in line with some previous studies indicating an increased risk of atherosclerosis among subjects with the LL genotype although, based on study II, it is still too early to conclude that PON1 with the LL genotype is the least efficient in destroying all kinds of lipid peroxidation products. Study II also confirms some previous findings indicating increased excretion of 8-iso-$PGF_{2\alpha}$ in type 2 diabetes patients. Smokers tended to have increased levels of 8-iso-$PGF_{2\alpha}$ in their urine, supporting the theory that systemic oxidative stress is enhanced among smokers.

F_2-isoprostanes are formed as a result of free radical-catalyzed peroxidation or autoxidation of arachidonic acid. Therefore, precautions must be taken to prevent artifactual formation of them by autoxidation during sample processing and storage. However, autoxidation is not considered as a problem in urine samples compared to plasma based assays, where the content of arachidonic acid is much greater (Pratico et al. 1998). Nevertheless, all immunoassays that measure 8-iso-$PGF_{2\alpha}$ should be

considered as semiquantitative indices of "8-iso-$PGF_{2\alpha}$-like immunoreactivity" due to some cross-reactivity with other F_2-isoprostanes or their metabolites.

In vitro susceptibility of LDL to oxidation and the autoantibody titer against ox-LDL. PON1 R/Q192 or M/L55 genotypes in study III were not associated with the *in vitro* susceptibility of LDL to oxidation or with the autoantibody titer against ox-LDL. This suggests that PON1 polymorphisms have no effect on LDL oxidation and, therefore, also the observed association between PON1 R/Q192 polymorphism and coronary vasomotion (study III) seems to be unrelated to LDL oxidation. We can, however, question how well the autoantibody titer against ox-LDL reflects the amount of ox-LDL in the intima and endothelium which regulate coronary vasomotion. Moreover, the autoantibody titer against ox-LDL is not comparable to the ability of HDL to prevent LDL oxidation and, therefore, the results of study III do not conflict with the results of previous studies which suggested that HDL isolated from persons with different PON genotypes has differential effects on *in vitro* LDL oxidation. Also, the observed higher concentration of antioxidants in the R192 allele carriers and LL homozygotes compared with QQ homozygotes and M55 allele carriers may have protected these subjects against lipid oxidation and concealed the possible differences between PON1 genotype groups in the ability to prevent oxidation.

Defense systems against oxidation. PON1 is obviously not the only component in the complex defense system against oxidation and atherosclerosis. All the enzymatic components of HDL, and possibly also apolipoproteins, may act in concert, in competition or even in opposite directions to eliminate the products of lipid oxidation. For example, apo J is well-known for its marked induction in a variety of pathological conditions including MI (Silkensen et al. 1998) and it has been suggested that the apo J/PON1 activity ratio may be a better predictor of atherosclerosis than the total cholesterol/HDL cholesterol ratio (Navab et al. 1997). In addition to the components of HDL, the role of the cytochrome P_{450} system has perhaps been underestimated. Cytochrome P_{450} catalyzes the formation of the highly toxic oxon forms of OP compounds in the liver that are subsequently hydrolyzed by PON1. Information about specific cytochrome P_{450} isoforms may thus be useful in addition to PON1 status in predicting a response to a specific compound.

Avoiding LDL oxidation at all costs? PON1, in tandem with apo AI has recently been suggested as a system that minimizes the accumulation of PC oxidation products through hydrolysis of PC isoprostanes and core aldehydes to lyso-PC (Ahmed et al. 2002). This observation is unexpected, since lyso-PC is believed to play a role, e.g., in the recruitment of monocytes and, therefore, might be considered as a pathogenic end product of lipid peroxidation (Hajjar and Haberland 1997). Although lyso-PC has several biological effects, its precursors, oxidatively truncated PCs, have more varied and potent effects on vascular cells (Tanaka et al. 1993). Many of the biological effects of ox-LDL, including the induction of SMC proliferation and of monocyte chemotactic protein-1 and adhesion molecules have been attributed to these short chain polar PCs (Goyal et al. 1997). The hydrolysis of these potentially deleterious products to lyso-PC can, therefore, be considered as a defensive mechanism that protects the vascular cells (Goyal et al. 1997). HDL is probably also a haven for the release of lyso-PC; HDL is apparently not cytotoxic, and it seem plausible that lyso-PC is metabolized and disposed further in HDL (Mackness and Durrington 1995).

