PERTTU PÖLLÄNEN

Matrix Metalloproteinase 3 and 9 Cis-regulatory Variation and Atherosclerotic Lesions

■

ACADEMIC DISSERTATION

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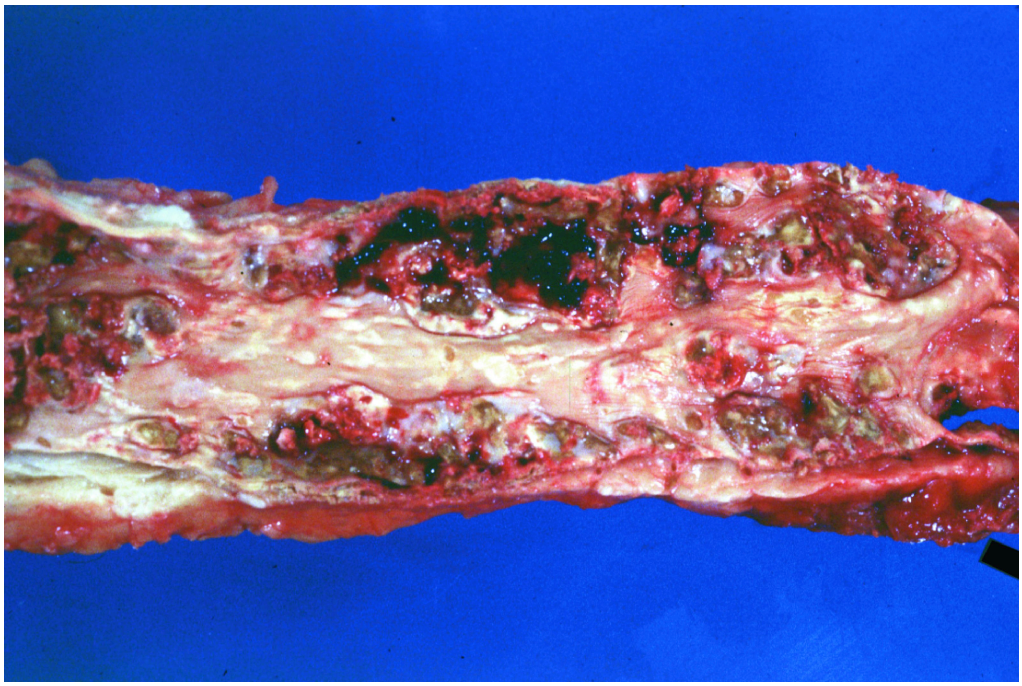
Genetic association studies for a complex disease



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Gross close-up view of typical advanced lesions

MATRIX METALLOPROTEINASE 3 AND 9 CIS-REGULATORY VARIATION AND ATHEROSCLEROTIC LESIONS

ACADEMIC DISSERTATION

Perttu Pöllänen

"We all labour against our own cure, for death is the cure of all diseases"

Sir Thomas Browne (1605-1682)

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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-IV.

I Pöllänen PJ, Karhunen PJ, Mikkelsson J, Laippala P, Perola M, Penttilä A, Mattila KM, Koivula T, Lehtimäki T. Coronary artery complicated lesion area is related to functional polymorphism of matrix metalloproteinase 9 gene: an autopsy study. *Arterioscler Thromb Vasc Biol* 2001; 21:1446-1450.

II Pöllänen PJ, Lehtimäki T, Ilveskoski E, Mikkelsson J, Kajander OA, Laippala P, Perola M, Goebeler S, Penttilä A, Mattila KM, Syrjäkoski K, Koivula T, Nikkari ST, Karhunen PJ. Coronary artery calcification is related to functional polymorphism of matrix metalloproteinase 3: the Helsinki sudden death study. *Atherosclerosis* 2002; 164:329-335.

III Pöllänen PJ, Lehtimäki T, Mikkelsson J, Ilveskoski E, Kunnas T, Perola M, Penttilä A, Mattila KM, Nikkari ST, Syrjäkoski K, Karhunen PJ. Matrix metalloproteinase 3 and 9 gene promoter polymorphisms: joint action of two loci as a risk factor for coronary artery complicated plaques. *Atherosclerosis* 2005; 180:73-78.

IV Pöllänen PJ, Karhunen PJ, Järvinen O, Sisto T, Mattila KM, Lehtimäki T. Matrix metalloproteinase 9 genetic diversity and vascular remodelling: association of the susceptibility locus with autopsy-verified defects in the internal elastic lamina (*submitted*).

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ABBREVIATIONS

AP-1	activator protein-1
AN(CO)VA	analysis of (co)variance
BMI	body-mass index
CA	celiac artery
CAD	coronary artery disease
DNA	deoxyribonucleic acid
EC(s)	endothelial cell(s)
ECM	extracellular matrix
ERK	extracellular signal regulated kinase
Ets	erytroblastosis twenty six
HSDS	Helsinki Sudden Death Study
IAP	International Atherosclerosis Project
IEL	internal elastic lamina
IL	interleukin
IMA	inferior mesenteric artery
JNK	c-jun N-terminal kinase
LAD	left anterior descending artery
LCX	left circumflex coronary artery
LSD test	Least Significant Difference post-hoc test
MAPK(s)	mitogen-activated protein kinase(s)
MI	myocardial infarction
MMP(s)	matrix metalloproteinase(s)
mRNA	messenger ribonucleic acid
NFκB	nuclear factor kappa B

PDGF	platelet-derived growth factor
PEA-3	polyoma enhancer A binding protein 3
PKC	protein kinase C
ROS	reactive oxygen species
SD	standard deviation
SMA	superior mesenteric artery
SMC(s)	smooth muscle cell(s)
SPBP	Stromelysin-1 PDGF responsive element binding protein
SPRE	Stromelysin-1 PDGF responsive element
TNF	tumor necrosis factor
TIMP(s)	tissue inhibitor(s) of matrix metalloproteinase
VSMC(s)	vascular smooth muscle cells
ZBP-89	zinc-binding protein-89

ABSTRACT

Matrix metalloproteinases (MMPs) are proteolytic enzymes which are expressed in atherosclerotic plaques and are thought to participate in the molecular mechanisms mediating atherosclerotic plaque disruption and rupture. Atherosclerosis is an inflammatory disease complicated by the development of rupture-prone atheromas, associated with the development of stenosis or thrombosis. The strictly regulated co-ordinated action of MMPs is primarily regulated at the level of transcription, the promoter cis-regulatory regions of the genes responding to stimuli including macrophage-derived growth factors and cytokines. Functional cis-regulatory variation in the promoters of matrix metalloproteinases leads to low- and high-transcription activity genotypes. To determine whether the *MMP3* and *MMP9* promoter genotypes predict the extent of atherosclerosis, the association of the *MMP* genotypes with different types of coronary lesions was investigated in an autopsy series of 300 middle-aged men (aged 35-65 years). In addition, the association with the number of defects in the internal elastic lamina (IEL) of the artery wall was investigated in the major abdominal arteries in an autopsy study of 123 subjects (90 male and 33 female, aged 18-93). Genotyping at these loci was performed by PCR, restriction enzyme digestion and minisequencing, areas of the coronary wall covered with atherosclerotic lesions were measured using computer-assisted morphometry, and the numbers of defects per millimetre in the IEL were measured by image analysis in samples from the arteries. Men with high promoter activity genotypes for *MMP3* had, on average, larger calcified lesion areas than those with the low promoter activity genotype, whereas men with high promoter activity genotypes for *MMP9* had larger complicated lesion areas. Men with high promoter activity genotypes for both loci had on average more than two times larger areas of complicated lesions compared with those men who had low promoter activity genotypes, but the loci showed no association with acute myocardial infarction. Men with high-activity genotypes for *MMP9* had a higher number of gaps per millimetre in the IEL of the right and left renal arteries than did those with the low-activity genotypes. These results suggest that the *MMP3* and *MMP9* cis-regulatory variants and combinations of genotypes at these loci may contribute to heterogeneity in the presentation of atherosclerosis. This may be mediated via effects on cell fate and the number of different cell types within the vessel wall.

Keywords: Atherosclerosis, Apoptosis, Matrix Metalloproteinases, MMPs, Single Nucleotide Polymorphism, Stromelysin, 92-kDa Gelatinase, SNPs

ABSTRACT IN FINNISH

MATRIKSIN METALLOPROTEINAASIEN 3 JA 9 CIS-VAIKUTTAVA VAIHTELU JA ATEROSKLEROOSILEESIOT

Matriksin metalloproteinaasit (matrix metalloproteinases, MMPs) ovat proteolyyttisiä entsyymejä, jotka ilmenevät ateroskleroottisissa leesioissa ja mahdollisesti osallistuvat ateroskleroottisen plakin repeytymistä välittäviin mekanismeihin. Ateroskleroosi on tulehdusperäinen sairaus, jolle on tyypillistä herkästi repeävien valtimon sisäkalvon pullistumien (ateroomien) kehittyminen verisuonen sisälle, ja johon liittyy ahtauman (stenoosin) ja verihyytymän (tromboosin) muodostuminen. Matriksin metalloproteinaasien toimintaa säädellään pääosin transkriptiotasolla promoottorien cis-säätelyalueiden avulla kasvutekijöiden ja sytokiinien vaikutuksesta. Matriksin metalloproteinaasien promoottorialueiden funktionaalinen cis-vaikuttava geneettinen vaihtelu johtaa matalan ja korkean promoottoriaktiivisuuden genotyyppeihin. Tutkimuksessa selvitettiin oikeuslääketieteellisissä avauksissa kerätyistä 300 miehen (ikä 35-65 vuotta) DNA- ja valtimonäytteistä, ovatko matriksin metalloproteinaasien 3 ja 9 (*MMP3* ja *MMP9*) promoottorien genotyypit yhteydessä ateroskleroosiin eri tyyppisissä ateroskleroosin muutoksissa. Lisäksi tutkittiin genotyyppien yhteyttä vatsan alueen suurten valtimoiden lamina elastica internan (internal elastica lamina) aukkojen lukumäärään 123 henkilön ruumiinavausaineistossa (90 miestä ja 33 naista, ikä 18-93 vuotta). Näiden kahden lokuksen genotyyppaus suoritettiin PCR-monistuksen, restriktioentsyymidigestion ja minisekvensoinnin avulla, ateroskleroottisten plakkien laatu ja määrä mitattiin tietokoneavusteisella morfometrialla ja mesenteriaalivaltimoiden lamina elastica internan aukkojen lukumäärä määritettiin valtimonäytteistä. Tutkimuksessa havaittiin, että miehillä, joilla on korkean promoottoriaktiivisuuden *MMP3* genotyyppi, on keskimäärin suurempi sepelvaltimon seinämän kalkkeutuneen vaurion pinta-ala kuin miehillä, joilla on matalan promoottoriaktiivisuuden *MMP3* genotyyppi. Vastaavasti miehillä, joilla on korkean promoottoriaktiivisuuden *MMP9* genotyyppi, havaittiin keskimäärin suurempi sepelvaltimon seinämän komplisoituneen vaurion pinta-ala ja keskimäärin enemmän lamina elastica internan aukkoja munuaisvaltimoissa kuin miehillä, joilla on matalan promoottoriaktiivisuuden genotyyppi. Myös *MMP3* ja *MMP9* genotyyppien yhteisvaikutus on merkittävä siten, että miehillä, joilla on molempien geenien korkean promoottoriaktiivisuuden genotyyppi, havaittiin suurempi komplisoituneitten plakkien pinta-ala, mutta yhteyttä akuuttiin infarktiin ei todettu. Matriksin metalloproteinaasien promoottorialueiden vaihtelu ja näiden kahden lokuksen genotyyppien yhdistelmät saattavat vaikuttaa ateroskleroosin ilmenemisen eroihin. Tämä voi johtua metalloproteinaasien vaikutuksesta eri solutyyppien määriin suonen seinämässä.

INTRODUCTION

The relationship between phenotype and genome variability constitutes a major challenge to post-genomic research. The major impetus for the genotyping of single nucleotide polymorphisms (SNPs) comes from the search for disease-susceptibility genes (Phillips *et al.* 2001, di Giusto and King 2003).

Atherosclerosis, the principal cause of heart attack, stroke and gangrene of the extremities, is responsible for 50 % of all mortality in Europe, in the USA and in Japan (Ross *et al.* 1999, Lusis *et al.* 2000). The hallmarks of atherosclerotic disease are the atherosclerotic lesions, which are characterized by SMC proliferation and lipid accumulation and extracellular matrix formation and deposition (Ross *et al.* 1993, Wernig and Xu *et al.* 2002). The lesions result from an excessive, inflammatory-fibroproliferative response to various forms of insult to the endothelium and smooth muscle of the artery (Ross *et al.* 1999). A large number of inflammatory growth factors, cytokines and vasoregulatory molecules participate in the process (Ross *et al.* 1993).

Forty years ago, Jerome Gross and colleagues described an "activity" which was present during metamorphosis in tadpoles and had the ability to degrade rigid rods of collagen (Gross and Lapiere 1962, Brinckerhoff and Matrisian 2002). This activity was interstitial collagenase, which was later shown to represent only a small component in the MMP repertoire (Brinckerhoff and Matrisian 2002). Various members of the MMP family have dedicated substrate specifities which permit them to degrade all components of the extracellular matrix in a concerted manner (Brinckerhoff and Matrisian 2002). The ability of this family to modify the structural integrity of tissues is essential for normal physiology, including embryonic development, cell migration and wound healing (Jones *et al.* 2003). Disregulation of MMP expression is a feature of numerous pathologic conditions such as tumor metastasis, rheumatic conditions and vascular remodeling (Jones *et al.* 2003).

The promoter cis-regulatory elements play an essential role in the co-ordinated regulation of *MMP* gene expression both at the basal level and in response to various stimuli, including the inflammatory cytokines and growth factors (Ye 2000). Naturally occurring genetic variation in the promoter regulatory regions of various *MMP*s have been shown to be associated with coronary artery disease and aneurysms (Ye 2000). In this thesis, the association between the promoter *MMP* genotypes and

atherosclerotic lesions was examined in coronary arteries and in major abdominal arteries in two autopsy series.

It is not all that long ago that the generally accepted views on the extracellular matrix suggested an entirely passive role for this ubiquitous component of any tissue or organ in the body (Bosman and Stamenkovic 2003). Only very recently has it been recognized that the right balance between tissue renewal and cell loss mediated by the matrix-degrading activities of proteases is not merely an incidental part of life but rather a highly controlled element of existence crucial for the decision between health and disease (Wernig and Xu 2002).

REVIEW OF THE LITERATURE

1. ATHEROSCLEROSIS

Macroscopical, visual examinations of the degree of atherosclerosis originated in the 1960s from the first large-scale autopsy survey by the International Atherosclerosis Project (IAP), which used validated methods and a detailed standard operating protocol to evaluate and quantify arterial lesions (Gutzman *et al.* 1968). Atherosclerosis was stained with Sudan IV and graded visually as fatty streaks, fibrous plaques and complicated lesions (see Table 1). The current classification of atherosclerotic lesions, as presented by the American Heart Association, divides them into six types based on their histological composition and structure (Stary *et al.* 1994; Stary *et al.* 1995). Atherosclerotic lesions are considered advanced by histological criteria when accumulations of lipid, cells and matrix components, including minerals, are associated with structural disorganization, repair and thickening of the intima, as well as deformity of the arterial wall (see Table 1).

1.1 EARLY LESIONS

Although formation of the intimal macrophage-rich fatty streak, the precursor of atherosclerotic lesions, appears to be ubiquitous in humans, not all fatty streaks evolve into advanced lesions, i.e., fibrous plaques or atheroma. Depending on the equilibrium between proatherogenic and antiatherogenic factors, some fatty streaks progress into atheroma while others may regress (Geng and Libby 2002).

The precursors of advanced lesions are divided into three morphologically characteristic types. Both type I and type II lesions represent small lipid deposits in the arterial intima, and type II includes those lesions generally referred to as fatty streaks. Type I lesions consist of macrophage-derived foam cells containing lipid droplets, type II contain both macrophages and smooth muscle cells (SMCs) with extracellular lipid deposits, and type III lesions consist of SMCs surrounded by extracellular connective tissue and lipid deposits (Zaman *et al.* 2000). Type III represents the stage which links type II to advanced lesions (Stary *et al.* 1994). Electron microscopy or histochemical assays for apoptosis may show evidence of ongoing cell death (Virmani *et al.* 2000).

Each type I and II lesion is focal and relatively small, and contains abnormal accumulations of lipoproteins and cholesterol esters. Increased numbers of cells, mainly macrophages, and accumulations of lipid droplets, mainly within macrophages, can be demonstrated microscopically. Changes

in the composition of the matrix and disruption of the intimal architecture are minimal or absent (Stary *et al.* 1994).

1.2 ADVANCED LESIONS

Advanced atherosclerotic lesions can be subdivided into three main histologically characteristic types: IV, V and VI (Stary *et al.* 1995). In type IV lesions a dense accumulation of extracellular lipid occupies an extensive but well-defined region of the intima. Complications such as defects on the lesion surface and thrombosis are not present. The type IV lesion is also known as atheroma (Stary *et al.* 1995) (see Table 1, page 25).

Type V lesions are lesions in which new fibrous connective tissue has formed. Here SMCs migrate into and proliferate within the plaque forming a layer over the lumina side of the core (Zaman *et al*. 2000). With these lesions, arteries are variously narrowed, generally more than with type IV. Histological studies of the lesions in large populations indicate that reparative connective tissue forms in and around regions of the intima in which large accumulations of extracellular lipid (lipid cores) disarrange or obliterate the intercellular matrix structure. Sometimes the new fibrous tissue accounts for more of the thickness of the lesion than does the underlying lipid accumulation (Stary *et al.* 1995).

Type IV plaques comprise confluent cellular lesions with a great deal of extracellular lipid, intermixed with fibrous tissues, whereas type Va possess an extracellular lipid core covered by a thin fibrous cap (Zaman *et al*. 2000). Type Va lesions may be multilayered: several lipid cores, separated by thick layers of fibrous connective tissue, are stacked irregularly one above the other. The term multilayered fibroatheroma can be applied to this morphology (see Table 1). Mechanical forces may play a role in the modeling of such lesions (Stary *et al.* 1995).

Lesions containing a large amount of calcium generally also have increased fibrous connective tissue, and often there is the underlying morphology of fibroatheroma. Lesions in which mineralization is the dominant feature may be termed type Vb (calcific) lesions (see Table 1, page 25). Mineral deposits may replace the accumulated remnants of dead cells and extracellular lipid, including entire lipid cores. The calcified lesion has also been labeled the type VII lesion (Stary *et al.* 1995) (see Table 1).

In Type Vc (fibrotic) lesions, the normal intima is replaced and thickened with fibrous connective tissue, while lipid is minimal or even absent. This

lesion is also referred to as the type VIII lesion (Stary *et al.* 1995) (see Table 1).

Morbidity and mortality from atherosclerosis is largely due to type IV and type V lesions in which disruptions of the lesion surface, hematoma or hemorrhage, and thrombotic deposits have developed. Type IV or V lesions with one or more of these additional features are classified as type VI and may also be referred to as complicated lesions. Type VI may be subdivided by the superimposed features. Thus, disruption of the surface may be labeled type VIa; hematoma or hemorrhage type VIb; and thrombosis type VIc. Type VIabc indicates the presence of all three features (Stary *et al.* 1995).

