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The Role of Interleukin 18 Gene Polymorphism in the Development of Atherosclerosis

ACADEMIC DISSERTATION
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LIST OF ORIGINAL PUBLICATIONS

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II Jussi A. Hernesniemi, Kaisa Anttila, Tuomo Nieminen, Mika Kähönen, Nina Mononen, Kjell Nikus, Väinö Turjanmaa, Jari Viik, Rami Lehtinen, Terho Lehtimäki. IL-18 gene polymorphism, cardiovascular mortality and coronary artery disease. Submitted to the European Journal of Clinical Investigation

III Jussi A. Hernesniemi, Pekka J. Karhunen, Riikka Rontu, Erkki Ilveskoski, Olli Kajander, Sirkka Goebeler, Leena E. Viiri, Tanja Pessi, Mikko Hurme, Terho Lehtimäki. Interleukin-18 promoter polymorphism associates with the occurrence of sudden cardiac death among Caucasian males: The Helsinki Sudden Death Study. Atherosclerosis. 2008;196:643-9

IV Jussi A. Hernesniemi, Pekka J. Karhunen, Niku Oksala, Mika Kähönen, Mari Levula, Riikka Rontu, Erkki Ilveskoski, Olli Kajander, Sirkka Goebeler, Leena E. Viiri, Mikko Hurme, Terho Lehtimäki. Interleukin 18 gene promoter polymorphism: a link between hypertension and pre-hospital sudden cardiac death – the Helsinki Sudden Death Study. European Heart Journal. 2009;30:2939-46

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AP	Angina pectoris
APC	Antigen presenting cell
ApoB	Apolipoprotein B
CAC	Carotid artery compliance (elacticity)
CAD	Coronary artery disease
Casp-1	Caspase 1
CHD	Coronary heart disease
CRP	C-reactive protein
CVD	Cardiovascular disease
DC	Dendritic cell
EC	Endothelial cell
ECM	Extracellular matrix
FMD	Flow-mediated dilatation
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome-wide association analysis
GWEA	Genome-wide expression analysis
HDL	High-density lipoprotein cholesterol
HSDS	Helsinki Sudden Death Study
ICAM-1	Intracellular adhesion molecule 1
IFN	Interferon
IL	Interleukin
IL-18R	Interleukin 18 receptor
IL-18BP	Interleukin 18 binding protein
IMT	Intima-media thickness
LDL	Low-density lipoprotein
Lp(a)	Lipoprotein a
LPS	Lipopolysaccharide
MCH	Major histocompatibility
MCP-1	Monocyte chemotactic protein 1
M-CSF	Macrophage colony stimulating factor
MI	Myocardial infarction

MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
NK cell	Natural killer cell
NKT	Natural killer T cells
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PPAR	Peroxisome-Proliferator-Activated Receptor
PR-3	Protease 3
PRR	Pattern-recognition receptor
ROS	Reactive oxygen species
SCD	Sudden cardiac death
SMC	Smooth muscle cell
SNP	Single nucleotide polymorphism
TGF- β	Tumour growth factor β
Th	T helper
TIMP	Tissue inhibitor of matrix metalloproteinases
TNF-α	Tumour necrosis factor α
TLR	Toll-like Receptor
TVS	Tampere Vascular Study
VCAM-1	Vascular cell adhesion molecule 1
QTL	Quantitative trait loci

ABSTRACT

Background: Atherosclerosis is an inflammatory disease. The process starts in childhood with the accumulation of lipid-laden macrophages (foam cells) onto the intima of the arteries. Atherosclerosis can be asymptomatic for decades, and the subsequent clinical manifestations of the disease vary from lower limb ischemia, myocardial infarction and stroke to sudden cardiac death (SCD). The subclinical development of atherosclerosis can be measured by carotid artery intima media thickness (IMT), carotid arterial elasticity (CAC) and brachial artery flow-mediated dilatation (FMD) which depicts the endothelial function. Within atherosclerotic plaques, antigen-presenting cells (i.e. macrophages) and T lymphocytes are the main players modifying the progression of the disease. Macrophages express interleukin 18 (IL-18) in response to inflammatory stimuli, and IL-18 further activates other cells. The main targets of IL-18 are the CD4+ T lymphocytes. IL-18 in combination with IL-12 stimulates the expression of the highly pro-atherosclerotic interferon-γ (IFN-γ). Both IL-18 and IFN-γ have been found to be expressed in human atherosclerotic lesions and linked with the development of atherosclerosis and unstable vulnerable atherosclerotic plaques in murine models and in humans.