If ox-LDL is atherogenic and if oxidation products derived from polyunsaturated fatty acids are deleterious to cardiovascular health, we face an interesting question. Should we avoid oxidation of fatty acids in every possible way that we can? At least in animal models, polyunsaturated fat is beneficial compared to saturated fat (Rudel et al. 1990), although it has an increased propensity to undergo oxidation. Similarly, the beneficial effects of moderate exercise in preventing cardiovascular disease are well known although exercise increases oxygen consumption, depletes antioxidants and increase the susceptibility of LDL to undergo oxidation (Wetzstein et al. 1998). If oxidation occurs, it may thus not necessarily lead to harmful effects since macrophages recognize these ox-LDL particles and forestall their damaging effects.

It is very unlikely that the antioxidant function of HDL has evolved to protect humans against atherosclerosis, a disease that has been prevalent for less than a century. HDL is the most abundant protein in tissue fluid and the only lipoprotein in the central nervous system. The ability of HDL to move around different tissues could be related to its function as a protector of all kinds of cell membranes against a variety of toxins. LDL thus perhaps only resembles cell membranes and therefore shares in this protection. It may thus be hypothesized that the natural organophosphate toxins and

numerous other exogenous and endogenous esters, such as homocysteine thiolactone, other lactones and cyclic carbonates which PON1 can detoxify by catalyzing their hydrolysis (Durrington et al. 2001), are in fact the natural substrates for PON1.

5. PON1 genotype and coronary function (III)

In study III, men that carried the R192 allele had lower coronary blood flow at rest than men that were QQ homozygotes, while adenosine-stimulated flow was similar. This observation implies that the PON R/Q192 genotype does not clearly contribute to the early changes in coronary reactivity, which stands in contrast to several traditional risk factors for CAD. The potential mechanisms underlying the difference in resting blood flow between PON1 genotype groups are only speculative since the regulation of vascular tone involves a number of complex metabolic, endothelial and neural mechanisms. Coronary flow, reflecting the events of the entire coronary vasculature, is regulated by the oxygen demand of the myocardium and it depends on vasomotor function of both epicardial arteries and the microcirculation.

Atherosclerosis influences arterial dilatation and tone. In its early stages, coronary reactivity is impaired due to increased amounts of ox-LDL (Raitakari et al. 1997). An increased production of superoxide anions by ox-LDL seems to be a major cause for this impairment (Harrison and Ohara 1995). In addition, reduced expression of endothelial nitric oxide synthase by ox-LDL may be involved (Liao et al. 1995). Ox-LDL increases also the effect of vasoconstrictor hormones (Galle et al. 1990). One may thus suggest that the PON1 genotype mediates coronary blood flow at rest by modulating the amount of ox-LDL in the vessel wall. This hypothesis is not supported by the findings in study III, which showed that the PON1 genotype is not associated with the *in vitro* susceptibility of LDL to oxidation or with the titer of autoantibodies against ox-LDL.

One previous study has examined the association between PON1 R/Q192 polymorphism and coronary vasomotor tone (Bauters et al. 2000). The subjects in that study had clinical manifestations of CAD and they underwent invasive testing by intracoronary administration of serotonin. Q192 homozygotes had a greater mean percentage reduction at proximal and distal segments than RQ heterozygotes, which