Lesion types IV, V and VI are sometimes associated with and appear to be the cause of localized dilatations of the part of the vascular wall they occupy. Distinct localized outward bulges or aneurysms are generally associated with type VI lesions in which the intimal surface is greatly eroded. Aneurysms often contain mural thrombi, both recent and old remnants. For aneurysms to occur, matrix fibers must be degraded and/or synthesized in new proportions. The expression of proteolytic enzymes would be expected to be increased in relation to wall destruction and remodeling (Stary *et al.* 1995).

Term	Histological Classification	* Other Terms
Type I Lesion	Initial lesion	*Fatty streak*
Type II Lesion		
IIa	Progression-prone type II lesion	*Fatty streak*
IIb	Progression-resistant type II lesion	
Type III Lesion	Intermediate lesion (preratheroma)	
Type IV Lesion	Atheroma	*Atheromatous plaque*
Va	Fibroatheroma (type V lesion)	*Fibrous plaque*
Vb	Calcific lesion (type VII lesion)	*Calcified plaque*
Vc	Fibrotic lesion (type VIII)	*Fibrous plaque*
Type VI Lesion	Lesion with surface defect and/or hematoma /hemorrhage and/or thrombotic deposit	*Complicated lesion*

Table 1. *Current American Heart Association AHA classification (modified from Virmani et al. 2000). * Macroscopical classification*

1.3 PLAQUE RUPTURE

Plaque disruption with resulting thrombus most frequently occurs where the fibrous cap is thinnest and most heavily infiltrated by lipid-filled macrophage foam cells. This is often the junction between the plaque and the adjacent less diseased vessel wall, i.e. the vulnerable shoulder of the plaque.

Coronary atherosclerosis without thrombosis is in general a benign disease; survival is good if thrombotic complications can be prevented (Falk *et al.* 1996). Plaque disruption is clinically very important, underlying about 75% of thrombi responsible for acute coronary syndromes (Falk *et al.* 1996).

Plaque rupture is defined by an area of fibrous cap disruption whereby the overlying thrombus is in continuity with the underlying necrotic core (Virmani *et al.* 2000). Ruptured lesions typically have a large necrotic core and a disrupted fibrous cap infiltrated by macrophages and lymphocytes; the smooth muscle cell content within the fibrous cap at the rupture site may be quite sparse (Virmani *et al.* 2000). The risk of plaque disruption is related to intrinsic properties (plaque vulnerability) and extrinsic forces acting on the plaques. Coronary plaques are constantly stressed by a variety of biomechanical and hemodynamic forces in the form of stretch and shear stress which may precipitate or trigger disruption of vulnerable plaques (Falk *et al.* 1996, Wernig and Xu 2002).

The mechanism of fibrous cap thinning is not known (Virmani et al. 2000). There is, however, evidence for extensive apoptosis of smooth muscle cells within the cap in advanced atherosclerosis, as well as in those cultured from plaques (Bennett et al. 1995, Geng et al. 1997). The German pathologist R. Virchow defined "apoptosis" in the plaque over a century ago: "Thus we have here an active process which really produces new tissues, but then hurries on to destruction in consequence of its own development" (Virchow [1858] 1989).

The most extensive hypothesis to explain rupture is that proposed by Libby and colleagues, originally described by Henney and colleagues (Henney *et al.* 1991). This hypothesis proposes that the critical effects of inflammation are the cytokines which drive the expression of proteases (Virmani *et al.* 2000). The principle limitation of the protease hypothesis is that we do not know when the inflammatory process becomes "critical", i.e. in experimental animals proteolytic activity may be elevated even in early lesions which do not rupture (Galis *et al.* 1995). Although atherosclerotic plaques do clearly evince protease activity, the time course of net activity in relationship to rupture will probably remain unknown (Virmani *et al.* 2000). The observations of Libby and colleagues suggest, however, that stromelysin-3

(MMP11) itself is able to digest proteolytic inhibitors and is present only in advanced human lesions, as opposed to xanthomata (Schonbeck *et al.* 1999).

2. MATRIX METALLOPROTEINASES (MMPS)

Four decades ago, a collagenase was discovered which was responsible for involution of the tadpole tail in amphibian morphogenesis. Today, the matrix metalloproteinases (MMPs) constitute a multigene family of over 25 secreted and cell surface enzymes which process or degrade numerous pericellular substrates (Sternlicht and Werb 2001). Their targets include other proteinases, proteinase inhibitors, clotting factors, chemotactic molecules, latent growth factors, growth factor-binding proteins, cell surface receptors, cell-cell adhesion molecules, and virtually all structural extracellular matrix proteins (Sternlicht and Werb 2001). There are also two other large families which have major roles in extracellular proteolysis, the ADAM family (A disintegrin and metalloprotease domain) and the ADAMTS family (A disintegrin-like and metalloprotease domain with thrombospondin type I repeats) (Somerville *et al.* 2003). The ADAMs contain a catalytic domain genetically related to that found in the MMPs (Chase and Newby 2003).

The degenerative eye disease Sorsby´s fundus dystrophy was the first human genetic disease to be associated with MMPs when it was found to be linked to a mutation in the TIMP3 gene (Weber *et al.* 1994). A form of inherited osteolysis in several Saudi Arabian families, or "vanishing bone" syndrome", was found to be associated with mutations in the *MMP2* gene (Martignetti *et al.* 2001). MMPs -2, -7, -8 and -11 have all been shown to contribute to tumor progression in studies using MMP-deficient mice (Nelson *et al.* 2000, Brinckerhoff and Matrisian 2002). Rheumatoid arthritis, a condition in which the collagen in the cartilage, bone and tendons surrounding joints is progressively and irreversibly degraded was one of the first links between MMPs and disease (Mudgett *et al.* 1998, Mountz *et al.* 2001, Brinckerhoff and Matrisian 2002). Krane and Jean-Michel Dayer described a soluble factor secreted by macrophages which induced collagenase in cultured human synovial cells; this factor was later identified as interleukin-1 (Dayer *et al.* 1977, Brinckerhoff and Matrisian 2002).

MMPs have always been considered to cleave components of the extracellular matrix (ECM) (Somerville *et al.* 2003). As the ECM was regarded as nothing more than a passive structure for cell attachment, MMPs were thought simply to remodel the ECM for its homeostasis. ECM is now known to contain growth factors, their binding proteins, and other bioactive molecules, as well as binding sites for cell-surface molecules, some of which are revealed only after proteolysis (Somerville *et al.* 2003).

2.1 BIOCHEMISTRY

The term "cysteine switch" describes the mechanism used by MMP family members to maintain enzyme latency (Van Wart and Birkedal-Hansen 1990). A cysteine-sulfhydryl group within the conserved "Pro-Arg-Cys-Gly-X-Pro-Asp" motif in the propeptide domain of latent MMPs forms a bridge with the catalytic zinc, thereby preventing enzymatic activity; activation occurs when this linkage is disrupted by physical or chemical means (Stamenkovic 2003). Most MMPs can be activated by other MMPs and a variety of serine proteases in vitro (Nagase and Woessner 1999). A proteolytic cascade regulates MMP activation, like the coagulation and thrombolytic systems; plasmin, which is produced by the action of a plasminogen activator on plasminogen, can activate several MMPs, including stromelysin-1 (MMP3, the "collagenase-activating protein"), by cleaving the pro-domain.

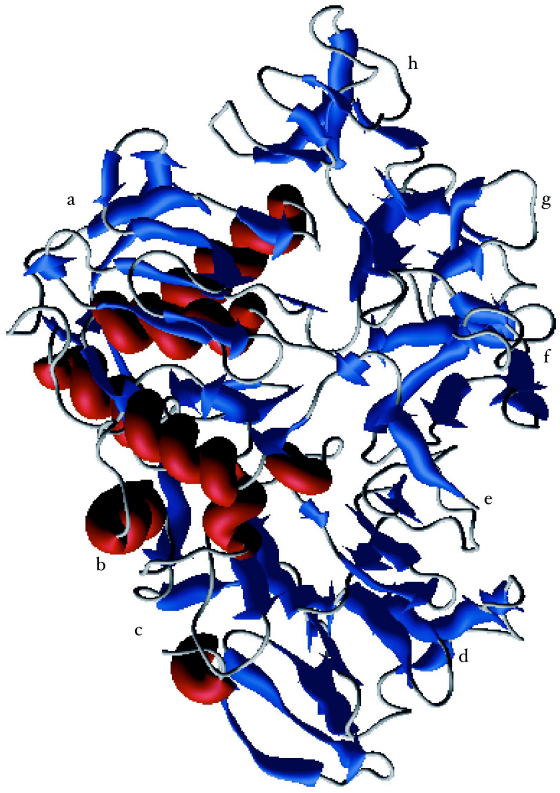

Figure 1. *3D structure of MMPs. a. Catalytic domain b. Propeptide c. COOH d. Hemopexin e. NH2 f. Fibronectin II g. FibII h. FibII. This figure was prepared with an iMol molecular viewer written by Piotr Rotkiewicz. The PDB file (PDB ID: 1CK7, Morgunova et al. 1999) was downloaded from the protein databank (Berman et al. 2000) http://www.rcsb.org.*

Proteolytic enzymes are classified as either exopeptidases or endopeptidases based on whether they cleave terminal or internal peptide bonds, respectively. Most endopeptidases are classified as serine, cysteine, aspartic or metalloproteases based on their catalytic mechanism and inhibitor sensitivities, and the metalloproteinases are further separated into five superfamilies based on sequence considerations (Sternlicht and Werb 2001). Of these, the metzincin superfamily is distinguished by a highly conserved motif containing three histidines which bind zinc at the catalytic site and a conserved methionine turn which sits beneath the active site zinc (Stocker *et al.* 1995). Their signature zinc-binding motif reads HEBXHXBGBXHZ, where histidine (H), glutamic acid (E) and glycine (G) residues are invariant, B is a bulky hydrophobic residue, X is a variable residue, and Z is a family-specific amino acid. The metzincins are further subdivided into four multigene families, the serralysins, astracins, ADAMs/adamalysins, and MMPs, based primarily on the identitity of the Z residue, which is serine in all but a few MMPs (Stocker *et al.* 1995).

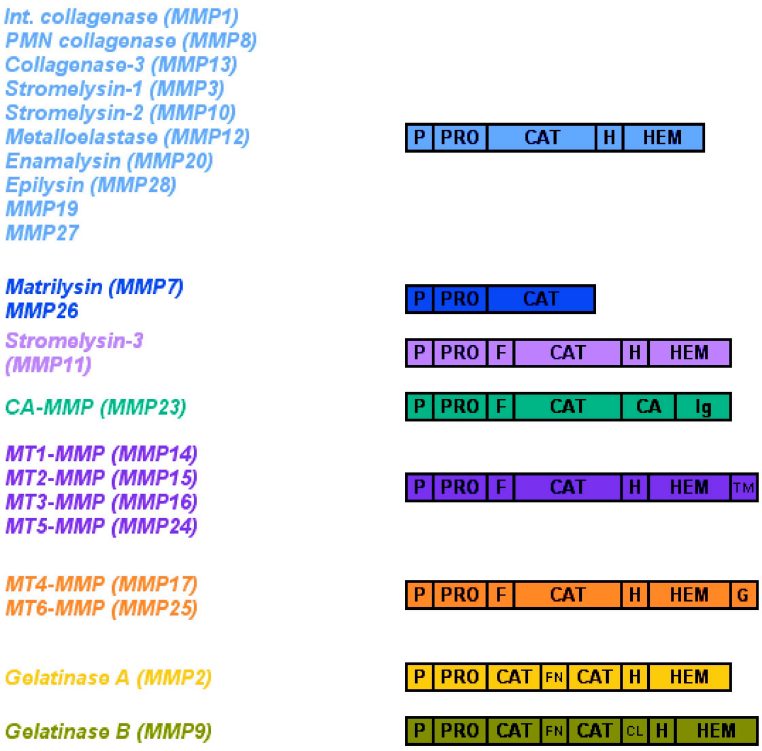

Figure 2. *The MMPs grouped by their domain structure. CA, cysteine array; CAT, catalytic domain; CL, collagen-like domain; F, furin cleavage consensus sequence; FN, fibronectin-like repeats; GPI, glycosyl phosphatidylinositol linkage signal; H, hinge domain; HEM, hemopexin domain; Ig, immunoglobulin-like domain; P, leader sequence; PRO, pro-domain; TM, transmembrane domain. Modified from Brinckerhoff and Matrisian 2002. This figure was prepared with ConceptDraw Med (Computer Systems Odessa Corporation)*

MMP activity is also regulated by an endogenous inhibitory activity known as a family of four "tissue inhibitors of metalloproteinases" (TIMPs), which acts by forming a 1:1 complex with the activated catalytic zinc in the MMPs (Brinckerhoff and Matrisian 2002). TIMPs differ in their expression patterns and affinity for the MMPs (Stamenkovic 2003). For example, the C-terminal domain of TIMP-1 binds the hemopexin domain of MMP9 more readily than it does the hemopexin domain of MMP2, whereas the C-terminal domain of TIMP2 preferentially binds the hemopexin domain of MMP2 (Murphy and Willenbrock 1995). There is a long history indicating that TIMPs exert growth-promoting or growth-suppressive activities, although the mechanisms of their growth-altering activities are not known (Sternlicht and Werb 2001). For example, a mutation which abolishes the MMP-inhibitory activity of TIMP3 also abolishes its ability to promote apoptosis (Bond *et al.* 2000). TIMPs are multifunctional, however, as many of their effects in biological processes cannot be explained by inhibition of MMP activity (Brinckerhoff and Matrisian 2002). The TIMP-1/pro-MMP9 complex is thought to recruit MMP3, forming a stable ternary complex which causes MMP3 inactivation (Kolkenbrock *et al.* 1995, Stamenkovic 2003). Thrombospondins, matricellular modulators of cell function, also regulate MMP proteolytic activity (Stamenkovic 2003, Bornstein *et al.* 2004).

The crystal structure of the human collagenase catalytic domain, a five-stranded β-sheet and three α-helices, which forms a spherical topology highly conserved among MMP family members, was published in 1994 (Lovejoy *et al.* 1994, Brinckerhoff and Matrisian 2002) (see Fig. 1, page 29). The close evolutionary relationship between MMPs is further reflected in the conservation of their domain structure and of their mechanisms of catalysis and regulation (Somerville *et al.* 2003) (see Fig. 2, page 30).

2.2 Molecular Biology

The first full-length complementary DNA of an MMP, transin/stromelysin-1, was identified in the mid-1980s (Whitham *et al.* 1986). The structural similarity of *MMP* genes indicates that they have evolved by duplication of a common ancestral gene followed by divergent evolution (Somerville *et al.* 2003). The concept of a gene family emerged when transin-1 (stromelysin-1/*MMP3*) and transin-2 (stromelysin-2/*MMP10*) genes were shown to be markedly similar (Breathnach *et al.* 1987). Like many other MMP family members, both genes were about 10 kb in length, contained 10 exons and 11 introns, and were localized in a cluster on chromosome 11q22.22, which is now known to consist of MMPs -1, -3, -7, -8, -10, -12, -13, -20 and 27 (Massova *et al.* 1998) (see Figs 3 and 4, pages 32 and 33). The gelatinases MMP2 and MMP9, however, were found to contain three extra exons which encode fibronectin-like repeats (see Fig. 2, page 30), and localized on chromosomes

16 and 20, respectively (see Fig. 3) (Collier *et al.* 1991, Brinckerhoff and Matrisian 2002).

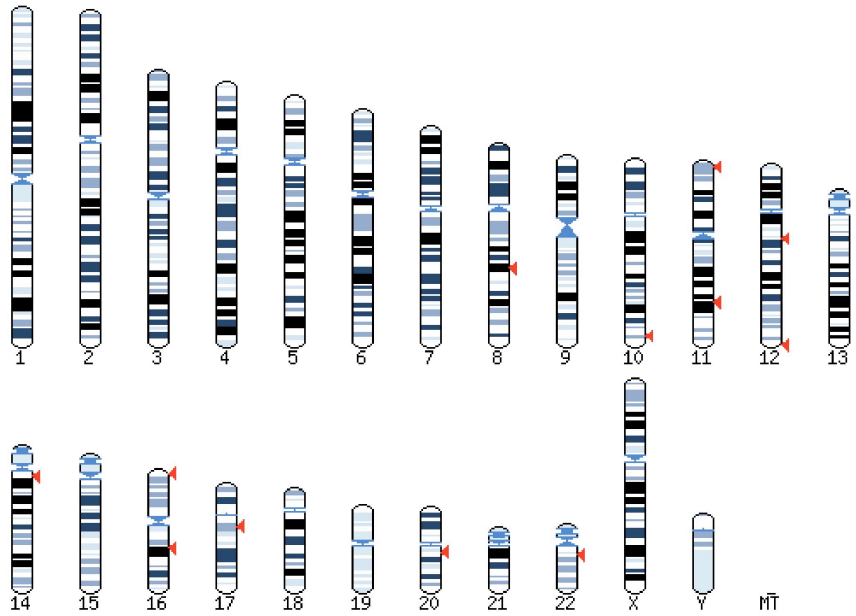

Figure 3. *Genes coding for precursor peptides of the MMP protein family, MMP16 (chr 8), MMP21 (chr 10), MMP1, -3, -10, -12, -13, -20, -26 and -27 (chr 11), MMP19 (chr 12), MMP17 (chr 12), MMP14 (chr 14), MMP25 (chr 16), MMP15 (chr 16), MMP2 (chr 16), MMP28 (chr 17), MMP9, -24 (chr 20) and stromelysin-3 (chr 22) are indicated with arrows. The protein family was generated using the MCL (Markov CLustering) package available at http://micans.org/mcl/. The application of MCL to biological graphs was initially proposed by Enright et al. 2002.*

MMP genes are "inducible", the effectors including growth factors, cytokines, chemical agents, physical stress etc., and gene expression may also be down-regulated by suppressive factors, e.g. transforming growth factor, retinoic acids or glucocorticoids (Nagase and Woessner 1999). The activator protein-1 (AP-1) enhancer element, which binds Fos-Jun heterodimers or Jun-Jun homodimers, located at about -70 bp, and another cis-acting sequence in close proximity to the AP-1 enhancer in the MMP-1 promoter, the Ets (erythroblastosis twenty six) site, are important in gene expression, and these elements turned attention towards the promoter region and regulation of gene expression (Brinckerhoff and Matrisian 2002). The Ets and AP-1 sites were shown to cooperate to enhance transcription, but even in cooperation were not sufficient to regulate *MMP* gene expression (Gutmann and Wasylyk 1990). Subsequent studies showed the importance of cooperation with several upstream elements in regulating gene expression and conferring tissue specificity in various *MMP* promoters (Vincenti 2001).