Aims: The present study aimed to investigate whether the variation of the IL-18 gene (SNPs rs1946519, rs360717, rs549908, rs4937100, rs5744292 and rs187238) affects ***1)*** the development of subclinical atherosclerosis among a healthy Finnish population (Study I); ***2)*** the expression of angiography-verified coronary artery disease (CAD) and the mortality for cardiovascular causes among a Finnish patient population (Study II); ***3)*** the occurrence of SCD and the artery wall areas of autopsy-verified coronary atherosclerotic lesions (Studies III and IV). Finally, the purpose was to examine ***4)*** whether the traditional risk factors interact with IL-18 gene polymorphism and modify the risk of SCD (Study IV).

Subjects and Methods: The study was based on three clinical and one autopsy study series comprising a total of over 5,000 subjects. In order to explore the frequencies of the most common haplotypes and 5 tag SNPs in the Finnish population, and to investigate the possible association between IL-18 gene polymorphism and the development of subclinical atherosclerosis, we used a study population of the Cardiovascular Risk in Young Finns Study comprising 2,282 young Finnish Caucasian adults selected randomly from the national population registry (Study I). In order to

investigate whether the same 5 IL-18 tag SNPs would affect the expression of CAD, we used a subpopulation of patients (n=461) who had undergone coronary angiography in the Finnish Cardiovascular Study. Follow-up data on the whole study population (n=2152) was used to investigate if these IL-18 gene polymorphisms associate with an altered risk of death due to cardiovascular causes (Study II). Furthermore, the material of an autopsy study of 663 men (The Helsinki Sudden Death Study, HSDS) was used to assess whether a known promoter region -137 G/C SNP (rs187238) affects the occurrence of SCD and CAD, and whether this effect is modulated by known risk factors (Studies III and IV). The Tampere Vascular Study, comprising 21 atherosclerotic and 5 control samples obtained from live patients, was further utilized to verify the functional interaction in reference to the expression of IL-18 and IFN-γ (Study IV).

Results: In the Cardiovascular Risk in Young Finns Study, one major haplotype (frequency, 0.24) associated significantly with lower IMT among young healthy Caucasian men. Among women there was no similar association. The sex-by-genotype interaction in relation to IMT was significant. Other polymorphisms or haplotypes were not seen to have any effect on IMT, CAC or FMD values (Study I). Among the patient population of the Finnish Cardiovascular Risk Study, the variation of the IL-18 gene was not observed to associate significantly with cardiovascular mortality. However, among patients who had undergone coronary angiography, one major haplotype (frequency, 0.27) had a different sex-dependent impact on the expression of CAD. Among men, the carriers of this haplotype had a lower occurrence of left main branch CAD, whereas among women the haplotype was not seen to associate with the expression of CAD (Study II). This major haplotype is also the only one carrying the minor T allele of the +127 C/T tag SNP. It is also the only haplotype carrying the minor C allele of the functional promoter region -137 G/C SNP (rs187238).

In the HSDS, the minor C allele of the -137 G/C SNP (rs187238) associated with a lower occurrence of SCD (Study III). Furthermore, hypertension interacts with the -137 G/C SNP, affecting the risk of SCD due to CHD and the development of coronary atherosclerosis. Hypertension was a major risk factor for both the risk of SCD and the development of coronary atherosclerosis among GG homozygotes. Among C allele carriers, we did not find any significant effect. Hypertension also affected the mRNA expression of IL-18 and IFN-γ in human atherosclerotic plaques obtained from live patients. This effect was also seen to be modulated by IL-18 genotype (Study IV).

Conclusions: IL-18 gene polymorphism was observed to affect the development of subclinical atherosclerosis as well as the occurrence of angiography-verified CAD among men but not among women. Furthermore, a functional IL-18 gene polymorphism (rs187238) was found to associate with the risk of SCD among men. Hypertension interacted with this promoter region polymorphism and modulated the artery wall areas of coronary atherosclerotic plaques and the risk of SCD due to CHD.

TIIVISTELMÄ

Tausta. Ateroskleroosi eli valtimonkovettumatauti on tulehduksellinen verisuonisairaus, joka alkaa nuoruusiässä rasvatäytteisten syöjäsolujen (vaahtosolujen) asteittaisella kertymisellä valtimoiden sisimpään kerrokseen (intimaan). Ateroskleroosilla on useita kliinisiä ilmenemismuotoja, joista tärkein, sepelvaltimotauti, voi johtaa sydänäkkikuolemaan. Taudin varhaisten, ei-kliinisten verisuonimuutosten kehittymistä voidaan seurata mittaamalla ultraäänitutkimuksen avulla kaulavaltimoiden sisäkerroksen (intima-media, IMT) paksuutta tai seinämän elastisuutta ja olkavaltimon laajenemisvastetta lisääntyneelle verenvirtaukselle. Olkavaltimon laajenemisvaste kuvastaa endoteelin toimintakykyä. Tärkeimpiä soluja, jotka vaikuttavat ateroskleroosin kehittymiseen suonen seinämässä, ovat sileiden lihassolujen ja endoteelisolujen ohella antigeenejä esittelevät solut (makrofagit) ja T-lymfosyytit. Makrofagit tuottavat interleukiini (IL) 18:aa, joka aktivoi muita soluja ja CD4+ T-lymfosyyttejä. IL-18 yhdessä IL-12:n kanssa stimuloi T-lymfosyyttejä tuottamaan interferoni gammaa (IFN-γ). Ihmisen ateroskleroosimuutoksista on löydetty sekä IL-18:aa että IFN-γ:aa, ja niiden on havaittu lisäävän repeämisalttiiden, herkästi komplisoituvien ateroskleroosimuutosten kehittymistä.