could be related to a higher synthesis or release of endothelium-derived relaxing factors in RQ heterozygotes. This study is, however, not comparable to study III, since serotonin induces vasodilatation of healthy human coronary arteries but acts as a vasoconstrictor in patients with CAD (Golino et al. 1991). Another study measured brachial artery reactivity at baseline and in the presence of acute hypertriglyceremia that was obtained by infusion of Intralipid®. Hypertriglyceremia has been shown to be a prooxidant factor and to impair endothelial response in healthy subjects (Lundman et al. 1997). Infusion of Intralipid® was associated with a significant reduction in the flow-induced change in diameter in all genotype groups, but the reduction was largest in RR homozygotes. The authors suggested that this was due to a lower antioxidant capacity and, consequently, to more severe endothelial dysfunction in RR homozygotes. Based on previous and the present studies, PON1 R/Q192 polymorphism, and possibly also M/L55 polymorphism, plays a role in the regulation of coronary vasomotor tone and coronary blood flow but, thus far, no solid evidence has been presented to support the hypothesis that lipid oxidation is involved in this action.

6. PON1 genotype and carotid artery intima-media thickness (IV)

In study IV, non-smoking L55 homozygotes had higher MMax IMT than M55 allele carriers. Logistic regression analysis showed also that the odds ratio for CAAD among non-smokers was higher in men with the LL genotype than in M55 allele carriers. This suggests that the LL genotype is a risk factor for CAAD. Based on this finding the association between the LL genotype and CAD remains obscure although a connection has been described between CAD and CAAD (Craven et al. 1990). Since CAAD is considered to be a major cause of ischemic stroke (Yatsu 1986), the LL genotype can be considered as a risk factor for stroke in non-smoking men. In line with this result, the LL genotype was found to be an independent predictor of carotid atherosclerosis in a previous study (Schmidt et al. 1998). In that report the R/Q192 genotype had no effect on carotid atherosclerosis and did not modulate the effect of the L55 allele. That study did not specifically address the question of the impact of smoking. We observed that in smokers, contrary to non-smokers, the M55 allele carriers tended to have higher MMax IMT values in the segments of the carotid artery than the LL homozygotes, suggesting

that the ability of PON1 to protect against CAAD is influenced by cigarette smoke and that the efficiency of this effect is somehow associated with the M/L55 genotype.

A limitation of study IV was the lack the stored serum samples from the participants and, therefore, it was not possible to measure PON1 concentration and activity to test the effect of M/L55 genotype and smoking on these parameters. Nor was the effect of the PON1 genotype on LDL oxidation studied.

7. PON1 genotype and early and advanced atherosclerotic lesions (V, VI)

Mesenteric arteries. In study V, male M55 allele carriers had more fatty streaks but simultaneously also less complicated lesions in their mesenteric arteries than LL homozygotes. In women, similar trends were seen but they were not statistically significant. This suggests that, in Finnish men, the PON1 LL genotype can be included into the risk factors for mesenteric atherosclerosis. The larger number of stenotic lesions in the SMA and thicker intima in IMA among male LL homozygotes compared with M55 allele carriers supports these findings. According to study V, R/Q192 polymorphism is not a risk factor for mesenteric atherosclerosis in the Finnish population. This observation is in line with a previous study reporting a lack of association between R/Q192 polymorphism and CHD in Finns (Antikainen et al. 1996) although these two studies are not totally comparable because the subjects in the previous study were survivors of coronary bypass surgery.

Aorta. The M/L55 genotype was not statistically significantly associated with atherosclerotic changes in the abdominal aorta in study V, although LL homozygotes tended to have more severe atherosclerotic changes compared with M55 allele carriers. The PON1 R/Q192 genotype seemed to affect the lesion area in aorta in males but the prevalence of fatty streaks and more advanced lesions was not different in R192 allele carriers and QQ homozygotes. In study VI, however, the MM homozygotes harbored larger fatty streak areas in their thoracic and abdominal aorta than did L55 allele carriers. Men under 54 years showed a more significant association, suggesting that the effect of the M/L55 genotype could be age-dependent and more clearly seen in younger men and in the early phase of the development of atherosclerosis. The fibrotic lesion area was not statistically significantly different between PON1 M/L55 genotypes,

although it tended to be smaller in MM homozygotes. The complicated lesion area in the abdominal aorta was smaller among MM homozygotes than among L55 allele carriers.