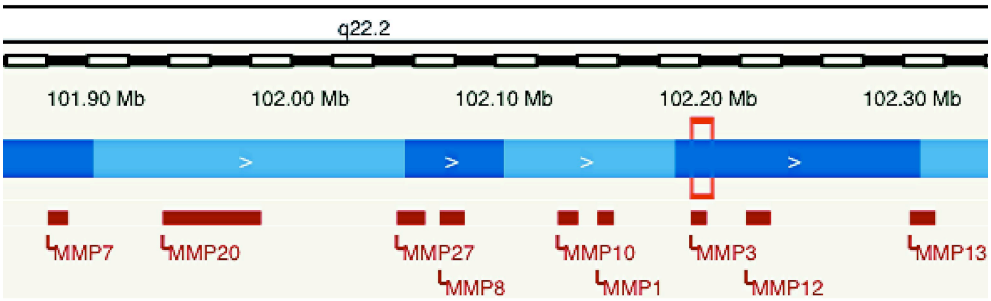

Figure 4. *Cluster of MMP genes on chromosome 11q22.22, which is known to consist of MMPs -1, -3, -7, -8, -10, -12, -13, -20 and 27 (Hubbard et al. 2005).*

2.3 BIOLOGY

The strictly regulated co-ordinated action of MMPs is essential for normal physiology, including embryonic development, morphogenesis and wound healing (Jones *et al.* 2003). MMPs mediate ovulation, are expressed in a coordinated fashion throughout the menstrual cycle and participate in homeostatic matrix remodelling of connective tissues (Jones *et al.* 2003). Although MMPs can cleave virtually all structural ECM molecules, they also cleave circulating, cell surface and pericellular proteins, which enables them to regulate cell behavior in numerous ways (Sternlicht and Werb 2001) (see Table 2, page 34).

Extracellular matrices regulate such basic processes as cell shape, movement, growth, differentiation and survival by controlling cell adhesion and the cytoskeletal machinery (Aumailley and Gayraud 1998, Lukashev and Werb 1998). Moreover, proteolytic ECM remodelling results in the release of modular breakdown products with biologic activity. Several MMPs are able to release growth factors by cleaving either the growth-factor-binding protein or the extracellular matrix molecule to which growth factors attach, and tender them able to bind their receptors and signal to the nucleus (Somerville *et al.* 2003). Most cells must adhere to a natural or provisional matrix to survive; thus MMP-mediated disruption of subcellular matrices can induce apoptosis in anchorage-dependent cells and plays an important role in normal physiologic cell death in tissues such as the involuting mammary gland (Sternlicht and Werb 2001).

MMPs can potentially influence cell behavior by cleaving cell-cell adhesion proteins, by releasing bioactive cell surface molecules, or by cleaving cell surface molecules which transduce signals from the extracellular environment. For example, MMP3 can cleave the adherens-junction protein E-cadherin, thus promoting cell invasion by disrupting cell-aggregation (Noe *et al.* 2001, Steinhusen *et al.* 2001) (see Table 2, page 34). MMP3 can release a soluble form of the adhesion molecule L-selectin from leukocytes (Preece *et*

al. 1996, Somerville *et al.* 2003) (see Table 2). In addition, MMP9 can cleave plasminogen and MMPs also cleave other serine proteinases such as urokinase-type plasminogen activator (Sternlicht and Werb 2001) (see Table 2). MMPs may regulate the availability of paracrine signals by inactivating them; for example, they can inactivate angiotensins I and II and limit their physiological effects (Sternlicht and Werb 2001). Virtually all MMPs can degrade fibrinogen, thereby suppressing normal clotting mechanisms (Hiller *et al.* 2000).

MMP	*Substrates	†Non-collagenous	†Non-structural
MMP3 Stromelysin-1 EC 3.4.24.17	Collagen types II, IV, V, VII, X, XI, XIV and gelatin (Reaction: Preferential cleavage where P1', P2' and P3' are hydrophobic residues‡)	Aggrecan, elastin, fibronectin, laminin, nidogen, proteoglycan, proteoglycan link protein, versican	α1-antichymotrypsin, antithrombin III, E-cadherin, fibrinogen, IGF-BP3, L-selectin, ovostatin, pro-IL-β, pro-MMP1, pro-MMP8, pro-MMP9, pro-TNFα
MMP9 Gelatinase-B EC 3.4.24.35	Collagen types IV,V, VII, X and XIV (Reaction: Cleavage of gelatin types I and V and collagen types IV and V)	Fibronectin, laminin, nidogen, proteoglycan link protein, versican	CXCL5, IL-1β, IL2-R, plasminogen, pro-TNFα, SDF-1, TGF-β

Table 2. *Substrates of human matrix metalloproteinases 3 and 9 (modified from Somerville et al. 2003). *Collagenous substrates † ECM Substrates ‡ Schechter and Berger nomenclature for the interaction of a substrate with a protease (Schechter and Berger 1967).*

3. Matrix metalloproteinases and atherosclerosis

In 1972, Ross and Glomset initially formulated the "response to injury" hypothesis, which focused on the behaviour of vascular smooth muscle cells and endothelial cells and took into account the discovery of peptide growth factors (Ross and Glomset 1973, Newby 2005). In his later reviews, Ross largely succeeded in unifying the various hypotheses by proposing oxidized lipids as an important protagonist of chronic vascular inflammation and hence response to injury (Ross 1999). By the beginning of the 1990s, a long list of inflammatory cytokines and growth factors had been identified which might mediate the response to injury; growth factors themselves were considered insufficient for this and it was understood that extracellular proteases might be basic in the process (Newby 2005).

Several lines of evidence now indicate that MMPs influence the process of atherosclerotic lesion formation (Jones and Sane 2003). The infiltration of smooth muscle cells and macrophages into the intima of the inflamed artery contributes to the formation of complicated atherosclerotic lesions (Jormsjö *et al.* 2002). Macrophages and SMCs produce a large number of proteases such as serine, cysteine, aspartic and MMPs (Jormsjö *et al.* 2002). Their activity may facilitate migration of vascular smooth muscle cells through the internal elastic lamina (IEL), the barrier between the vascular layers intima and media, into the intimal space, where SMCs proliferate and contribute to plaque formation (Jones and Sane 2003). On the other hand, MMP-mediated remodelling may constitute one of the key elements in plaque stability and/or rupture (Jones and Sane 2003). Matrix degradation is a prerequisite for the recruitment of monocytes and migration of SMCs, because these cells have to traverse the extracellular barriers as well as interstitial collagen (Watanabe and Ikeda 2004). Despite their overlapping substrate repertoires, studies using various genetic manipulations in animal models have been used to determine which MMPs are relevant in the progression of atherosclerosis. Knockout of MMP3 in apolipoprotein E (ApoE)-knockout mice increased atherosclerotic plaque size and collagen content, decreased plaque macrophage content and reduced the incidence of aortic aneurysms (Silence *et al.* 2001). Among the gelatinases, knockout of MMP2 produced a mild phenotype and knockout of MMP9 impaired VSMC migration and possibly also proliferation (Itoh *et al.* 1997).

MMPs Expressed in Vascular Tissue		
Collagenases	MMP1 MMP8 MMP13	intersitial collagenase neutrophil collagenase collagenase-3
Gelatinases	MMP2 MMP9	gelatinase A gelatinase B
Stromelysins	MMP3 MMP10 MMP11 MMP7	stromelysin-1 stromelysin-2 stromelysin-3 matrilysin
MT-MMPs	MMP14 MMP15 MMP16 MMP17	MT1-MMP MT2-MMP MT3-MMP MT4-MMP
Others	MMP12	metalloelastase

Table 3. *MMP family members expressed in vascular tissues (modified from Chase and Newby 2003).*

The serine proteases, cysteine proteases (chatepsins) and MMPs (see Table 3) have been analyzed in association with arterial changes (Henney *et al.* 1991, Galis *et al.* 1994, Galis *et al.* 1995, Jormsjö *et al.* 2002). Increased expression of MMPs has been observed in diseased human arteries; conversely, in non-diseased human arteries no in situ MMP enzymatic activity is detectable (Galis *et al.* 1994b, Galis *et al.* 1995). Macrophage-derived foam cells in the necrotic cores of plaques and in the cap regions express proteases such as chatepsins (Jormsjö *et al.* 2002). Intracellular accumulation of lipid, characteristic of macrophages residing in atherosclerotic plaques, or in vitro incubation with oxidized lipoproteins, increases MMP expression in macrophages as well as vascular cells (Galis *et al.* 1995, Huang *et al.* 1999). The presence of foam cell macrophages further enhances the oxidative stress through increased production of ROS, which can trigger the activation of latent MMP zymogens stored in the vessel wall (Rajagopalan *et al.* 1996, Galis and Khatri 2002) (see Fig. 5, page 37).

Macrophage-derived foam cells, characteristic of unstable plaques, have been identified as a major source of MMPs and MMP-associated activity in human and experimental lesions (Galis *et al.* 1994b, Galis *et al.* 1995). The discovery of local MMP overexpression and in situ matrix-degrading activity in the vulnerable shoulders of human atheroma, later found to coincide with areas subjected to the highest mechanical stress, has provided a potential mechanistic insight into the action of MMPs, especially in the vulnerable shoulders (Galis and Khatri 2002). Upregulation of MMP secretion by cytokines and growth factors implies an association between increased matrix turnover and inflammation (Bond *et al.* 2001). Activated macrophages also secrete cytokines which upregulate *MMP* gene expression in vascular cells (Galis *et al.* 1994b). Simultaneous expression of PDGF, IL-1α, and TNF-α is known to occur in atherosclerosis, and may help to explain the presence and activity of MMP1, MMP3 and MMP9 in lesions (Galis *et al.* 1994b, Galis *et al.* 1995, Zaltsman and Newby 1997, Bond *et al.* 2001, Shah and Galis 2001). The preponderant location of MMP1, MMP3 and MMP9 in the shoulder regions of plaques, where genetically programmed cell death may occur, and where there are abundant inflammatory cells, is also consistent with an influence of locally released growth factors and inflammatory cytokines (Galis *et al.* 1994b, Nikkari *et al.* 1995, Lee *et al.* 1996, Ross *et al.* 1999, Bond *et al.* 2001). The similar pattern of regulation of MMP1, MMP3 and MMP9 protein secretion implies that concerted turnover of all extracellular matrix components may occur during injury and inflammation (Bond *et al.* 2001).

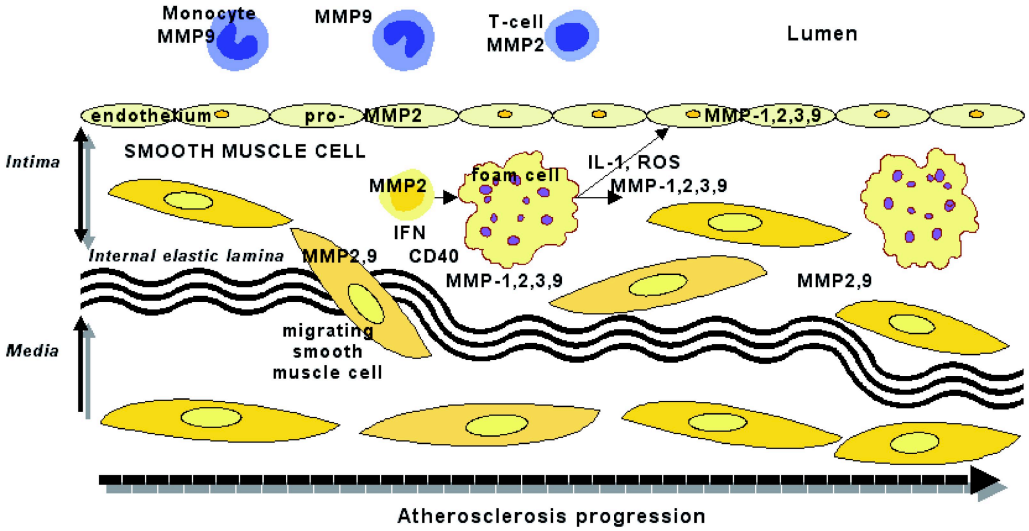

Figure 5. *The expression of MMPs is increased in atherosclerotic lesions. The spectrum of MMPs is diversified through the presence of inflammatory cells, stimulation by soluble factors, cell-cell, and cell-matrix interactions (Galis et al. 2002). Modified from Galis et al. 2002. This figure was prepared with ConceptDraw Med (Computer Systems Odessa Corporation).*

4. Regulation of Matrix Metalloproteinase Transcriptional Activity

The co-ordinated upregulation of *MMP* gene expression within the vessel wall is a highly regulated functioning of the promoter cis-regulatory elements, trans-acting DNA-binding nuclear regulator proteins, and signalling events connecting the nuclear regulator proteins with signals from outside the nucleus (Vincenti 2001, Hussain *et al.* 2002)

Vascular smooth muscle cells (VSMCs) constitutively express MMP2 rather than MMP9 (Galis *et al.* 1994a). Mechanical stretch further upregulates MMP2 via production of reactive oxygen species (ROS) (Grote *et al.* 2003). Both MMP2 activation and MMP9 induction are rapidly triggered by injury to the vessel wall (Bendeck *et al.* 1994). *MMP1*, *MMP3* and *MMP9* are inducible by inflammatory cytokines and growth factors (Galis *et al.* 1994a, Yanagi *et al.* 1991) and combinations of IL-1 or TNF with either bFGF or PDGF synergistically increase expression of *MMP1*, *MMP3* and *MMP9* in human and rabbit VSMCs (Fabunmi *et al.* 1996, Bond *et al.* 2001).

In blood monocytes, MMP9 is rapidly upregulated after contact with the endothelial surface, although peripheral blood monocytes do not express MMPs (Amorino and Hoover 1998, Chase and Newby 2003). Inflammatory activation is a second stage in macrophage MMP upregulation and activation by immune cells is a potent mechanism which may represent a third stage of MMP recruitment; MMPs colocalize with CD40 expression within

atherosclerotic plaques (Mach *et al.* 1998, Chase and Newby 2003). Another candidate effector of MMP upregulation in atherosclerotic lesions is the effect of ROS (Galis *et al.* 1998, Nelson and Melendez 2004).

MMP regulation in endothelial cells (ECs) shows many similarities with that in VSMCs (Chase and Newby 2003). Co-ordinated upregulation of MMP1, MMP3 and MMP9 occurs in ECs in response to TNF-α and IL-1α (Hanemaaijer *et al.* 1993). ECs also express both CD40 and CD40 ligand, and consistent with observations in VSMCs and macrophages, CD40 ligation upregulates MMP1, MMP3 and MMP9 (Mach *et al.* 1999).

4.1 Cis-regulatory targets, nuclear proteins and signalling events

The realization that even a complete description of proteins will not suffice to understand the conditions under which they are expressed has focused attention on cis-regulatory regions, loci of programmatic control for gene expression (Rodriguez-Trelles et al. 2003). MMP substrate specificities overlap, and the action of MMPs is largely dictated by the regulatory loci, which encode the cis-acting regions (Sternlicht and Werb 2001).

MMP expression is regulated by several cytokines and growth factors, including interleukins, interferons, PDGF, TNF-α, and EMMPRIN, an immunoglobulin superfamily member (Sternlicht and Werb 2001). Many of these stimuli induce the expression and/or activation of c-fos and c-jun proto-oncogene products, which heterodimerize and bind activator-protein-1 (AP-1) sites within several *MMP* promoters (see Fig. 6, page 39). Despite numerous advances in the understanding of *MMP* gene regulation, the cross-talk between the many signalling pathways, trans-acting nuclear regulator proteins, and promoter cis-regulatory elements which regulate *MMP* gene expression remains to be elucidated (Sternlicht and Werb 2001).

The essential cis-regulatory elements involved in the regulation of *MMP* gene expression include the activating protein-1 (AP-1) element, located approximately at position -70, which binds the Fos and Jun families of transcription factors, and the polyoma enhancer A binding protein 3 (PEA-3) site, which interacts with the Ets family of transcription factors (Vincenti *et al.* 2001). Closely spaced PEA-3/ets and AP-1 elements are a common motif throughout the MMP promoters (Benbow and Brinckerhoff 1997) (see Fig. 6).

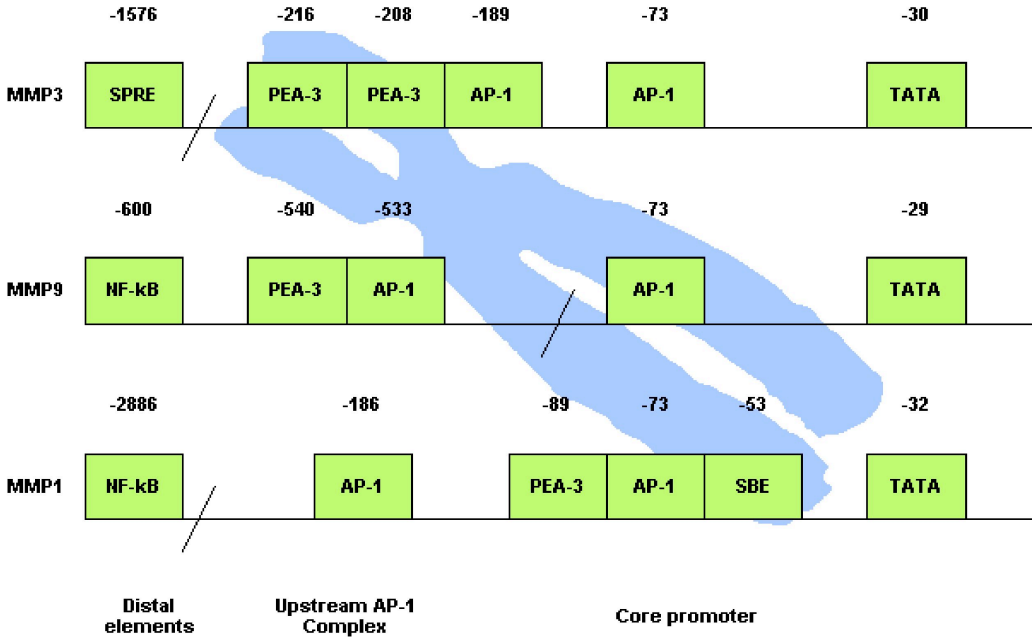

Figure 6. *Simplified promoters of MMP3 and MMP9 as well as MMP1. The binding sites for NF-kB, AP-1 and PEA-3, and stromelysin PDGF responsive element (SPRE) are shown. Modified from Chase and Newby, 2003. This figure was prepared with ConceptDraw Med (Computer Systems Odessa Corporation)*

The proximal AP-1 element plays a major role in the transcriptional regulation of MMP1, MMP3 and MMP9 gene expression, as shown by mutation, which dramatically reduces the basal activity and responsiveness of the promoters to external stimuli in vitro (Benbow and Brinckerhoff 1997). Overexpression of Ets transcription factors enhances MMP1 (Westermarck *et al.* 1997), MMP3 (Buttice and Kurkinen 1993) and MMP9 (Gum *et al.* 1996) promoter activity. AP-l and NF-kappaB transcription factors synergistically regulate *MMP3* and *MMP9*, although only *MMP9* has a well-defined NF-kappaB regulatory element in its proximal promoter (Bond *et al.* 2001). An NF-kB consensus sequence is present in the proximal MMP9 promoter (at -600 bp) and may be responsible for the synergistic effects between cytokines and growth factors (Bond *et al.* 1998); inhibition of NF-kB results in complete suppression of MMP1 and MMP3 protein and mRNA expression in rabbit and human VSMCs (Bond *et al.* 2001) and macrophages (Chase *et al.* 2002).