Tavoitteet. Tutkimuksen tarkoituksena oli tutkia, vaikuttaako IL-18-geenin polymorfismi eli monimuotoisuus (pistemutaatiot, single nucleotide -polymorfismit (SNP) rs1946519, rs360717, rs549908, rs4937100, rs5744292 ja rs187238): ***1)*** edellä kuvattujen varhaisten ei-kliinisten valtimomuutosten kehittymiseen suomalaisilla nuorilla aikuisilla (tutkimus I, LASERI-tutkimus), ***2)*** sepelvaltimoiden varjoainekuvauksella diagnosoidun sepelvaltimotaudin ilmenemiseen ja kardiovaskulaarikuolleisuuteen suomalaisilla sairaalapotilailla (tutkimus II, FINCAVAS-tutkimus) sekä ***3)*** ruumiinavauksissa mitattuihin erityyppisten ateroskleroosimuutosten pinta-aloihin ja sydänäkkikuoleman riskiin (III ja IV, HSD-tutkimus). Lisäksi selvitettiin, ***4)*** onko IL-18-geenin ja riskitekijöiden interaktioilla (yhteisvaikutus) vaikutusta sydänäkkikuoleman riskiin ja geenien ilmenemiseen (IV).

Aineisto ja menetelmät. Tutkimus perustui kolmeen kliiniseen (LASERI, TVS, FINCAVAS) ja yhteen ruumiinavausaineistoon (HSDS), joista tutkittiin yhteensä yli 5000 henkilöä. IL-18-geenin yleisempien haplotyyppien ja 5 pistemutaation yleisyys suomalaisessa populaatiossa määritettiin Lasten sepelvaltimotaudin riskitekijät -tutkimuksen (LASERI) kansallisesta rekisteristä sattumanvaraisesti valitun

tutkimuspopulaation perusteella. LASERI-tutkimukseen kuuluu 2282 suomalaista tervettä aikuista (tutkimus I). LASERI-tutkimusaineistosta tutkittiin myös, vaikuttaako IL-18-geenin variaatio valtimonkovettumataudin varhaisten, ei-kliinisten muutosten kehitykseen. FINCAVAS (The Finnish Cardiovascular study) -tutkimuksessa on seurattu yliopistollisen sairaalan 2152 potilaan tilaa kliinisen rasituskokeen jälkeen. Osalle potilaista (n=461) on myös tehty sepelvaltimoiden varjoainekuvaus. FINCAVAS-aineiston avulla tutkittiin, vaikuttaako IL-18-geenin variaatio sepelvaltimotaudin ilmenemiseen ja kardiovaskulaarikuolleisuuteen (II). IL-18-geenin promoottorialueen funktionaalisen -137 G/C (rs187238) -pistemutaation vaikutus sydänäkkikuoleman esiintyvyyteen selvitettiin käyttäen oikeuslääketieteellistä ruumiinavausaineistoa (The Helsinki Sudden Death Study, HSDS) (III ja IV). HSDS koostuu 663 miehen ruumiinavauksesta saaduista tiedoista. Saman ruumiinavaussarjan avulla tukittiin, muokkaako ko. pistemutaatio (rs187238) perinteisten riskitekijöiden vaikutusta sydänäkkikuoleman esiintyvyyteen (IV) eli onko tällä IL-18 -geenivaihtelulla interaktiota riskitekijöiden kanssa. Lisäksi tässä osatyössä tutkittiin IL-18 ja IFN-γ mRNA -ekspressiota pitkälle edenneissä ateroskleroosimuutoksissa (n=21) ja terveissä valtimonäytteissä (n=5). Näytteet ilmenemistutkimuksiin saatiin menossa olevasta TVS-tutkimuksesta (Tampere Vascular Study) .