Coronary arteries. In study VI, non-smoking carriers of the MM genotype had less advanced plaques harboring larger areas of fatty streaks in their LAD and RCA but there were no differences between the M/L55 genotype groups in smokers. This suggests that the effect of M/L55 polymorphism on coronary atherosclerosis is dependent on smoking. Again, the association between fatty streaks and M/L55 genotype was observed especially among younger men. In the coronary arteries, as was the case in the aorta, the areas of fibrotic and complicated lesions were not significantly different among the PON1 M/L55 genotype groups, although the fibrotic lesion areas tended to be smaller in MM homozygotes.

Why are PON1 genotypes associated with atherosclerotic lesion areas? The association between PON1 M/L55 genotype and fatty streak area in studies V and VI is in line with the current knowledge that PON1 plays a role in LDL oxidation and in the development of atherosclerosis. In studies V and VI, MM homozygotes had more fatty streaks in their arteries compared with L55 allele carriers; only in the mesenteric arteries did the LL homozygotes have more advanced lesions. This association was absent regarding the coronary arteries. This raises an interesting question: Is PON1 with the M55 allele more efficient in preventing LDL oxidation in all circumstances and thus more effective in preventing atherosclerosis?

Most of the epidemiological studies thus far have not considered the variability of the levels of PON1 protein within each PON1 genotype group in sufficient detail although this variability is probably as important as the genotype alone and can have a significant impact on LDL oxidation and on the development of atherosclerosis (Furlong et al. 1989). The importance of the PON1 level is supported by a recent experimental study showing that PON1 protects against atherosclerosis *in vivo* in a dose-dependent manner (Tward et al. 2002): Human PON1 transgenic mice that had a two to four fold increased plasma PON1 levels were protected more effectively against both the early fatty streaks and the intermediate to advanced types of lesions than wild-type mice. In humans, M/L55 polymorphism influences the level of serum PON1: the M55 allele is associated with a lower serum concentration of the PON1 enzyme than the

L55 allele (Garin et al. 1997), which is due to a lower expression of M55 type mRNA in the liver (Leviev et al. 1997). Considering only the concentration of PON1, it is not surprising that MM homozygotes have an increased risk of MI compared to men who do not carry the M55 allele (Salonen et al. 1999). This finding could be related to an increased amount of fat-containing, thin-walled and rupture-prone coronary plaques in these subjects. Some experimental evidence suggests also that a decrease in serum PON1 activity may occur as a part of an inflammatory response. What if also acute inflammatory conditions reduce PON1 activity and increase LDL oxidation? This could lead to foam cell generation in a critical part of a preexisting atheroma, which could weaken its fibrous cap and predispose to plaque rupture and acute MI (Durrington et al. 2001).

PON1 is also known to be partially inactivated by lipid peroxides (Aviram et al. 1999), and serum PON1 activity is decreased in CHD and in type 1 and type 2 diabetes (McElveen et al. 1986, Abbott et al. 1995), conditions well known to be associated with increased concentration of lipid peroxidation products (Liu et al. 1992). These findings suggest that in certain conditions the PON1 concentration might be a more important factor in determining the progression of atherosclerosis than the PON1 activity. This could explain the association of the MM genotype with a larger fatty streak area, an observation which could be regarded as unexpected since the HDL with the PON1 MM genotype should retard LDL oxidation more effectively than HDL with the PON1 LL genotype (Mackness et al. 1998a).

On the other hand, L55 allele carriers in study VI tended to have more fibrotic plaques in their coronaries and aorta, which is in line with study V and with the fact that MM homozygotes have increased *in vitro* activity against lipid peroxidation compared with L55 allele carriers. Thus, in MM homozygotes the elements of repair in artery walls, including PON1, might be strong enough to delay the process of atherosclerosis, since the amount of advanced lesions among MM homozygotes tends to be smaller than in other genotype groups while the amount of fatty streaks lesions is increased. Since the amount of advanced lesions in the coronaries was not different in PON1 genotype groups, other factors than PON1 might be more important for the development of plaque to a more advanced type. This obviously leaves the question of whether the

M/L55 genotype of PON1 is of clinical importance unanswered, since clinically relevant cardiovascular events are often associated with advanced lesions.