The PDGF-responsive SPRE element (stromelysin PDGF-responsive element) is located at position -1571 to -1576 (AT-rich palindromic ACTAGT) in the promoter of the *MMP3* gene. A 9-bp cis-regulatory target site for a repressor protein (GCGCAC/TGCC) is located at position -1567 to -1559 in the promoter of the *MMP9* gene (Zhang *et al.* 1999). At a more considerable distance from the transcription start site, the cis-regulatory target site for an IL-l inducible repressor protein, stromelysin IL-l responsive

element or SIRE (G(T)TTTTTCCCCCCATCAAAG), is located at position -1614 to -1595 in the promoter of the *MMP3* gene (Borghaei *et al*. 1999) and the GC box within the same sequence interacts with the transcription factor ZBP-89 (Ye *et al* . 1999).

Transcriptional regulation also requires the concerted action of a large number of trans-acting regulatory proteins, many of which act in multiprotein complexes (Rekdal *et al.* 2000). A polymorphism in the promoter of *MMP3* (dbSNP rs3025058) falls in a region bound by different protein complexes, which contain the zinc-finger transcription factor ZBP-89 and the NF-kappaB dimers p50/p50 and p50/p65 (Rockman *et al.* 2004). The transcriptional effects of these proteins are mediated by protein-protein interactions between SPBP (stromelysin-l PDGF-responsive element binding protein) (AR1), which binds an adjacent element, and Jun, which binds in the proximal promoter, near the transcription start site (Kirstein *et al.* 1996). SPBP (stromelysin-l PDGF-responsive element binding protein), which binds to the PDGF-responsive element (SPRE) in the immediate vicinity of the polymorphism, is a large 220-nuclear factor with homology to the DNA basic domains of Fos and Jun (Rekdal *et al.* 2000). SPBP co-activates such diverse transcription factors as Spl, c-Jun and Ets-l and may constitute a coactivator for several structurally and functionally dissimilar transcription factors binding to distinct target sequences in promoters/ enhancers (Rekdal *et al.* 2000). SPBP does not function by increasing translation or increasing protein stability but most likely acts on the transcriptional initiation step, either at the chromatin/DNA level or in the recruitment of factors necessary to the initiation state (Rekdal *et al.* 2000). Fos and Jun proteins interact with NF-kappaB, which in part may explain its apparent functional cooperativity with AP-l sites (Crawford and Matrisian 1996).

The signalling events by which inflammatory growth factors and cytokines integrate with trans-acting nuclear regulator proteins in order to regulate *MMP* gene expression involve mitogen-activated protein kinases (MAPKs) and isoforms of protein kinase C (PKC) (Hussain *et al.* 2002). Inhibitors of ERK1/2, JNK and p38 have all been shown to inhibit MMP production in a variety of cell types (Simon *et al.* 1998, Chase and Newby 2003). Specific PKC isoforms are involved in the pathways leading to the induction of *MMP9* expression by inflammatory cytokines (Esteve *et al.* 2002). Selective inhibitors of PKC block upregulation of *MMP1*, *MMP3* and *MMP9* in rabbit VSMCs (Fabunmi *et al.* 1996). Production of *MMP1*, *MMP3* and *MMP9* is reduced by adeno-virus-mediated over-expression of a dominant negative (DN) PKCζ, which inhibits NF kappaB DNA binding (Hussain *et al.* 2002). Inhibition of NF-kappaB may not be the only effect of (DN) PKCζ, as this isoform has also been shown to be specifically and critically involved in the activation of *MMP3* expression through the *MMP3*

promoter PDGF-responsive SPRE element to which SPBP binds (Sanz *et al.* 1994, Sanz *et al.* 1995, Hussain *et al.* 2002).

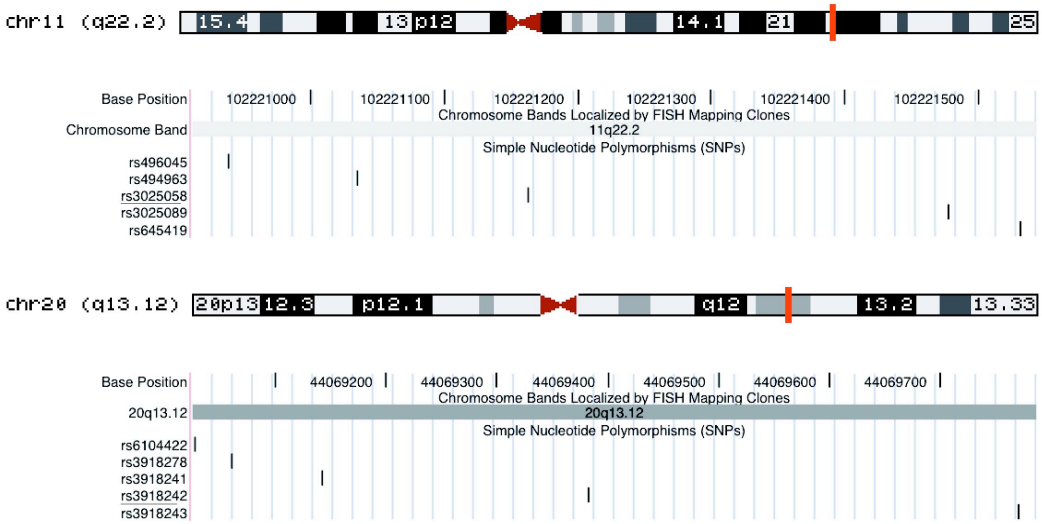

Figure 7. *Chromosomal location of the polymorphisms in MMP3 (11: 102221162-102221161) and MMP9 (20: 44069383-44069383). This figure was prepared with UC Santa Cruz UCSC Genome Browser http://genome.ucsc.edu (Kent et al. 2002, Karolchik et al. 2003). The Human May 2004 Genome Assembly was used to produce the image.*

4.2 Cis-regulatory polymorphisms in the promoters of *MMP*s

The single nucleotide polymorphisms (SNPs) occur once every 100 to 300 bases in the human genome and, due to a low mutation rate in mammalian genomes, are inherited. In the process of completion of the human genome (3 x 109 base pairs) in the year 2003, it became clear that the extent of genetic variation was much greater than had been estimated (Lander *et al.* 2001, Venter *et al.* 2001). Currently 10,054,521 (5,054,675 validated) SNPs have been reported (dbSNP build 124, January 06, 2005). Most of the SNPs are silent and may not alter the function or expression of a gene (see Fig. 8, page 43). Allelic differences in transcription, however, translate into measurable differences in phenotypes.

The cis-regulatory target site variants in the promoters of *MMP*s receive informational inputs through recruitment of trans-acting nuclear regulator proteins and convey regulatory instructions to the transcription apparatus (see Fig. 7). Common bi-allelic SNPs which influence the rate of transcription have been identified within *MMP* gene promoters (Ye 2000).

Population	Alleles A	Alleles -
European*	0.333	0.667
European† (Southern Italy)	0.58	0.42
African*	0.795	0.205
Ethiopian†	0.730	0.270
Pacific†	0.980	0.020
Chinese†	0.830	0.170

Table 4. *Allele frequencies (MMP3) per population for rs3025058 (dbSNPbuild124). *SeattleSNPs. NHLBI Program for Genomic Applications, UW-FHCRC, Seattle, WA URL: http://pga.mbt.washington.edu † Wray LaB, Department of Biology, Duke University, Box 90338, Durham, NC 27708, URL: http://www.biology.duke.edu/wraylab/, data presented with kind permission from Greg Wray email: gwray@duke.edu.*

Population	Alleles T	Alleles C
European*	0.130	0.870
African*	0.188	0.812

Table 5. *Allele frequencies (MMP9) per population for rs3918242 (dbSNPbuild124). *SeattleSNPs. NHLBI Program for Genomic Applications, UW-FHCRC, Seattle, WA URL: http://pga.mbt.washington.edu*

The *MMP3* SNP (-/T) is located 1612 bp upstream of the transcription site and contains a run of five or six adenosines (dbSNP rs3025058) (Ye 2000). This polymorphism is one of the best-characterized functional variants in humans, both at the biochemical and at organism level (Rockman *et al.* 2004). In transient transfection assays, a *MMP3* promoter construct with 6A at the polymorphic site was found to express less of the reporter gene than a construct containing 5A (Ye *et al.* 1996). The 5A allele binds with lower affinity than the 6A allele to at least one transcription complex, now identified as the p50/p50 NF-kappaB dimer, which would suggest that NF-kappaB is a repressor of cytokine-induced expression of *MMP3* (Ye *et al.* 1996, Borghaei *et al.* 2004). The *MMP9* SNP (T/C) contains either a cytidine or thymidine 1562 bp upstream of the *MMP9* transcription start site (dbSNP

rs3918242) (Zhang *et al.* 1999). Here, nuclear protein complexes bind the C allele most readily, and the less common T allele is more potent than the C-allele, as shown in transient transfection experiments (Zhang *et al.* 1999). These cis-regulatory variants in the *MMP3* and *MMP9* genes have been validated (by non-computational methods) (see Tables 4, 5, page 42). There is interaction of one or more nuclear trans-acting transcriptional regulatory proteins with the DNA sequences at both of these polymorphic sites, as revealed by electrophoretic mobility shift assays and DNase I footprinting (Ye *et al.* 1996, Zhang *et al.* 1999). The *MMP3* polymorphism is often also reported to be located at -1171 (Quinones *et al.* 1989), or at -1608 (Borden *et al.* 1994).

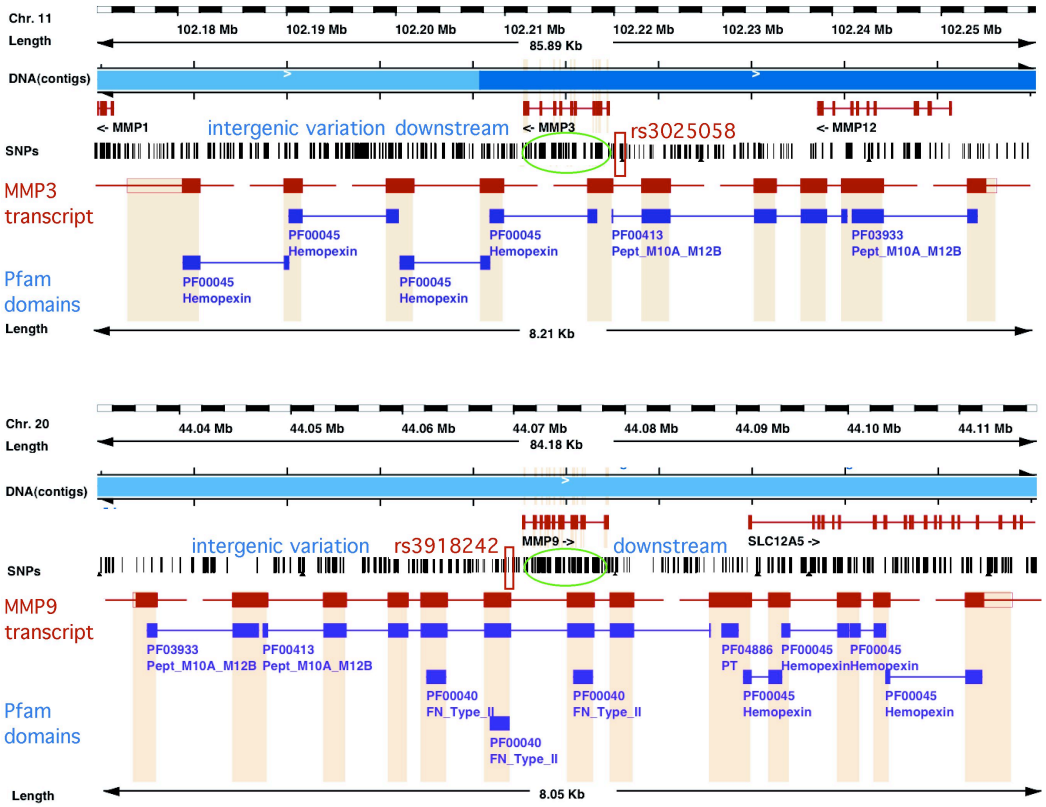

Figure 8. *Genetic variation information of MMP3 and MMP9 in genomic context. Vertical bars represent Single Nucleotide Polymorphisms. Gene transcripts are shown below the 'DNA (contigs)' bar, which is a representation of the genomic sequence. At the bottom, a summary for exon sequences of transcripts is provided. In addition to promoter genetic variation, sequence changes in the introns (intergenic variation) may affect the production of mRNA via splicing. MMP3: Exons: 10, Transcript length: 1821bp, Translation length: 477 residue. MMP9: Exons: 13, Transcript length: 2335bp, Translation length: 707 residue. (Birney et al. 2004, Bateman et al. 2004).*

An *MMP1* SNP contains one or two guanidines 1607 basepairs upstream of the transcription start site (dbSNP rs1799750) (Rutter *et al.* 1998). Here, the insertion of an extra G creates a functional Ets-binding site immediately

adjacent to an AP-1 site and, as a result, transcription is enhanced. Another SNP located 1306 bp upstream of the *MMP2* transcription start site contains either a cytidine or thymidine, such that the less common T allele disrupts an otherwise functional Sp1-binding site and diminishes promoter activity (Price *et al.* 2001). An adenine-to-guanine substitution at position -181 and a cytosine-to-thymidine substitution at position -153 upstream of the *MMP7* transcription start site interact, such that a significant increase in promoter activity is only achieved when the two rare alleles are combined, i.e. with the -181G/-153T construct; the -181 G site coincides with a putative binding site for a transcription factor (Jormsjö *et al.* 2001). An adenine-to-guanine transition exists 82 bp upstream of the *MMP12* transcription start site, such that the A allele has higher AP-1 binding affinity and higher promoter activity than the less common G allele (Jormsjö *et al.* 2000).

5. CIS-REGULATORY POLYMORPHISMS IN THE PROMOTERS OF *MMP*S AND ATHEROSCLEROSIS

The genomic variants with allele-specific effects on the transcriptional activities of *MMP*s have been shown to be associated with susceptibility to coronary heart disease and cancers (Ye S 2000). The relationship between the *MMP* promoter polymorphisms and atherosclerosis was first detected in the *MMP3* gene in 1995 (Ye *et al.* 1995, Ye S 2000) (see Table 6, page 46). In a study by McGlinchey and colleagues, *MMP3* genotype was not associated with ischemic heart diseases (McGlinchey *et al.* 2004). According to Terashima and colleagues, prevalence of the 5A/6A+5A/5A genotype is significantly more frequent in patients with acute myocardial infarction than in control subjects (Terashima *et al.* 1999). Yoon and colleagues found no association of *MMP9* genotypes with aneurysms, but the frequency of the 5A *MMP3* allele was somewhat higher in the group with abdominal aortic aneurysm (Yoon *et al.* 1999).

In a study by Cho and colleagues, the single nucleotide polymorphism at position -1562 in the promoter regulatory region of *MMP9* gene was related to severity of coronary atherosclerosis and restenosis after percutaneous coronary intervention; C/C homozygosity emerged as a potential genetic protective factor for coronary artery disease in Koreans (Cho *et al.* 2002) (see Table 6, page 46). In a study by Blankenberg and colleagues, the *MMP9*/C-1562T polymorphism was not associated with cardiovascular events, but concentrations of MMP9 were significantly higher among patients who experienced a fatal event (Blankenberg *et al.* 2003).

According to Medley and colleagues, *MMP9* genotype influences large artery stiffness through effects on aortic gene and protein expression (Medley *et al.* 2004). Jones and colleagues found genotypes containing the T allele of this polymorphism to be significantly more common in patients with abdominal aortic aneurysm compared with control subjects (Jones *et al.* 2003).

In a study by Ghilardi and colleagues involving combined *MMP1* and *MMP3* genotypes, homozygosity for the 6A allele of the *MMP3* promoter in combination with *MMP1* 2G homozygosity predicted an increased risk of internal carotid artery stenosis (Ghilardi *et al.* 2002) (see Table 6, page 46). Haplotypic analysis of the *MMP9* gene in relation to coronary artery disease indicated that the C-G-C haplotype (-1562C, +279Q, and +6C) was associated with a protective effect against atherosclerosis (Morgan *et al.* 2003). The *MMP12* promoter polymorphism (A to G substitution at position -82) was associated with coronary artery luminal dimensions in diabetic patients with manifest coronary artery disease (Jormsjö *et al.* 2000).

Table 6. *Matrix metalloproteinase genes with functional cis- regulatory polymorphisms, references for transcription (protein binding) experiments and references for associations of the polymorphisms with atherosclerosis. * Position denoted in relation to the transcription start site. ↑Increased risk ↓Decreased risk † Haplotype ‡ Combined genotype*

Gene	Position*	Reference	Phenotype/ Event	Association	Reference
MMP1	-1607 indel (rs1799750)	Kanamori *et al.* 1999	Coronary Heart Disease	2G↓	Ye *et al.* 2003
(CLG)	-1607 (*MMP1*) -1612 (*MMP3*)	"	Carotid Artery Stenosis	6A6A 2G2G ↑	Ghilardi *et al.* 2002 ‡
MMP3	-1612 indel (rs3025058)	Ye *et al.* 1996, 1999	Coronary Atherosclerosis	6A ↑	Ye *et al.* 1995
(STR1)	"	"	Acute Myocardial Infarction	5A6A/6A6A ↑↓	de Maat *et al.* 1999
	"	"	Acute Myocardial Infarction	5A6A/5A5A ↑	Terashima *et al.* 1999
	"	"	Myocardial Infarction	5A ↑	Beyzade *et al.* 2003
	"	"	Abdominal Aortic Aneurysm	5A ↑	Yoon *et al.* 1999
	"	"	Carotid Artery Stenosis	6A6A ↑	Ghilardi *et al.* 2002
	"	"	Coronary Aneurysm	5A↑	Lamblin *et al.* 2002
	Haplotype -1612, -376, +45	"	Ischaemic Heart Disease	5A ↑	McGlinchey *et al.* 2004
	-1612 (rs3025058)	"	Myocardial Infarction	-1612 5A -376G +45K↑	Zhou *et al.* 2004 †
	"	"	Acute Myocardial Infarction	No association	Gao and Li 2004
	"	"	Large Artery Stiffness	5A5A ↑	Kingwell *et al.* 2001
	"	"	Large Artery Stiffness	5A5A ↑	Medley *et al.* 2003
	"	"	Coronary Artery Disease	5A5A ↓	Humphries *et al.* 1998
MMP7	Haplotype -181, -153	Jormsjö *et al.* 2001	Coronary Artery Dimensions	-181G -153T ↓	Jormsjö *et al.* 2001 †
MMP9	-1562 C/T (rs3918242)	Zhang *et al.* 1999	Coronary Artery Disease	CC ↓	Cho *et al.* 2002
(GELB)	"	"	Myocardial Infarction	No association	Zhang *et al.* 1999
	"	"	Coronary Artery Disease	T T/TC ↑	Zhang *et al.* 1999
	"	"	Cardiovascular Death	No association	Blankenberg *et al.* 2003
	"	"	Abdominal Aortic Aneurysm	CT/TT ↑	Jones *et al.* 2003
	"	"	Coronary Aneurysm	No association	Lamblin *et al.* 2002
	"	"	Large Artery Stiffness	T ↑	Medley *et al.* 2004
	Haplotype -1562, +279, +6	"	Coronary Artery Disease	-1562C +279Q +6C↓	Morgan *et al.* 2003 †
	-1562 (rs3918242)	"	Intracranial Aneurysm	No association	Krex *et al.* 2004
	"	"	Cerebral Hemorrhagic Events	No association	Montaner *et al.* 2003
MMP12	-82 A/G	Jormsjö *et al.* 2000	Coronary Artery Dimensions	-82 A ↓	Jormsjö *et al.* 2000
(MME)	"	"	Coronary Aneurysm	No association	Lamblin *et al.* 2002

6. Extracellular matrix-dependent cell proliferation and apoptosis in the development of atherosclerosis

The extracellular matrix, main components of which are collagen (main framework of the artery wall) and elastin (organized in sheets separating the vascular layers, e.g. IEL) surrounded by a viscoelastic gel consisting of proteoglycans, hyaluronan, glycoproteins and water, was previously commonly conceived as an inert collection of structural macromolecules (Libby and Lee 2000) and the commonly held view was that of a tissue component interconnecting other (important) tissues; hence the term connective tissue, the cement, the glue between the important elements (Bosman and Stamenkovic 2003). The extracellular matrix homeostasis, however, is highly regulated and depends on communication, i.e. signal transduction, between the organic structure and its environment (Wernig and Xu 2002). A large body of evidence now supports a central role of the extracellular matrix in the control of cellular functions, i.e. as a substrate for cell adhesion, source of survival signals (antiapoptosis), reservoir for growth factors and site for arterial lipoprotein binding, and as a determinant of tissue mechanics (Libby and Lee 2000).