Limitation of studies V and VI. Studies V and VI were based on post-mortem examinations and, therefore, it was not possible to collect samples for assessment of PON1 activity and concentration. The lack of activity and concentration data makes it impossible to conclude that PON1 genotypes are associated with atherosclerotic lesion areas through their different abilities to hydrolyze oxidized lipids. The interpretation of the results has to take into account the possibility that M/L55 and R/Q192 are not the functional mutations but may mark a functional mutation present in either *PON1* (promoter polymorphisms) or a nearby gene (*PON2, PON3*). It is more likely, however, that there is a net of interactions and linkages between all *PON* genes and their polymorphisms.

8. Smoking as an acquired PON1 modifying factor (IV, VI)

The interaction between genotype and environment is a poorly understood biological phenomenon, and because of its complexity, it is usually ignored when the associations between genetic variation and interindividual phenotypic variation are interpreted. However, especially in multifactorial diseases, such as CAD, the impact of a particular allelic variation in a gene depends on the environment in which the gene is expressed (Zerba and Sing 1993).

Smoking is known to roughly double the lifetime risk of CHD by several mechanisms, one of which is the increase in oxidative stress and lipid peroxidation (Heitzer et al. 1996). Because of dietary habits, smokers have also usually lower levels of antioxidants, which may favor the oxidation of LDL (Fickl et al. 1996). In two previous studies (Nishio and Watanabe 1997, James et al. 2000a), but not in one (Bielicki et al. 2001), PON1 activity was inhibited by cigarette smoke, suggesting that PON1 may play a role in the smoking-associated CHD risk. In studies IV and VI, the association between smoking and PON1 M/L55 genotype and atherosclerosis was studied. In study IV, non-smoking men with the LL genotype had an increased risk of carotid artery intima-media thickening compared to men carrying the M55 allele. Among smokers, in turn, PON1 M55 carriers had an increased risk of intima-media

thickening; the effect of M/L55 genotype on carotid atherosclerosis was almost opposite among smokers and non-smokers. In study VI, the carriers of the MM genotype had a larger mean percentual area of fatty streaks in their coronary and aortic plaques than carriers of the L55 allele, but this difference was not present in smokers. The significant interaction between M/L55 genotype and smoking on the area of fatty streaks in coronaries implies, however, that the effect of the M/L55 genotype on coronary atherosclerosis is dependent on smoking. Based on our studies, the smoking status might be an important factor explaining the disparate results in previous studies concerning PON1 genotypes and CHD risk, since smokers and non-smokers have not usually been analyzed separately. The interaction between smoking and the M/L55 genotype might also explain the controversy between three previous studies concerning the action of cigarette smoke on PON1 activity. With regard to the effects of PON1 at the population level, the genetic history of the subjects and the presence of different risk factors for CHD seem to play a key role in determining the association between PON1 genotype and CHD. Therefore, carriers of a certain PON1 genotype may be protected against some risk factors but not against some others. The effect may not be the same on all arterial segments.

9. Future prospects

Several well-documented environmental variables are currently known that influence serum lipid levels and increase the risk of CHD, including dietary fat, smoking, alcohol consumption and low exercise level (Talmud and Humphries 2002). The effect of some of these factors on the activity or concentration of PON1 has already been tested, but there is a growing need to know more about the variables that modify the actions of PON1, especially in a PON1 genotype related manner. Gene-environment interaction studies provide new ideas in the field of genetic studies and despite several challenges such studies are urgently needed (Wallace et al. 2000). Several simultaneous gene-gene and gene-environment interactions, i.e., PON1, smoking and alcohol use, will, however, complicate the interpretation of the results.

The search for alleles that are more frequently associated with a particular stage of atherosclerosis than would be expected by chance is not new, and given the current

excitement over polymorphisms and DNA chips it is likely that even more association studies will be performed in the future. In such studies it may, however, be insufficient to focus only on a particular genetic polymorphism. The term "PON1 status", which incorporates both PON1 genotype (usually R/Q192) and plasma PON1 level (determined in part by M/L55 polymorphism), was introduced several years ago (Li et al. 1993) but it has not been widely used in atherosclerotic research. Because the level and activity of PON1 are substantially related to dietary, lifestyle and environmental factors, the PON1 status may be more informative in association studies examining the role of PON1 in susceptibility to vascular diseases than the genotype alone.

Based on the present study, it would be interesting to elucidate if subjects with a particular PON1 haplotype will especially benefit from certain antiatherosclerotic therapy, such as lipid-lowering medication or antioxidant supplementation. It might also be useful to determine if the PON1 haplotype affects the catalytic efficiency of some antiatherosclerotic drugs and whether there is a correlation between the drug half-lives and PON1 status (i.e., combined effect of polymorphisms and plasma PON1 levels). In the future, it may also be productive to focus on the antioxidant properties of PON1. Random and site-specific mutagenesis has been tested to engineer PON1 variants with a high catalytic efficiency to hydrolyze nerve agents and OP insecticides (Furlong et al. 2002). It remains to be determined whether recombinant human PON1 can be used for the treatment of vascular diseases in the future. Given the importance of environmental influences and the complex genetic etiology of atherosclerosis, it is, however, unlikely that such therapies will be available very soon. Before such sophisticated therapies are available, the contribution of each potential antiatherosclerotic enzyme system in appropriately genetically-modified strains of mice or in *in vitro* models will be needed to be known. An understanding of such enzyme systems will hopefully help us to clarify the specific roles of different pathways in xenobiotic metabolism as well as in normal physiological processes.

SUMMARY AND CONCLUSIONS

Three clinical and two autopsy studies were performed to elucidate the association between the M/L55 and R/Q192 genotypes of PON1 and serum lipid levels, changes in lipid concentrations during pravastatin treatment, oxidation of lipids, regulation of coronary vasomotor tone and reactivity, intima-media thickening and the development of early and advanced atherosclerotic plaques in coronary arteries, the aorta and mesenteric arteries. The purpose was also to study whether smoking can modify the association between PON1 genotypes and manifestations of atherosclerosis. The main findings and conclusions of this thesis are:

1. PON1 genotypes were not consistently related to lipid or apolipoprotein concentrations in healthy men with no lipid medication (study I and IV). Both R/Q192 and M/L55 genotypes were, however, significant predictors of the apo AI and HDL cholesterol concentration changes during pravastatin therapy. The increases in apo AI and HDL cholesterol levels were greatest in men carrying the R192 allele and they might thus benefit especially much from pravastatin therapy.

2. Subjects carrying the PON1 LL genotype had higher concentration of a urinary oxidative stress marker 8-iso-$PGF_{2\alpha}$ than subjects carrying the M55 allele (study II). This finding may reflect the impact of PON1 M/L55 polymorphism on the regulation of lipid oxidation which leads to the formation of 8-iso-$PGF_{2\alpha}$. This result is in line with the findings showing more plaques and complicated lesions in the mesenteric arteries (study V) and higher MMax IMT in the carotid arteries (study IV) in non-smoking subjects with the LL genotype. The PON1 R/Q192 or M/L55 genotypes were, however, not associated with the other indices of LDL oxidation, i.e., the susceptibility of LDL to oxidation and the autoantibody titer against ox-LDL (study III). R192 allele carriers and LL homozygotes had higher concentration of antioxidants compared with QQ homozygotes and M55 allele carriers, which may have protected these subjects against peroxidation and hampered the interpretation of the results.