In the cardiovascular system, blood vessels are dynamically subjected to mechanical forces where biomechanical stress initiates signalling pathways leading to proliferation or differentation, or to cell death. Hypertension is one of the most important risk factors in both coronary heart disease and cerebrovascular accidents (Wernig and Xu 2002). Overstretching leads to hypertrophy, hyperplasia and cell migration, which have been considered the key events in the development of atherosclerosis (Zou *et al.* 1998, Li and Xu 2000). Mechanosensors include platelet-derived growth factor (PDGF) receptors, integrin receptors and G-protein-coupled receptors (such as receptors for angiotensin II or endothelin) (Hu *et al.* 1998, Cattaruzza *et al.* 2000). After transduction of the stretch signal, protein kinase C and mitogen-activated protein kinases (MAPKs) are activated (see Fig. 9, page 48), leading to increased c-fos and c-jun gene expression and enhanced transcription factor AP-1 DNA binding activity (Li and Xu 2000, Wernig and Xu 2002). At the same time, overstretching induces signalling pathways leading to growth inhibition and apoptosis (Mayr *et al.* 2000).

6.1 Matrix metalloproteinases and cell proliferation

Cell proliferation is a highly regulated process controlled by numerous communication networks. In recent years it has been recognized that proliferation and cell death are connected and considerable overlap exists

between the components which execute both processes (Wernig and Xu 2002, Li *et al.* 2003). The endothelial injury or dysfunction resulting from multiple stresses (including biomechanical stress) induces responses which alter the homeostatic properties of the endothelium (Bendeck *et al.* 2002).

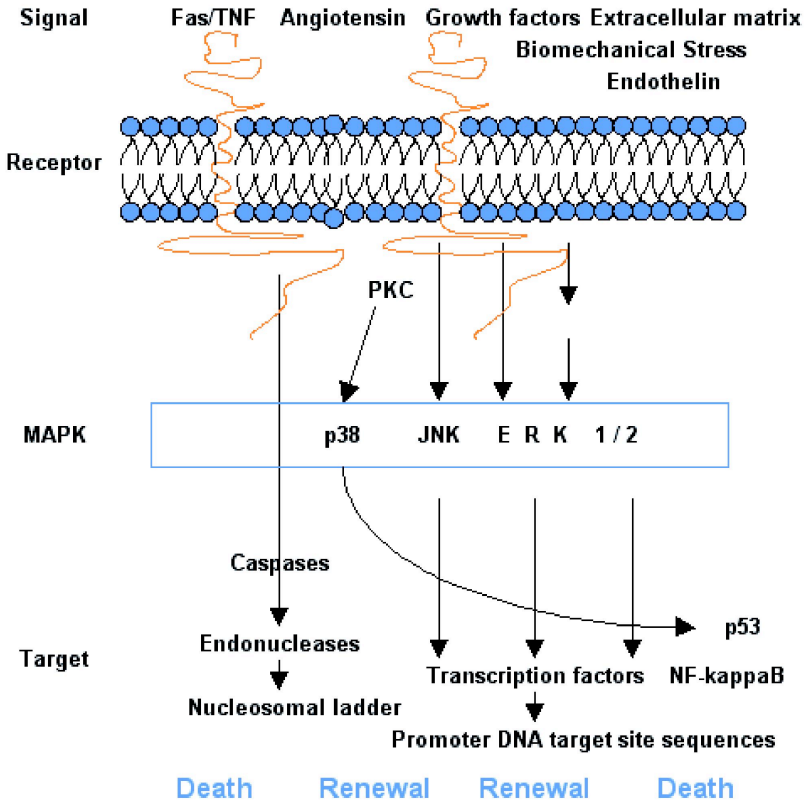

Figure 9. *Signal transduction pathways mediating apoptosis in atherosclerosis. The death receptor pathway is initiated by members of the death receptor superfamily. A variety of external signals such as cytokines, extracellular matrix composition, growth factors and biomechanical stress activate directly or indirectly MAPK, which are able to mediate apoptosis via p53 or via phosphorylation and activation of nuclear regulatory proteins, which induce the expression of genes involved in the regulation of cell proliferation and apoptosis. Isoforms of PKC also appear to be essential in mechanical stress-mediated apoptosis in atherosclerosis. Modified from Wernig and Xu 2002. This figure was prepared with ConceptDraw Med (Computer Systems Odessa Corporation).*

MMPs may potentially regulate any of the processes required for cells adhering to the ECM to change from an adhesive to a migratory phenotype, i.e. modulation of adhesive sites and cell-surface adhesive molecules, clearing of ECM to break down physical barriers, and the presence of chemoattractants to guide migration (Vu and Werb 2000). "Physiologically" invasive cells include trophoblasts and osteoclasts; MMP inhibitors block migration of osteoclasts through collagen gels, and anti-MMP9 antibody can abrogate ECM invasion of trophoblasts in vitro (Behrendtsen and Werb

1997, Sato *et al.* 1998). A role for MMPs during development is suggested by the necessity of cell migration, sometime over long distances, during tissue morphogenesis (Vu and Werb 2000).

Tissue homeostasis depends on a dialogue between cell types operating within a dynamic three-dimensional tissue ECM microenvironment (Mannello *et al.* 2005). It is thus not surprising that MMPs can alter cell behavior through their action on the ECM (Vu and Werb 2000). Studies on the roles of MMPs in cell behavior are few but have shown effects on cell proliferation, survival, apoptosis, differentiation and organization (Vu and Werb 2000) (see Table 7, page 50). The major issue is whether the spatial organization of a cell within the 3D tissue microenvironment can modulate cell responsiveness to regulate cell fate decision (Mannello *et al.* 2005)

Several mechnanisms have been proposed to explain MMP-mediated cell growth. One of these is the cleavage of matrix proteins associated with growth factors; MMPs can affect growth factor-mediated cell proliferation by regulating their bioavailability and/or activity (George and Divedi 2004).

6.2 Matrix metalloproteinases and apoptosis

Apoptosis, programmed cell death, is the *conditio sine qua non* for any change in tissue composition or renewal (Wernig and Xu 2002). It is a genetically regulated cellular suicide mechanism which plays a crucial role in development and in the defence of homeostasis in many tissues; physical stimuli such as mechanical stress must be sensed and transmitted through intracellular signal transduction pathways, e.g. MAPK kinases, to the nucleus (Cryns and Yuan 1998, Wernig and Xu 2002, Littlewood and Bennett 2003) (see Fig. 9, page 48).

Recent studies indicate that activation of caspase proteases does not always lead to cell death, and instead might be important for cell differentiation (Meier and Silke 2003, Mannello *et al.* 2005). In both physiological and pathological conditions, many other proteases in addition to caspases (including granzymes, cathepsins, proteasomal proteases and MMPs) display activation which can effectively trigger cell death (Lockshin and Zakeri 2004, Mannello *et al.* 2005).

Pro-apoptotic	References	Anti-apoptotic	References
MMP1	*Conant et al. 2004*	*MMP2*	*Kwan et al. 2004*
MMP2	*Schönbeck et al. 1998*	*MMP3*	*Wetzel et al. 2004*
MMP3	*Witty et al. 1995*	*MMP7*	*Powell et al. 1999*
MMP7	*Powell et al. 1999*	*MMP9*	*Bergers et al. 2000*
MMP9	*Vu et al. 1998*	*MMP11*	*Baserga et al. 2000*
MMP11	*Ishizuya-Oka et al. 2000*		
MMPs/ADAMs	*Herren et al. 1998*		

Table 7. *Selected references for opposing apoptotic and anti-apoptotic functions of MMPs. Several MMPs are reported to be involved in the processes of programmed cell death through paradoxical contrasting action modes. Modified from Mannello et al. 2005.*

Proteolytic damage by any of the proteases can trigger cell death, even though activation of a protease is not necessarily synonymous with apoptosis (Green and Evan 2002, Abraham and Zhaman 2004, Mannello *et al.* 2005). There is increasing evidence for the involvement of MMPs, with MMP3 and MMP9, in apoptosis phenomena; in atherosclerotic lesions these effects could be mediated through precise degradation of the most common ECM glycoproteins in the vascular wall, e.g. laminin, fibronectin, thrombospondin and osteopontin (Chintala *et al.* 2002, Witty *et al.* 1995). MMPs can also promote apoptosis via "Anoikis", a type of programmed cell death which is induced by inadequate/altered cell-matrix interactions. MMPs affect cell survival and proliferation both positively and negatively through proteolysis of several biologically active intra/ and/or extra-cellular molecules in a context-dependent manner (Egeblad and Werb 2002, Mannello *et al.* 2005).

Proteolytic cleavage also occurs in the nucleus in processes like the regulation of the cell cycle, nuclear matrix protein degradation and apoptosis (Martelli *et al.* 2002, Mannello 2005). In addition to the nuclear translocation and localization of MMP3 and TIMP-1, the presence of MMP2 and MMP9 in the nuclei suggests that these MMPs may have functions like those of caspases in the nucleus (Ritter *et al.* 1999, Mannello 2001, Soldani and Scovassi 2002, Si-Yayeb *et al.* 2003, Mannello 2005).

Virchow recognized cell death in atherosclerotic lesions 150 years ago and described atherosclerotic plaques as a transitional lesion turning proliferating cells into death (Isner *et al.* 1995, Han *et al.* 1995, Geng and Libby 1995, Geng 2001, Virchow [1858] 1989). In the normal vessel wall, vascular cells evince very low rates of turnover. Physical force or stress, essential to develop organic shape and function and to maintain the functional phenotype throughout life, initiates signalling pathways leading to proliferation or differentiation, or to cell death (Wernig and Xu 2002). Apoptosis-inducing stress signals from outside the nucleus include MAPK cascades and protein kinase C (PKC) (Wernig and Xu 2002). ERK activation of transcription factors tends to favor survival, whereas JNK and p38 MAPK activation of nuclear regulator proteins seems to contribute to growth arrest and cell death (Wernig and Xu 2002). NF-kappaB transcription factor, important for both prevention and pathology of atherosclerotic disease, translocates to the nucleus after its activation by reactive oxygen species (ROS) and may protect from apoptosis (Wernig and Xu 2002). Other chemical and physical signals triggering apoptosis include proteins from the tumor necrosis factor cytokine family (Fas-ligand, TNF-alpha), oxygen radicals (e.g. NO), angiotensin, growth factors and loss of extracellular matrix attachment (Wernig and Xu 2002).

Whereas cell death is apparent in all of the major types of cells within the atherosclerotic lesion, the contribution of the apoptosis of different cell types to the progression of the lesion is unclear (Kockx and Herman 2000, McCarthy and Bennett 2000, Littlewood and Bennett 2003). The transition of a fatty streak into an atherosclerotic plaque is characterized by the appearance of focal and diffuse regions of cell death (Kockx *et al.* 1998). The presence of apoptotic cell death in human and experimental atherosclerotic plaques has been demonstrated (Isner *et al.* 1995, Geng and Libby 1995, Han *et al.* 1995, Kockx *et al.* 1996a, Kockx *et al.* 1996b) and is associated with macrophage infiltration (Han *et al.* 1995, Björkerud and Björkerud 1996, Kockx *et al.* 1996b), whereas lesions consisting only of SMCs present very little apoptosis, as demonstrated by the TUNEL technique.

Advanced atherosclerosis is often associated with dystrophic calcification, which may contribute to plaque rupture and thrombosis (Bini *et al.* 1999). The initiation sites for calcification in cartilage and bone are cellular products called matrix vesicles. Similar structures have been found in calcified arteries and it has been suggested that these may be derived from apoptotic cells. Apoptosis may constitute a crucial initiating event for vascular calcification (Proudfoot *et al.* 2001). Initial calcium deposits associated with elastic fibers have been shown to be accompanied by elastin degradation, disorganization of aortic extracellular matrix, and moderate levels of vascular cell apoptosis, indicating a correlation between MMP-

mediated elastin degradation and vascular calcification (Basalyga *et al.* 2004). MMP-knockout mice were resistant to $CaCl_2$-mediated aortic injury and did not develop elastin degeneration or calcification (Basalyga *et al.* 2004).

OUTLINE OF THE STUDY

The motivation for this study originated from results showing that naturally occurring sequence variations in the promoters of *matrix metalloproteinases 3* and *9* may be associated with susceptibility to coronary heart disease and aneurysms. Furthermore, it had been realized that the expression of matrix metalloproteinases 3 and 9 is increased in atherosclerotic plaques.

In line with the above, the aims of the present study can be separated into two, albeit interconnected subjects, as follows:

1) To study the association between the *matrix metalloproteinase* genotypes and the area of autopsy-verified atherosclerotic lesions in the coronary arteries.

2) To study the association between the *matrix metalloproteinase* genotypes and autopsy-verified defects in the continuity of the internal elastic lamina.

SUBJECTS AND METHODS

1. Autopsy series

1.1 The Helsinki Sudden Death Study (I, II, III)

The Helsinki Sudden Death Study (HSDS) was designed to investigate the background and risk factors predisposing to sudden out-of-hospital death in Finnish middle-aged men living in Helsinki and its environs. The original study population comprised a series of 300 Caucasian males whose mean age at their time of death was 53 years (range 35 to 69 years). The autopsy series comprising out-of-hospital deaths were collected during 1991-1992 at the department of Forensic Medicine in the University of Helsinki. This single autopsy series covered 28% of all deaths of males within this age group in the Helsinki area during the study period in question. The reason for the medicolegal autopsy was an unexpected sudden death occurring outside hospital.

For collection of data on CAD risk factors, a relative or a close friend of the deceased was interviewed within two weeks postmortem. The detailed questionnaire used consisted of more than 50 detailed questions and included a review of past and recent smoking and drinking habits as well as previous illnesses (Karhunen and Penttilä 1990). Body mass index (BMI) and age were verified as part of the routine autopsy protocol. The ethics committee of the Department of Forensic Medicine, University of Helsinki, approved the study.

1.2 Consecutive autopsy series (IV)

Initially, the autopsy material was collected from 81 forensic and 42 medical postmortem examinations performed in Tampere University Hospital in 1993 to evaluate atherosclerosis in abdominal arteries. Subjects were both men (n=90) and women (n=33) aged 18-93 years. Their mean age was 62 years (range 18 to 93 years). The histories of cardiovascular diseases and classical risk factors for atherosclerosis were collected from the hospital records of each subject. The Ethics Committee of Tampere University Hospital approved the study.

2. Measurement of atherosclerosis at autopsy

2.1 Macroscopic classification (I, II, III)

The areas of atherosclerotic lesions were measured from the left anterior descending (LAD), right coronary artery (RCA), left circumflex coronary artery (LCX) and from the thoracic and abdominal aorta. At autopsy, coronary angiography was performed using vulcanizing liquid silicone rubber mixed with lead oxide as the contrast medium. The arteries were dissected free, opened, attached to cardboard and fixed in 10 % buffered formalin, and stained for fat by the Sudan IV staining method. 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. Any flat or slightly elevated intimal lesion which was stained distinctly by Sudan IV and showed no other type of change was classified as a fatty streak. A raised lesion which did not exhibit hemorrhage, necrosis or thrombosis was regarded as a fibrous plaque. The X-ray positive areas in the radiologic examination of the vessels were regarded as areas of calcification. The areas of different types of lesions were expressed in percentages (%). The presence of MI in the series was confirmed by a macroscopic and histologic examination of the myocardium. The presence/absence of neutrophil granulocytes was considered diagnostic of an acute MI and the presence/absence of fibrous scar tissue diagnostic of an old MI.

2.2 Histological classification (IV)

For histological examinations of the mesenteric arteries with light microscopy, 0,5-cm long segments of the arteries 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 circumference (length) of the IEL and the thickness of the intima were measured in millimetres by the MOP3 image-analysis system (Reichert-Jung, Eching, 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). Finally, the number of gaps in the IEL was counted, divided by the circumference of the IEL and expressed as gaps per millimetre.

3. DNA EXTRACTION AND *MMP* GENOTYPING

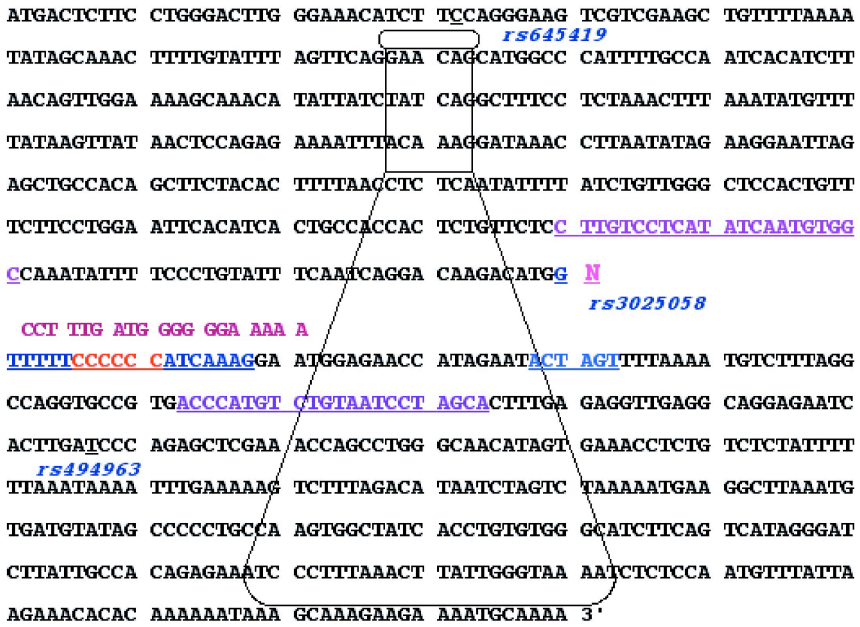

Figure 10. *Sequence region of SNP ID: rs3025058. Target sites for forward primer 5´ CTT GTC CTC ATA TCA ATG TGG C 3´ and reverse primer 5´ TGC TAG GAT TAC AGA CAT GGG T 3´ are indicated by underlined nucleotides. Minisequencing detection primer 5´ CCT TTG ATG GGG GGA AAAA 3´ is shown as a separate string. Stromelysin IL-1 responsive element or SIRE (G(T)TTTTTCCCCCCATCAAAG) (Borghaei et al. 1999), the GC box within the same sequence (Ye et al. 1999) and the PDGF-responsive SPRE element (AT-rich palindromic ACTAGT) are indicated by underlined nucleotides. Some neighbouring SNPs and their dbSNP identifiers are shown. N denotes the site of the polymorphism (-/T in-del) at promoter position -1612 (dbSNP rs3025058). This figure was prepared with ConceptDraw Med (Computer Systems Odessa Corporation).*

DNA was isolated from pieces of cardiac muscle by a standard phenol-chloroform method (*Studies I, II, III*). In study IV, DNA was isolated from paraffin-embedded samples of the mesenteric arteries by QIAamp DNA Tissue Kit (Qiagen Inc., USA).