3. In the PET study III, the PON1 R/Q192 or M/L55 genotype did not clearly contribute to the changes in coronary reactivity and CFR. However, male R192 allele carriers had lower coronary blood flow at rest compared to QQ homozygotes, suggesting that R/Q192 polymorphism may play a role in the regulation of baseline coronary blood flow and vasomotor tone. The study did not confirm the hypothesis that the ability of PON1 to affect lipid oxidation is involved with this finding.

4. In a random sample of Finnish middle-aged men (study IV), non-smoking subjects carrying the PON1 LL genotype had higher MMax IMT of their carotid arteries than subjects carrying the M55 allele. This suggest that the LL genotype is a risk factor for CAAD and perhaps also for stroke.

5. In the autopsy studies V and VI, non-smoking MM homozygotes of PON1 M/L55 polymorphism had more fatty streaks in their mesenteric and coronary arteries and in their aorta compared with L55 allele carriers. In the mesenteric arteries, the LL homozygotes had more plaques and complicated lesions compared with the M55 allele carriers, which in line with the results of studies II and IV. A similar statistically significant association between advanced plaques and LL genotype was not the case regarding the coronaries although the fibrotic lesion area in the coronary arteries tended to be smaller among the MM homozygotes.

6. Among middle-aged men (study IV) smoking M55 allele carriers seemed to have an increased IMT in the carotid arteries compared to smoking LL homozygotes. Interestingly, the effect of the M/L55 genotype on carotid atherosclerosis tended to be almost the opposite among smokers and non-smokers indicating significant gene-environment interaction. In study VI, non-smoking carriers of the MM genotype had a larger mean percentual area of fatty streaks in their coronary arteries than carriers of the L55 allele, but this difference was not present in smokers. These findings imply that the effect of the M/L55 genotype on carotid and coronary atherosclerosis is dependent on smoking.

Table 6 summarizes the antiatherosclerotic and proatherosclerotic effects of the M/L55 and R/Q192 alleles of PON1 that were found in this thesis. In conclusion, this series of studies shows that both the M/L55 and the R/Q192 genotype of PON1 seem to be important genetic factors related to the development of atherosclerosis, which may be due to differential abilities of PON1 isozymes to destroy certain ox-LDL species. The effects of PON1 genotypes are more clearly seen in the very early stages of atherosclerosis and in the development of fatty streak lesions. The impact of PON1 genotypes on the manifestation of atherosclerosis is, however, substantially related to smoking, and possibly also to other dietary, lifestyle and environmental factors, and may not be the same in all arteries or in different anatomical locations of the same artery. By understanding the risk factors, including genetics and gene-environment interactions, it might be possible to develop new strategies for the prevention of atherosclerosis and its complications.

Table 6. Summary of antiatherosclerotic, proatherosclerotic and negative findings of this thesis of the M/L55 and R/Q192 alleles of PON1 among non-smoking, non-diabetic male subjects

	Antiatherosclerotic findings	Proatherosclerotic findings	No effect on atherosclerosis
M55 allele	Lower level of urinary 8-iso-$PGF_{2\alpha}$*, lower IMT in carotid arteries*, less advanced lesions in mesenteric arteries.	More fatty streaks in coronaries*, aorta and mesenteric arteries.	Genotypes were not consistently related to serum lipid levels. The susceptibility of LDL to oxidation and the autoantibody titer against oxidized LDL was similar in PON1 genotype groups. No statistically significant effect on coronary flow reserve or the area of advanced lesions in coronaries was detected.
L55 allele	Less fatty streaks in coronaries*, aorta and mesenteric arteries.	Higher level of urinary 8-iso-$PGF_{2\alpha}$*, higher IMT in carotid arteries*, more advanced lesions in mesenteric arteries.	
Q192 allele	Higher coronary blood flow at rest.		
R192 allele	HDL and apo AI levels increased most effectively during pravastatin therapy.	Lower coronary blood flow at rest.	

ABBREVIATIONS: IMT, intima-media thickness; 8-iso-$PGF_{2\alpha}$, 8-iso-prostaglandin $F_{2\alpha}$.

*In smokers, such an association was not detected or it was reversed.

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