The polymorphism at position -1612 relative to the start of the transcription site in the *MMP3* gene (dbSNP rs3025058) was determined by PCR and solid-phase minisequencing (Syvänen 1994) (*Studies II, III*) (see Fig. 10). The promoter region of the *MMP3* gene was amplified by PCR in 50 µl of Perkin-Elmer GeneAmp® 10 × buffer (Roche Molecular Systems, Inc., Branchburg, New Jersey, USA), containing each of the four deoxynucleotides, PCR primer 5´ TGC TAG GAT TAC AGA CAT GGG T 3´, 5´-biotinylated PCR primer 5´ CTT GTC CTC ATA TCA ATG TGG

C 3´, $MgCl_2$ and the Perkin-Elmer AmpliTaq Gold™ DNA polymerase (Roche Molecular Systems, Inc.). Genomic DNA extracted from frozen cardiac muscle samples taken at autopsy was used in each of the amplifications.

Figure 11. *Sequence region of SNP ID: rs3918242. Target sites for nested forward primer 5' AGC ATA TAA AAT GCC TGGCA 3', nested reverse primer 5' GCC TCC CTC ACT CCT TTC 3, forward primer 5' GCC TGG CAC ATA GTA GGC CC 3', reverse primer 5' CTT CCT AGC CAG CCG GCATC 3' and repressor protein (GCGCAC/TGCC) (Zhang et al. 1999) are indicated by underlined nucleotides. N denotes the site of the polymorphism (T/C) (dbSNP rs3918242). The final amplicon was cleaved into fragments with restriction endonuclease Bbu I (Bacillus sp. Bu17091), which cleaved the T allele at the -1562 polymorphic site, generating 2 fragments, but did not cut the C allele. This figure was prepared with ConceptDraw Med (Computer Systems Odessa Corporation).*

After 12 min of preheating at 95 °C, 40 cycles of amplification were carried out in a thermal cycler (PTC-225, DNA Engine™ Tetrad MJ Research Inc., Watertown, Massachusetts, USA): 95 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min and final extension at 72 °C for 8 min followed by cooling to 8 °C. The PCR-amplified biotin-labeled PCR products were bound to streptavidin-coated microwells (Scintistrip™ Wallac Oy (EG&G), Turku, Finland). NaOH was added to the wells for 5 min at room temperature followed by washing and drying of the plate to make the target single-stranded. Minisequencing reactions were performed, where the detection primer was allowed to anneal to the biotinylated strand and was extended with a single labeled nucleoside triphosphate complementary to the nucleoside at the polymorphic site in a volume of 100 µl containing 0.5 U Dynazyme™ II polymerase (Finnzymes Oy, Helsinki, Finland), 20 pmol

detection primer 5´ CCT TTG ATG GGG GGA AAA A 3´, 2 pmol ^{3}H-labeled dATP or alternatively 2 pmol ^{3}H-labeled dCTP (Amersham Life Science, Buckinghamshire, England). The excess PCR reagents were removed by washing the wells with buffer (4 mM Tris-HCl, pH 8.0-8.8, 0.1 mM EDTA, 5 mM NaCl, Tween 20) in an automatic microtitration plate washer. The amount of radioactivity was measured with a scintillator counter (Microbeta®, Wallac Oy, Turku, Finland). The genotypes of the samples were defined by the ratio between incorporated ^{3}H-labeled nucleotides. The ratio between values (R-value) for the two ^{3}H-labeled nucleotides for each sample reflected the ratio between the two sequences in the original sample.

A cytosine (C)-to-thymidine (T) promoter polymorphism at position -1562 relative to the start of transcription in *MMP9* gene (dbSNP rs3918242) was determined by polymerase chain reaction and restriction enzyme BbuI digestion (Promega, Inc.) (*Studies I, III, IV*) (see Fig. 11, page 58). Thirty-five cycles of amplification were carried out in the following conditions: denaturation at 97 °C for 1 min, annealing at 65,7 °C for 1 min and extension at 72 °C for 1 min. After the BbuI enzyme digestion, fragments were separated by electrophoresis on 2.5 % agarose gel and visualized with ultraviolet light.

4. Statistical methods

The means of continuous variables in different genotype groups were compared by analysis of covariance (ANCOVA). Non-normally distributed data were analyzed in either square root or arc sin square root transformed form but the results were expressed as crude data. Stepwise linear regression analysis was used to identify independent risk factors for lesions (*Studies I, II, IV*). In the case of a significant main effect or interaction, least significant difference (LSD) post-hoc tests were performed to compare differences between groups (*Study III*). Analyses of the effect of the *MMP* genotype on MI, acute MI and thrombosis were based on logistic regression (SPSS 10.0 for Windows). The Spearman Rank Order Correlation matrix was used to calculate correlation (*Study IV*). Pearson's $\chi 2$-test was used to compare the genotype distributions. The criterion for statistical significance was set at $p<0.05$.

5. DATABASES

KEGG, Kyoto Encyclopedia of Genes and Genomes, Japan http://www.genome.jp/kegg/
PubMed, DbSNP, National Center for Biotechnology Information (NCBI), USA http://www.ncbi.nlm.nih.gov
The peptidase database (MEROPS), UK
http://merops.sanger.ac.uk/
Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB)
http://www.chem.qmw.ac.uk/iubmb/enzyme/EC34/
SNPper
http://snpper.chip.org/
GAD Genetic Association Database
http://geneticassociationdb.nih.gov/
HapMap Project http://www.hapmap.org/
JSNP Database http://snp.ims.u-tokyo.ac.jp/
SNP Concortium http://snp.cshl.org/
SeattleSNPs http://pga.gs.washington.edu/
Human Genome Variation Database http://hgvbase.cgb.ki.se/
Brenda The Comprehensive Enzyme Information System http://www.brenda.uni-koeln.de/
TRANSFAC® http://www.gene-regulation.com/
cisRED: Regulatory Element Database http://www.cisred.org/

RESULTS

All the observed genotype frequencies based on the experimental allele frequencies (see Tables 9 and 10, pages 64 and 65) were in agreement with Hardy-Weinberg (Hardy 1908) expectations (*Studies I, II, III and IV*).

1. *MMP9* GENOTYPE AND AREA OF ATHEROSCLEROTIC LESIONS

Men aged ≥53 years with a high promoter activity genotype had a 153.7% larger area of complicated lesion than did those with the low-activity genotype (P=0.008) *(Study I, Table 2)*. When analyzed by linear regression, age, *MMP9* genotype, BMI, hypertension, diabetes and smoking being used as independent variables, the significant variables were age (P=0.001, standardized regression coefficient 3.43) and *MMP9* genotype (P=0.012, standardized regression coefficient -2.56). The entire model explained 20.2% of the variation in lesion area (P<0.005). For other types of lesions there were no significant differences between the genotype groups. Men aged ≥53 years with a promoter high-activity genotype had, on average, a 57.0% larger area of calcified lesion compared with those with the promoter low-activity genotype (P=0.07, borderline significance) *(Study I, Table 2)*.

Compared with carriers of the promoter low-activity genotype, carriers of the high-activity genotype had, on average, a 74.0% larger area of complicated lesion in the LAD, a 419.0% larger area in the LCX, and a 51.4% larger area in the RCA. Carriers of the high-activity genotype had, on average, a 65.8% larger area of calcified lesion in the LAD, a 31.7% larger area in the LCX, and a 67.6% larger area in the RCA. There were no significant differences between the genotype groups with respect to other types of lesion. The percentage of coronary narrowing was not significantly associated with *MMP9* genotype in any of the 3 coronary arteries. There was no significant association between *MMP9* genotype and severity of atherosclerosis as assessed by numbers of coronary arteries with a stenosis >50%.

The *MMP9* promoter high-activity genotype increased the risk of MI significantly (odds ratio [OR] 1.95, 95% CI 1.03 to 3.67; P=0.04), as did the older age group (OR 3.47, 95% CI 1.84 to 6.54; P=0.001). The occurrence of acute MI, i.e., the presence/absence of neutrophil granulocytes (OR 1.10, 95% CI 0.48 to 2.53; P=0.82), or coronary thrombi (OR 0.79, 95% CI 0.28 to 2.25; P=0.66) was not significantly associated with the *MMP9* genotype.

2. *MMP3* GENOTYPE AND AREA OF ATHEROSCLEROTIC LESIONS

The *MMP3* genotype was associated with calcified atherosclerotic lesions in men ≥53 years, and in this subgroup the carriers of the 5A5A and 5A6A genotypes evinced on average a 55% increase in the area of calcified lesion involvement of the most severely affected coronary artery when compared with carriers of the 6A6A genotype (P=0.029) (*Study II, Table 2*) (see Fig. 12). The significant predictors for the mean calcified lesion area of the coronary arteries were age, *MMP3* genotype and number of affected vessels, when analyzed by stepwise regression using age, BMI, *MMP3* genotype, hypertension, diabetes, number of affected vessels and smoking as independent variables; multiple R^2 was 0.307.

In the LAD, LCX and RCA the carriers of the 5A5A and 5A6A genotypes in the subgroup of men with two- or three-vessel disease tended to have on average a 100% (P=0.09), 350% (P=0.005) and 277% (P=0.005) larger area of calcified lesions, respectively, when compared with carriers of the 6A6A genotype (*Study I, Table 3*). In stepwise regression analysis with independent variables as above, the significance of the *MMP3* genotype as an independent predictor of calcified lesion area persisted in the LCX (P=0.018) and RCA (P=0.024) (*Study I, Table 4*); multiple R^2 was 0.299 for LCX and 0.245 for RCA.

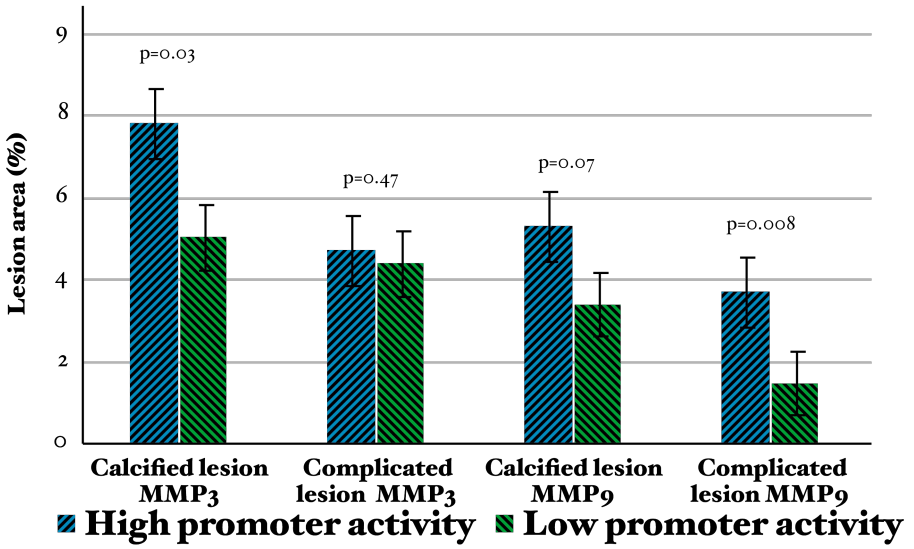

Figure 12. *Mean per cent areas of calcified and complicated atherosclerotic lesions in the most severely affected artery by MMP3 and MMP9 genotype in men ›53 years.*

The occurrence of healed MI (presence of fibrous scar tissue) (OR=1.12, 95% CI: 0.54-2.47, P=0.71), acute MI (presence of neutrophil granulocytes)

(OR=1.15, 95% CI: 0.48-2.76, P=0.75) or coronary thrombi (OR=0.70, 95% CI: 0.25-1.90, P=0.48) was not significantly associated with *MMP3* genotype.

	HIGH[a]/HIGH[b]	LOW[a]/HIGH[b]	HIGH[c]/LOW[d]	LOW[c]/LOW[d]
Area of Complicated Plaque %	6,19	4,11*	2,00†	2,54‡

Table 8. *Significant associations between mean per cent areas of complicated lesion (largest lesion in LAD, LCX or RCA) and combined matrix metalloproteinase MMP3 and MMP9 high and low promoter activity genotypes. a High promoter activity (5A/6A; 5A/5A) genotype. b High promoter activity (C/T; T/T) genotype. c Low promoter activity (6A/6A) genotype. d Low promoter activity (C/C) genotype. P-values *0.048, †0.002 and ‡0.008 (LSD post hoc).*

3. *MMP3-MMP9* COMBINED GENOTYPE STATUS AND AREA OF ATHEROSCLEROTIC LESIONS

In ANCOVA with age, body mass index, hypertension, diabetes, smoking and alcohol consumption as covariates, a significant interaction between the combined *MMP3* and *MMP9* genotypes was observed on the area of complicated lesions (P=0.012). Men with high-activity genotypes for both loci had, on average, a 250 % larger area of complicated lesion in the most severely affected coronary artery when compared with subjects not carrying high-activity genotypes (P=0.008) (*Study III, Figure 1*) (see Table 8). In addition, men with high-activity genotypes for both loci also had, on average, a 301 % larger area of complicated lesion in the most severely affected coronary artery when compared with subjects who carried the high-activity genotype for *MMP3* (P=0.002) (*Study III, Figure 1*) and a 150 % larger area of complicated lesion when compared with subjects who had high-activity genotype for *MMP9* (P=0.048) (*Study III, Figure 1*).

No significant interactions between the *MMP3* and *MMP9* genotypes were observed in a similar analysis for fatty streaks, fibrotic lesions or calcified lesions. However, men with high-activity genotypes for both loci tended to have, on average, a 200 % larger area of calcified lesion in the most severely affected coronary artery when compared with subjects who had no high-activity genotypes, but this difference was of borderline significance only (P=0.066). No significant differences were found between genotype groups in any of the main coronary arteries for fatty streaks or fibrotic lesions. The percentage of coronary narrowing was not significantly associated with the combined *MMP3-MMP9* genotype.

In a logistic regression analysis with covariates as above, the presence of coronary thrombi (p=0.99) or acute myocardial infarction (p=0.90) was not significantly associated with the combined *MMP3-MMP9* genotype.

Alleles A	Alleles -
0.59	0.41

Alleles T	Alleles C
0.14	0.86

Table 9. *Allele frequencies in studies I, II and III.*

4. *MMP9* genotype and the continuity of the internal elastic lamina

Among the male subjects, carriers of high-activity genotypes had a 2.3-fold and a 2.2-fold higher number of gaps per millimetre in the right renal artery (p=0.05) and in the left renal artery (p=0.02), respectively, as compared to men with low-activity genotypes (*Study IV, Figure 1*). A similar trend was observed for SMA and IMA. When women were included in the analyses, carriers of high-activity genotypes had a 2.7-fold higher number of gaps per millimetre in their left renal artery (p=0.001) and in addition, a 1.8-fold higher number of gaps per millimetre in the IMA (p=0.05) (*Study IV, Figure 1*). The autopsy cases with the high-activity genotype also tended to have a higher number of gaps per millimetre in their right renal artery and a similar trend was observed for SMA, but these differences did not reach statistical significance (*Study IV, Figure 1*). The number of gaps in the IEL of the celiac trunk did not vary according to the *MMP9* polymorphism.

In the group with intimal thickening, the mean number of gaps in the IEL was significantly higher than in the group with no considerable thickening; the p-values for differences in the left and right renal arteries were p<0.0001 and p<0.002, respectively (ANCOVA). In the celiac artery and in the SMA, the intimal thickness was also significantly associated with the IEL defects (p<0.0001 and p<0.0002, respectively). However, in these vessels no association of the *MMP9* genotype with the number of IEL defects was observed. The overall correlation coefficient between the number of gaps and the degree of intimal thickening in the CT/TT group were 0.71 for the left renal artery (P<0.0001) and 0.54 for the right renal artery (P=0.007). For the C allele carriers the corresponding correlation coefficients were 0.60 (P<0.0001) and 0.47 (P<0.0001) (*Study IV, Table 2*).

ALLELES T	ALLELES C
0.15	0.85

Table 10. *Allele frequencies in study IV.*

The best model by linear regression analysis identified gender (p=0.048, standardized regression coefficient −2.027) and the *MMP9* genotype (p=0.037, standardized regression coefficient −2.138) as the significant risk factors for the number of gaps/mm in the left renal artery in all subjects independent of smoking, hypertension, diabetes mellitus, BMI and sex (*Study IV, Table 3*). The entire model explained 25.0 % of the variation in the number of gaps/mm (p=0.032).

DISCUSSION

1. STUDY SUBJECTS

In the studies undertaken for this thesis (I-IV), subjects from two autopsy series were examined. In studies I, II and III, the victims of sudden death or trauma were subjected to medicolegal autopsy, and in study IV, a medical or forensic autopsy was performed on the subjects. Both series were independent and all study subjects were unrelated. In autopsy studies, the measurement of atherosclerotic variables can be performed directly from the arteries and the stage of CAD, the percentage of coronary stenosis and the presence of MI as well as the presence of defects in the internal elastic lamina can be reliably determined, circumventing many of the problems encountered in phenotyping clinical patients. Conventional studies can also easily lead to survival bias, because only patients with non-fatal events can be studied. However, owing to the sudden nature of death, the possible confounding effect of serum cholesterol levels in the deceased could not been taken into account and furthermore, autopsy studies may carry selection bias, since the subjects may have more severe atherosclerosis than subjects selected randomly. The allele frequencies were comparable to those reported for Caucasian and European populations (see Tables 4 and 5, page 42), and it is therefore plausible that the subjects here were representative, making it possible to use the data to draw conclusions regarding the Finnish population and to be fairly confident in them. On the other hand, only men were involved in studies I, II and III, and thus the results cannot be extrapolated to women.

2. METHODOLOGICAL CONSIDERATIONS

2.1 CANDIDATE GENES AND GENOMIC VARIATION

Association studies can be performed without assuming one region of the genome to be more likely to harbor the associated genetic factor (genome-wide approach) or by using some biological knowledge to prioritize the parts of the genome for the study (candidate gene approach) (Kwok 2001). In this thesis (I-IV), the candidate gene approach was used to investigate the *MMP3* and *MMP9* genotypes as determinants of atherosclerosis lesion phenotype. At present, knowledge of common genetic variation is incomplete for the majority of genes; therefore, marker selection for association analysis is in effect random (Collins *et al.* 1997, Carlson *et al.* 2004). Although the number

of common variants per gene is limited, the throughput of current genotyping technologies is inadequate for genotyping all existing common variants in all but the smallest of genes (Nickerson *et al.* 2000, Carlson *et al.* 2004). Candidate genes can be selected from any of the pathways involved in atherosclerosis, for example lipoprotein metabolism, coagulation and thrombolysis, platelet function, regulation of vascular tone, development and maintenance of structural components of the arterial wall, or regulation of cell proliferation and death, as in this thesis (Hegele 2002). Here *MMP3* and *MMP9* were chosen as candidate genes in view of the importance of MMPs in the pathology of atherosclerosis. One of the most important preconditions in association studies is the proper characterization of the phenotype of the atherosclerotic lesion. In each of the studies (I-IV), the extent of atherosclerosis, i.e. lesion area and number of gaps in the IEL, was measured as a continuous variable.

In the studies for this thesis, as in most candidate-gene studies, analysis was made of the association between disease status and a small number of candidate SNPs which have known or predicted functional consequences (Collins *et al.* 1997; Botstein and Risch 2003, Carlson *et al.* 2004). The fundamental question in association studies is, what should constitute the basic genetic component to be considered for association with a complex disorder (Neale and Sham 2004). In this thesis, association referred to allelic association, implicating the allele as the basic unit of analysis. With increasing marker density, association is often considered at the haplotypic level. Both analyses are potentially problematic in the context of replication. Furthermore, the impact of a single gene on atherosclerosis depends on the context created by environment and other genes; the effect of a single gene on the disease process is most likely to be indirect, proceeding through its effect on intermediate quantitative phenotypes of atherosclerosis such as the kinetics of cellular growth (Hegele 2002, Freimer and Sabatti 2003).

In addition, the frequency of SNPs and the extent of linkage disequilibrium (LD) may vary significantly between populations (Erichsen and Chanock 2004). Owing to historical isolation, major racial/ethnic groups differ in allele frequencies (Botstein and Risch 2003). In Europe, for example, the evidence suggests that positive selection played a role in increasing the frequency of the *MMP3* 5A-allele (Rockman *et al.* 2004) (see Table 4, page 42). It is also well known that African populations have greater genetic diversity than other populations, more haplotypes and less LD (shorter haplotype blocks) (Botstein and Risch 2003). Although atherosclerosis is considered a modern phenomenon, it is possible that the diet of Ice Age Europeans, rich in the atherogenic fats of large mammals, could have contributed to the development of atherosclerosis and constituted a selective advantage for the high expression 5A allele, or the arterial phenotype constitutes a side-effect of selection on other currently

unidentified consequences of upregulated *MMP3* expression (Rockman *et al.* 2004).

The use of association studies for the genetic analysis of complex disorders has given rise to controversy concerning study design, statistical analysis and interpretation of findings (Lander and Schork 1994, Risch 2000, Marian 2001, Neale and Sham 2004). Points of criticism often include the lack of functional studies on protein levels and activity (Taegtmeyer and Barton 2002). It can be argued that if the interest lies solely in genetic risk-factor profiling, knowledge of protein levels or activity, although essential to understanding mechanisms, does not add to the strength of the tests adopted (Taegtmeyer and Barton 2002). In autopsy studies, it is not possible to measure the activities of proteins. A frequent source of difficulty is the matter of sample size and statistical power. In the case of the *MMP3* polymorphism, the frequencies of the alleles confer good power when subjects are of European ethnicity, even if the materials in the studies for this thesis should have been larger and older. While genetic association studies have limitations, they sometimes represent the only practical approach in beginning to address a particular hypothesis (Hegele 2002). The autopsy series, for example, is the only type of material in which the association between complicated lesion area and *MMP* genetic variation can be studied. Given the limited ability of current clinical imaging methods to visualize the vessel wall, we are highly dependent on autopsy material (Virmani *et al.* 2000); such analyses are not possible in clinical patient series. For example, lesions in current animal models rarely progress beyond the stage of atheroma (i.e., a well-developed fibrous cap overlying a necrotic core) (Virmani *et al.* 2000).

There are also problems related to the use of SNPs themselves as genetic markers; isolating a true regulatory variant is complicated by linkage disequilibrium (LD) in the human genome (Pastinen and Hudson 2004). Single nucleotide polymorphisms in the same chromosomal region are not randomly or independently distributed, but tend to be associated with one another based on genetic inheritance (Botstein and Risch 2003, Erichsen and Chanock 2004). When an SNP is selected as marker for a locus, there may be no functional basis for a relationship with the phenotype (Hegele 2002). Instead, the SNP may be in linkage disequilibrium with an unmeasured functional variant (Hegele 2002). Consequently, the issue of how to select a maximally informative set of common polymorphisms for association analyses is generating considerable interest (HapMap Project); quantitative methods have been described which minimize the number of SNPs required for this task (Johnson *et al.* 2001, Zhang *et al.* 2002, Meng *et al.* 2003, Carlson *et al.* 2004). Indirect analysis does not require a priori identification of functional SNPs; rather, it assumes that genotypes at unassayed, risk-related

SNPs will be correlated with one or more assayed SNPs (Collins *et al.* 1997, Carlson *et al.* 2004). Statistical power to detect unassayed, disease-associated polymorphisms depends on the correlation (r2) between the unassayed site and an assayed site (Carlson *et al.* 2004) (see Fig. 13). Only when genotypes are perfectly correlated does r2 = 1, which can be described as "perfect LD" (Carlson *et al.* 2004).

SNPs: bp position	rs3848722: 44067227									
rs4810482: 44067957	1.000	rs4810482: 44067957								
rs3761159: 44068364	1.000	1.000	rs3761159: 44068364							
rs6065913: 44068510	1.000	1.000	1.000	rs6065913: 44068510						
rs6104420: 44068765	-	-	-	-	rs6104420: 44068765					
rs3918240: 44069023	0.912	0.912	0.912	0.912	-	rs3918240: 44069023				
rs3918278: 44069061	0.119	0.119	0.119	0.119	-	0.109	rs3918278: 44069061			
rs3918241: 44069142	0.256	0.256	0.256	0.256	-	0.233	0.465	rs3918241: 44069142		
rs3918242 **44069383**	0.256	0.256	0.256	0.256	-	0.233	0.465	1.000	***rs3918242*:** **44069383**	
rs3918243: 44069771	-	-	-	-	-	-	-	-	-	rs3918243: 44069771
rs3918245: 44070317	-	-	-	-	-	-	-	-	-	-
rs3918247: 44070825	-	-	-	-	-	-	-	-	-	-
rs3918249: 44071543	1.000	1.000	1.000	1.000	-	0.912	0.119	0.256	0.256	-
rs3918250: 44071759	-	-	-	-	-	-	-	-	-	-
SNPs: bp position	rs3848722: 44067227	rs4810482: 44067957	rs3761159: 44068364	rs6065913: 44068510	rs6104420: 44068765	rs3918240: 44069023	rs3918278: 44069061	rs3918241: 44069142	***rs3918242*:** **44069383**	rs3918243: 44069771

Figure 13. *Linkage Disequilibrium (LD) centered in the genomic position of the SNP rs3918242 in the MMP9 gene (LD measures the correlation between two neighbouring genetic variations in a specific population). Pairwise r2 values for LD values were calculated by a pairwise estimation between SNPs genotyped in the same individuals. The maximum likelihood of the proportion each possible haplotype contributed to the double heterozygote was estimated. Linkage disequilibrium describes the relationship between genotypes at a pair of polymorphic sites; genotypes at common SNPs <10 kb apart tend to be correlated. Only when genotypes are perfectly correlated does r2 = 1, which can be described as "perfect LD". Population: North America (Hubbard et al. 2005)*

Transcriptional regulation of *MMP*s may include competitive binding of proteins, cooperative binding, chromatin bending and other complex, non-linear, often strongly context dependent molecular interactions (Wray *et al.* 2003) and furthermore, the structural and functional properties of cis-regulatory elements are not always reflected in the nucleotide sequence (Dermitzakis *et al.* 2003, Rodriguez-Trelles *et al.* 2003). An additional problem is that nucleotides not directly involved in transcription-factor binding specificity (eventually 80-90% of all nucleotides in the promoter) can influence transcription-factor binding in ways not yet well understood; for example through changes in the local conformation of DNA, or in the spacing between binding sites (Rodriguez-Trelles *et al.* 2003). Conserved sequence blocks in promoter alignments do not always coincide with

functionally characterized binding sites (Bergman and Kreitman 2001, Rodriguez-Trelles *et al.* 2003).

The massive amount of single nucleotide polymorphism (SNP) data stored at public Internet sites provides unprecedented access to human genetic variation (Wjst 2004) (see Fig. 15, page 78). Although current genomic databases contain information on several million SNPs and are growing at a very fast rate, with the increasing attention towards whole-genome linkage studies and the interest in defining the haplotype structures across the genome, the true value of SNPs is a function of the quality of the annotations which characterize it. Retrieving and analyzing such data for a large number of SNPs represents a major bottleneck in the design of association studies (Riva and Kohane 2004). SNP databases contain redundant, incomplete and even wrong information and data are constantly changing during a large genotyping project (Wjst 2004). Annotation is often outdated, incomplete or fragmented among different websites (Wjst 2004). As a result the US National Human Genome Research Institute is planning to identify all functional elements in the human genome sequence in the ENCODE (Encyclopedia of DNA Elements) project (Wjst 2004, Encode 2004).

2.2 Classification of atherosclerotic lesions at autopsy.

The classification of the lesions in studies I, II and III was based on visual inspection after staining of arterial samples with Sudan IV and protocols of two international studies: the International Atherosclerosis Project, Standard Operating Protocol 1962 (Guzman *et al.* 1968) and the WHO Study Group in Europe (Uemura *et al.* 1964). The area of lesion involvement was measured in square millimetres by planimetry. A complicated lesion was characterized by plaques with ulceration or thrombosis, whereas an uncomplicated lesion is composed of fatty streak or elevated fibrous plaque erosion. Some of the fatty streaks developing in regions with adaptive intimal thickening are deeper under the endothelium, and may not become visible upon staining (Stary *et al.* 1994). Most fatty streaks, however, stain well with the Sudan fat-staining method. Some regions of adaptive intimal thickening may also project into the lumen after death and can be mistaken for raised lesions (Stary *et al.* 1992). The new standardized histological classification method (Stary *et al.* 1992, Stary *et al.* 1994, Stary *et al.* 1995) was not available at the time of collection of the series of the HSDS but should be used in future studies.

2.3 SNP GENOTYPING

Almost all SNP genotyping methods are based upon one or more of four fundamental molecular biological processes: hybridization, polymerization (nucleotidyl transfer), ligation and nucleolysis (Kwok 2001, Syvänen 2001). Major impetus for the popularity of single nucleotide polymorphism (SNP) genotyping comes from the search for disease-susceptibility genes and from the promise of pharmacogenomics and personalized medicine for health care (Phillips *et al.* 2001, Di Giusto and King 2003). Polymerization is a particularly powerful tool, with applications ranging from full Sanger sequencing through limited pyrosequencing (Ronaghi et al. 1998) to single base extension or 'minisequencing' methods which identify a single allelic nucleotide immediately adjacent to a defined primer terminus (Syvänen 1999). Minisequencing has proved particularly attractive for its simplicity (the minimal implementation contains only three major added components: primer, polymerase and nucleoside triphosphate substrate) and for its adaptability to various detection formats (Di Giusto and King 2003).

A number of genotyping principles are available to meet the needs of different study designs. These include hybridization, primer extension, ligation, invasive cleavage and, as in this thesis, minisequencing and restriction enzyme digestion. No ideal genotyping method exists, however. The perfect genotyping method according to Pui-Yan Kwok (Kwok 2001) possesses the following attributes: the assay must be easily developed from sequence information; the cost of assay development must be low in terms of marker-specific optimization; the reaction must be robust, such that even suboptimal DNA samples yield reliable results; the assay must require minimal hands-on operation; the data analysis must be simple, with automated, accurate genotype calling; the reaction format must be flexible and scalable; and once optimized, the total assay cost per genotype must be low (Kwok 2001).

To be able to resolve the issue of *MMP* gene function in vascular disease, simultaneous examination of several cis-regulatory variations in several *MMP* genes would be needed. A promising approach towards high-throughput genotyping of single nucleotide polymorphisms (SNPs) is to use arrays of immobilized oligonucleotides in miniaturized assays (Lindroos *et al.* 2002). Microarray-based technology (RNA-expression arrays) for analyzing the expression levels of thousands of genes per single experiment is well established (Pastinen *et al.* 2000); in contrast, analyses of known sequence variants (predefined human SNPs) on microarrays are less common in the literature (see Fig. 16, page 79). DNA polymerase-assisted single nucleotide primer extension methods (minisequensing) discriminate genotypes better than e.g. hybridization with oligonucleotide probes; DNA-modifying enzymes such as DNA polymerases, based on their high-sequence specifity,

may provide better genotyping power as compared with allele-specific oligonucleotide hybridization (Pastinen *et al.* 2000).

In MALDI-TOF MS- (matrix assisted laser desorption/ionization time-of-flight mass spectrometry) the primer extension products are separated due to their differences in mass. SNPs can also be determined on the RNA level with the same methods as on the DNA level.

3. CIS-REGULATORY POLYMORPHISMS IN THE PROMOTERS OF *MMP3* AND *MMP9* AND ATHEROSCLEROTIC LESIONS

3.1 *MMP3* AND *MMP9* GENOTYPES AND AREA OF ATHEROSCLEROTIC LESIONS

Study I

Earlier reports based on angiographically measured atherosclerosis and immunostaining led us to hypothesize that a larger lesion area involvement would be due to increased or decreased degradation of the extracellular matrix (Brown *et al.* 1995, Zhang *et al.* 1999). It could be hypothesized that the *MMP9* -1562 C and T alleles, which are associated with transcription rate *in vitro*, would be associated with coronary artery lesion area and risk of MI. The finding that this polymorphism was not associated with MI or coronary thrombus but with advanced lesion area only within the subgroup of elderly men suggests that this polymorphism has a role in clinically silent plaque ruptures/erosion; on the other hand, only macroscopic thrombi were involved in the classification. It is very important to realize that the presence of a plaque rupture does not imply a causal association with the thrombus which occluded the lumen (Virmani *et al.* 2000). Since we studied only men, we were not able to anticipate any role of *MMP9* variation in elderly women.

Morgan and colleagues have demonstrated an association between the C-G-C (-1562C, +279Q, +6C) haplotype and a protective effect against atherosclerosis; since other haplotypes containing the -1562C allele did not show a protective effect, it seems likely that +279Q (located in the catalytic domain in the fibronectin-like inserts which play an important role in substrate binding) or some other variant located within or near the *MMP9* gene, also contributes to the observed effect (Morgan *et al.* 2003).

Study II

The finding of an association between the *MMP3* polymorphism and calcified lesion area is somewhat surprising. However, different mechanisms leading to MMP-mediated calcification can be hypothesized. This was in agreement with reports showing that calcified atherosclerotic plaques are associated with the presence of mast cells and macrophages in carotid arteries (Jeziorska *et al.* 1998) and that MMP3 is co-localized with calcium deposits in advanced carotid lesions (Bini *et al.* 1999). The mechanisms underlying vascular calcification are obscure, but apoptosis of VSMCs is currently suspected to be a key factor in the process (Trion *et al.* 2004).

The reason for the absence of an association between the *MMP3* genotype and acute myocardial infarction (AMI) is more difficult to explain. The measurements of the coronary artery wall covered with atherosclerotic lesions were performed independently of the presence of myocardial infarction. Zhou and colleagues demonstrated an association between the 5A-G-Lys haplotype (-1612 5A, -376G and 45C) and susceptibility to MI in a Chinese population (Zhou *et al.* 2004). Beyzade and colleagues also demonstrated an association between the 5A/5A and 5A/6A genotypes and an increased risk of MI; the common haplotype and two rare haplotypes, all containing the 5A allele, were associated with MI susceptibility (Beyzade *et al.* 2003). Terashima and colleagues found in a Japanese population that the prevalence of the 5A/6A+5A/5A genotype was significantly more frequent in patients with AMI than in control subjects (Terashima *et al.* 1999). In our study there were no allele-specific effects of the *MMP3* polymorphism on acute MI, occurrence of healed MI (presence of fibrous scar tissue) or coronary thrombi.

This discrepancy may be attributable to the fact that complicated lesions may develop following erosion or small rupture in the cap of the plaques clinically silently and only a minor part of AMIs are due to rupture of an atheroma with a resulting thrombus. Most of our AMIs were due to severe coronary atherosclerosis, in which case the pathogenesis of MI is probably involved with white platelet microthrombosis. There is ample evidence that nonfatal lesions can contain areas of rupture (Virmani *et al.* 2000). Advanced plaques may undergo many ruptures nonfatal and moreover, the existence of nonruptured but fatal lesions suggests that the equation of rupture with death may be overly simplistic (Virmani *et al.* 2000).

The most severe lesion is presented because it is the lesion which clinically best determines the functional capacity of that particular artery. Since we studied only men, we were not able to anticipate any role of *MMP3* variation in elderly women. The present data do not allow any conclusion to

be drawn as to whether MMP3 actually participates in calcification of the arteries or how this function is accomplished. Furthermore, it is not known whether calcification of the plaque is beneficial or detrimental in respect of acute coronary syndromes.

Study III

To analyze whether the combined *MMP3* and *MMP9* polymorphisms are associated with coronary artery disease, 300 Caucasian males were genotyped and genotype groups were compared with respect to atherosclerotic lesion areas and MI. No previous studies on joint effects of *MMP3* and *MMP9* loci in respect of coronary artery lesion area and plaque rupture had been reported.

A significant interaction between the *MMP3* and *MMP9* genotypes was observed in respect of area of complicated lesions. The effect of *MMP9* seemed to weigh more than that of *MMP3*. In a similar analysis for calcified lesions, no significant interactions between the *MMP3* and *MMP9* genotypes were observed. In respect of the independency of these two studied phenotypes (% of complicated and % of calcified plaque), or the association between them, a positive correlation between the complicated and calcified lesions was observed (Spearman´s correlation coefficient r=0.51). However, in several cases where complicated plaques can be found, little or no calcification can be identified. Both complicated plaques and calcified lesions develop in association with advanced atherosclerosis and they are thus not fully independent phenotypes. We also excluded cases with complicated plaques from the analysis and no associations between the combined genotype and stenosis, fatty streak or raised (fibrotic) lesions were found.

Although this report does not include any information on whether the sequence variations in the promoter cis-regulatory elements are involved with apoptosis in atherosclerotic lesions, our data support the notion that they could be (see Fig. 14, page 75). With the current understanding of the diverse roles of matrix metalloproteinases, their direct or indirect relevance for the early steps during programmed cell death, it has become clear that the strictly co-regulated *MMP* gene expression, via cis-regulatory elements in the promoter regulatory sequences, can affect cell survival and proliferation. The occurrence of acute myocardial infarction and the presence of coronary thrombi were not associated with single *MMP* genes alone, nor with the combined *MMP3* and *MMP9* genotype. The finding that the polymorphisms, both of which are associated with transcriptional activity in

vitro, were not associated with MI suggests an effect on clinically silent complicated plaque ruptures/erosions. The absence of an association between the co-expressed genes and coronary thrombi also suggests that other influencal factors may be required in the development of the thrombotic complications underlying acute syndromes, plaque rupture commonly taking place at the shoulders of the lesions where macrophages accumulate and where genetically programmed cell death may occur.

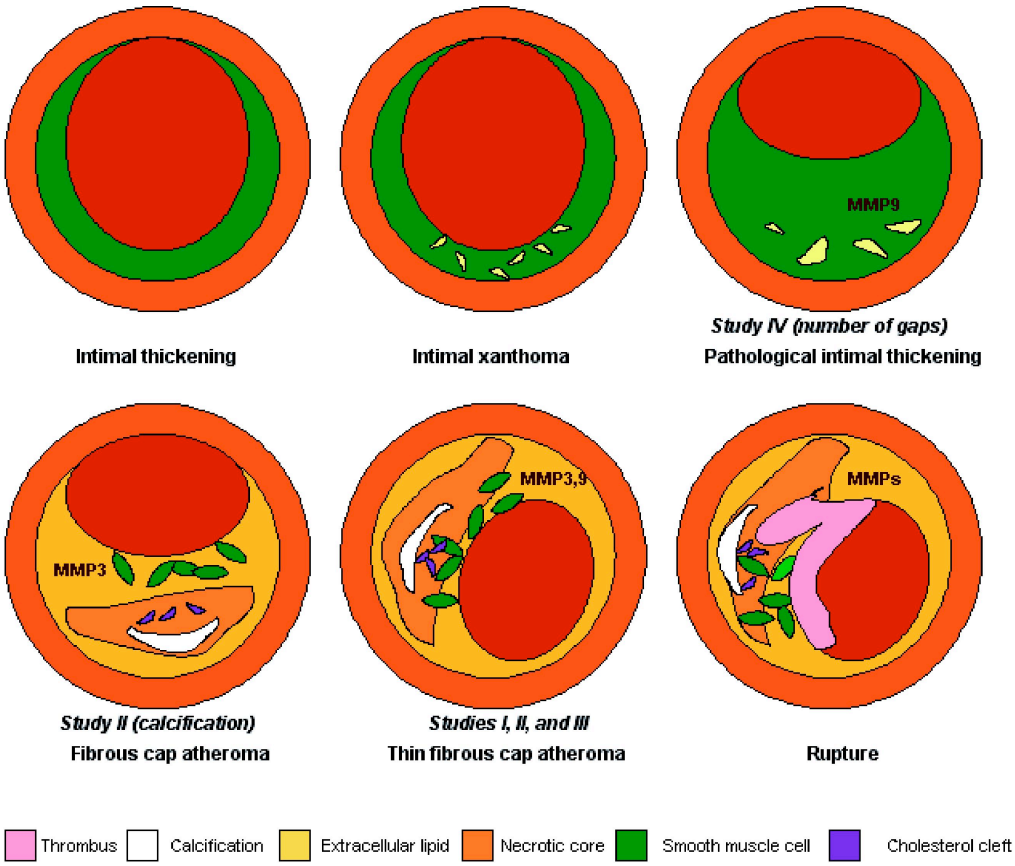

Figure 14. *The potential role of MMP3 and 9 cis-regulatory variation in various forms of atherosclerosis. MMP expression is stimulated during the formation of intimal xanthomas and is further induced by macrophage-derived cytokines and growth factors. Intimal thickening (mainly smooth muscle cells) and intimal xanthoma (fatty streak, macrophage-derived foam cells) are present in all populations, although intimal xanthomas are more prevalent with exposure to a Western diet. Pathological intimal thickening ("intermediate lesion") has no true necrosis and there is no evidence of cellular death (Study IV, number of gaps). Fibrous cap athroma has a necrotic core and consists of smooth muscle cells in a proteoglycan-collagen matrix, with a variable number of macrophages (Study II, calcified lesion). Thin fibrous cap atheromas, often referred to as "vulnerable plaques", are lesions with large necrotic cores containing numerous cholesterol clefts; the overlying fibrous cap is thin and heavily infiltrated by macrophages. This lesion does not necessarily develop rupture (Studies I, II and III). Ruptured plaques are thin fibrous cap atheromas with luminal thrombi. Modified from Virmani et al. 2000. This figure was prepared with ConceptDraw Med (Computer Systems Odessa Corporation).*

3.2 *MMP9* GENOTYPE AND CONTINUITY OF THE INTERNAL ELASTIC LAMINA

Study IV

In study IV, carriers of the promoter high (C/T, T/T) activity genotypes had a higher number of gaps per millimetre in the left renal artery and in the inferior mesenteric artery, as compared to carriers of low-activity genotypes. In men, the *MMP9* genotype was significantly associated with the number of caps in the IEL of the left and the right renal artery. The finding that no significant associations prevailed between the *MMP9* genotype and the number of gaps in the IEL of SMA or CA suggests that flow parameters such as velocity and pressure and hemodynamic conditions of the vessels, which initiate diverse signalling pathways and lead to proliferation or to cell death, may regulate the extracellular matrix homeostasis in the vascular wall.

The intimal thickness in this study was associated with the number of IEL defects. MMP9 cleaves the IEL and the SMC basement membranes and is known to be expressed by human macrophages prior to migration of SMCs into the intima, which suggests that the regulation of *MMP9* gene expression may be critical for SMC migration and intimal thickening, and MMP-induced degradation of the IEL may be required for proliferation and migration of SMCs (Pauly *et al.* 1994, Dollery *et al.* 1999, Lepidi *et al.* 2001, Sho *et al.* 2002, Johnson *et al.* 2004, Sho *et al.* 2004) (see Fig. 14, page 75). In a previous study by Jones and colleagues, genotypes containing T allele of this polymorphism were significantly more common in patients with abdominal aortic aneyrysm compared with control subjects; the greatest difference between groups was observed in male patients (Jones and Phillips *et al.* 2003).

Another important fact to consider is that we cannot prove that the increased transcriptional activity associated with the T allele actually results in increased proteolytic activity or defects in the internal elastic lamina in vivo; no MMP9 protein levels of the autopsy material could be measured. Analysis should include mRNA levels and protein levels as well as enzymatic activity to resolve the mechanism.

4. FUTURE PROSPECTS

There is a potential redundancy in the regulation of matrix metalloproteinase function; gene knockout models may not be transferable to humans, i.e. if matrix metalloproteinases are genetically knocked out in mice, other MMPs can take their place, and equally, clinical treatments designed to inhibit matrix metalloproteinases may fail while other proteases step in to fill the role of one which was targeted. The complex cascade-like networks for pathways involving proteases, regulating "all-or-nothing" biological systems such as blood clotting and programmed cell death, may have many entrances and may utilize systems of parallel pathways.

Similarly, complex traits may not be mediated via chains of events but through networks of relationships. The distance between an *MMP* genotype and a phenotype is a function of the complexity of the network of components linking them (Jeong *et al.* 2000, Alm and Arkin 2003). The less complex a network is, the more it resembles a tree or a chain (pathway), and it is possible to understand the weak functional consequences of genetic variability on distal features, i.e. phenotypes.

The principal control point in *MMP* gene expression is translational initiation (Rodriguez-Trelles *et al.* 2003, Wray *et al.* 2003). The initiation of transcription is regulated by an interaction, in a sequence-specific manner, between transcription-factor proteins and short stretches of DNA surrounding the target gene, called cis-regulatory DNA (Rodriguez-Trelles *et al.* 2003). A change in either the spatial distribution or concentration of trans-acting transcription factors, or the sequence of the cis-regulatory DNA, can alter *MMP* gene expression. Because a given transcription factor usually interacts with many promoters, a change in its specificity can cause changes at many loci; it seems therefore more likely that modifications of *MMP* gene expression evolve by changes in cis-regulatory promoter sequences (Rodriguez-Trelles *et al.* 2003).

To be able to resolve the issue of matrix metalloproteinase function in vascular disease, simultaneous examination of proteases at genetic level would be needed. Cis-regulatory elements in the promoters of *MMP3* and *MMP9* can be thought of in very different ways (Bolouri and Davidson 2002). They can be regarded as pieces of DNA sequence which contain clustered arrays of brief but specific target site sequences recognized by DNA-binding proteins. Alternatively, they can be considered as staging platforms for the assembly of multiprotein machines, of which the DNA-binding parts serve merely as anchors, and the functional meaning lies in the biochemical transactions which cause transcription or its repression (Bolouri and Davidson 2002). Cis-regulatory elements can also be thought of as genetically hardwired information processors; each such element receives

informational inputs which determine its activity, and it produces an information output in the form of the regulatory instructions it conveys to the basal transcription apparatus (Bolouri and Davidson 2002). The inputs are carried into the intergenic regulatory system (or "cis-regulatory network") by the transcription factors which bind at target site sequences; the output is never identical to the input (Bolouri and Davidson 2002).

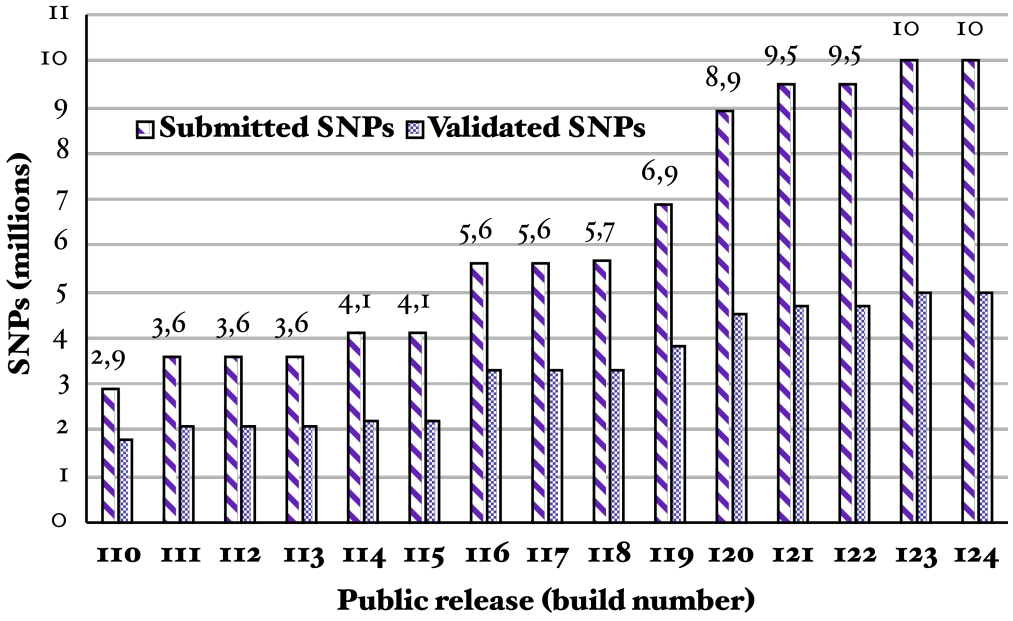

Figure 15. *Submitted and validated SNPs (Homo Sapiens) from build 110 (January 2003) to build 124 (January 2005) (NCBI dbSNP).*

Cis-regulatory information processing is important in functions like embryonic development and tissue remodelling, because these processes depend fundamentally on 3D spatial as well as temporal control of *MMP* gene expression (Bolouri and Davidson 2002). The commonplace conception that development and programmed cell death are "programmed" at the genomic level is now interpretable at the structure-function level of As, Cs, Gs and Ts (Howard and Davidson 2004). The heart of such an intergenic network would consist of the cis-regulatory elements controlling the expression of genes (Davidson *et al.* 2002). Each of these cis-regulatory elements receives multiple inputs from other genes in the network; these inputs are the transcription factors for which the element contains the specific target site sequences and which themselves may be due to cis-regulatory variation (Davidson *et al.* 2002). For example, cis-regulatory

elements active in spatial specification often use "and" logic, in that two different transcription factors must be bound to the cis-regulatory DNA at once in order for transcription to be activated (Bolouri and Davidson 2002, Davidson *et al.* 2002).

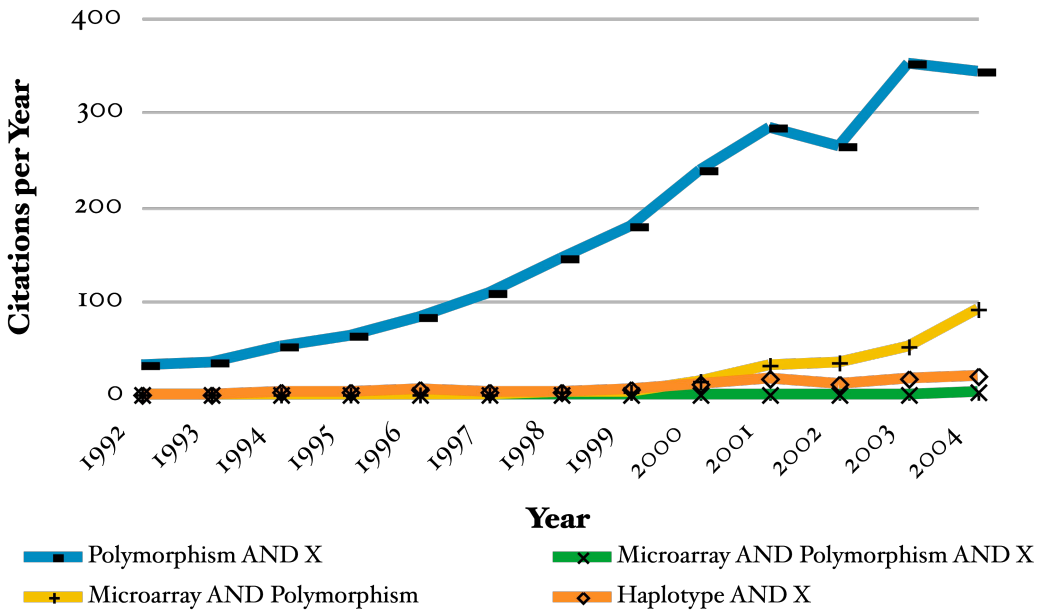

Figure 16. *The results of a PubMed search using the terms "microarray", "haplotype" and "polymorphism", where X is "(atherosclerosis OR vascular biology OR thrombosis). The number of articles published each year for each combination of search terms is shown.*

The question what is the relationship between remodelling and vascular diseases, or whether the matrix metalloproteinases in the atherosclerotic tissues are "good" or "bad", is more complex than initially thought. The cis-regulatory architecture lies at the heart of fundamental biological questions regarding matrix metalloproteinases and their co-ordinated action in atherosclerotic tissues. During this present undertaking, the human genome was completed and the number of available SNPs increased dramatically (see Fig. 15, page 78). The development of high-throughput genotyping technologies will accelerate research on polygenic atherosclerosis susceptibility (see Fig. 16). The search for polymorphic candidates in promoter cis-regulatory sequences faces obstacles due to the high number of potentially promising SNPs and the difficulties involved in the identification of gene-disease interactions.

CONCLUSIONS

The aim of this study was to examine the potential role of *matrix metalloproteinase* promoter cis-regulatory variation in the development of atherosclerosis.

1. In the coronary arteries the *MMP9* promoter genotype associated with complicated atherosclerotic lesions and the carriers of the high promoter activity *MMP9* genotypes evinced an increase in the area of complicated lesions. The high promoter activity *MMP9* genotype was associated with old infarct scarring.

2. In the coronary arteries the *MMP3* promoter genotype was associated with calcified atherosclerotic lesions in elderly men, and in this subgroup the carriers of the high promoter activity *MMP3* genotypes evinced an increase in the area of calcified lesion involvement. The *MMP3* genotype was not associated with coronary thrombi or myocardial infarction.

3. In the coronary arteries there was a significant interaction between the combined *MMP3* and *MMP9* promoter genotype status on the area of complicated lesions. Men with high-activity genotypes for both loci evinced an increase in the area of complicated lesions as compared with subjects who carried the high-activity genotype for *MMP3* or the high-activity genotype for *MMP9*. The combined *MMP3-MMP9* genotype status was not associated with coronary thrombi or myocardial infarction.

4. In the mesenteric arteries carriers of high promoter activity *MMP9* genotypes evinced an increase in the number of gaps per millimetre in the right renal artery and in the left renal artery. The number of gaps in the IEL of the celiac trunk, SMA and IMA did not vary according to the *MMP9* polymorphism.

The results from the present studies indicate that *matrix metalloproteinases* are implicated in the mechanisms leading to vascular disease. Taken together, the findings suggest that genetic variation in the promoters of individual proteases may exert important effects on extracellular matrix-mediated lesion development.

EPILOGUE

Eric Achrelius (1604-1670) was designated as the first Professor of Medicine in Turku in 1641. He was also chosen rector at the newly founded university. In his time, Eric Achrelius got through his entire academic career without a Doctor's Degree and despite the fact that he never published anything in his field. Since the days of Achrelius, the volume of scientific research has increased and massive amounts of data (clinical, genomic, proteomic, etc) are available in heterogeneous sources and formats. Genomic variations, as reflected in this thesis, are expected to form the backbone of the genetics of common diseases.

In our time, no single person can claim to have exclusive control over access to medical knowledge, as was the situation in the days of Achrelius. The system of scientific communication has become a monopoly of a couple of large international enterprises, and the scholarly communication system, originally built to assist career development, now carries primary responsibility for stockholders, not for academics. Thousands of peer-reviewed journals constitute a moneymaking device, where the transmission and visibility of publicly funded science is not the primary concern of scholarly journals but only secondary to the lucrative business, sky-scraping subscription prices and, subsequently, an ever-increasing number of new journals. Despite open access to many biomedical databases, access to scholarly knowledge, the keystone of scientific authority ever since the days of Achrelius, is facing a profound revolution.

In order to draw valid inferences from genetic variation data, or to answer questions such as what are the genetic risk factors for common diseases, biomedical information must be converted to understanding. Although we are 99.9 per cent identical at the genetic level, SNP variations are responsible for much of the differences between individuals, and are important in that they help to elucidate why one person is more susceptible to atherosclerotic disease than another. I was overwhelmed by the amount of biological information available and asked myself "What does all this mean?" The democratic ethos of scientists derives from the assumption that scientific results become valuable if they are made public. In the future, once we understand more of the diversity in the genetic code, access to organized biological knowledge will involve issues concerning democracy and social equality, individuals calling for participation in the processes which impact their lives. In the end, however, all the democratic justifications which accompany discussions on science and education only become valid if we decide whether scholarly knowledge is for the benefit of humanity, or whether it is for the benefit of small scientific and business elites.

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and sons of farmers. Their memory and example has encouraged me in all my endeavours.

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Forssa, Kuusto, March 2005

Perttu Pöllänen

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