Tulokset. LASERI-tutkimuksessa havaittiin, että yhden yleisen haplotyypin (frekvenssi 0.24) mieskantajilla oli merkitsevästi pienempi karotisvaltimon sisäkalvon (IMT) paksuus verrattuna muihin miehiin. Naisilla ei vastaavaa yhteyttä havaittu. Tämä ero haplotyypin vaikutuksessa karotisvaltimon sisäkalvon paksuuteen naisten ja miesten välillä oli merkitsevä. Muuten IL-18-geenin variaatio ei merkitsevästi vaikuttanut valtimokovettumataudin subkliinisiin ilmenemismuotoihin (IMT, elastisuus, endoteelifunktio) (tutkimus I). FINCAVAS-tutkimuksessa ei havaittu yksiselitteistä yhteyttä tutkitun IL-18-geenin variaation ja kardiovaskulaarikuolleisuuden välillä. Kuitenkin yhden yleisen IL-18-geenin haplotyypin mieskantajilla (frekvenssi 0.27) oli merkitsevästi pienempi todennäköisyys sairastua sepelvaltimoiden päärungon (left main) tautiin. Naisilla ko. haplotyyppi ei vaikuttanut sepelvaltimotaudin ilmenemiseen. Interaktio sukupuolen ja IL-18-haplotyypin kantajuuden välillä on merkitsevä (tutkimus II). Kyseinen haplotyyppi on ainoa IL-18-geenin haplotyyppi, joka sisältää +127 C/T -pistemutaation (rs360717) harvinaisemman T-alleelin. Samoin myös funktionaalisen IL-18 -137 G/C (187238) -promoottorialueen pistemutaation harvinaisempi C-alleeli esiintyy

ainoastaan tässä haplotyypissä. HSDS aineistossa C-alleelin kantajilla sydänäkkikuoleman esiintyvyys oli merkitsevästi alhaisempi verrattuna GG-homotsygootteihin. Lisäksi tämän funktionaalisen pistemutaation havaittiin muokkaavan korkeaan verenpaineeseen liittyvää kohonnutta sydänäkkikuoleman riskiä ja sepelvaltimoiden ateroskleroosimuutosten kehittymistä. GG-homotsygooteilla korkea verenpaine lisäsi voimakkaasti sydänäkkikuoleman esiintyvyyttä ja edisti sepelvaltimoiden ateroskleroosimuutosten kehittymistä. C-alleelin kantajien keskuudessa vastaavaa vaikutusta ei havaittu. Lisäksi korkean verenpaineen havaittiin vaikuttavan IL-18:n ja IFN-γ:n mRNA-ekspressioon (ilmentymiseen) ateroskleroottisissa plakeissa, ja IL-18-genotyypin havaittiin muokkaavan tätä vaikutusta (IV).

Johtopäätökset. IL-18-geenin variaatio vaikuttaa subkliinisen valtimonkovettumataudin kehittymiseen ja sepelvaltimotaudin ilmenemiseen miehillä. Lisäksi havaittiin merkitsevä yhteys funktionaalisen IL-18-polymorfian (rs187238) ja sydänäkkikuoleman riskin välillä. Tämä pistemutaatio (rs187238) muokkaa myös korkean verenpaineen vaikutusta sepelvaltimoiden ateroskleroosivaurioiden kehittymiseen ja sydänäkkikuoleman esiintyvyyteen.

INTRODUCTION

Atherosclerosis is a multifactorial disease that develops from childhood and ultimately can lead to death. The clinical manifestations of atherosclerosis include myocardial infarction (MI), stroke and sudden cardiac death (SCD). The prevalence of the disease is high, and approximately 50% of all deaths globally can be attributed to atherosclerosis (Lusis 2000).

The development of atherosclerosis begins with the accumulation of lipid particles into the intima, which is the innermost layer of the arteries. The intima is separated from the vessel lumen only by the endothelium. This process starts in early childhood and adolescence (Enos et al. 1953, Insull 2009, McGill and McMahan 1998, Newman et al. 1986). In the next phase, the accumulated lipids – or, more precisely, low-density lipoprotein (LDL) cholesterol – are oxidized and engulfed by the accumulating macrophages, forming lipid-filled foam cells. This denotes the formation of an early fibroatheroma. Fibroatheromas begin to form in persons in their teens and early twenties (Enos et al. 1953, Insull 2009, McGill and McMahan 1998, Newman et al. 1986). From this point on, the progression of the disease is marked by accumulating lipids and increasing inflammatory activity in the plaques. The inflammatory activity is caused by the interplay of accumulating macrophages, T lymphocytes, dendritic cells, and also by other cells capable of modulating the inflammatory reaction (Ross 1999). This process develops further, and generally by the 6th decade of life, the arteries already contain fibroatheromas capable of causing clinical manifestations of the disease (Insull 2009). The whole disease process is driven by two main entities – the accumulation of lipids due to excessive concentrations of circulating LDL cholesterol and the inflammatory reaction within the atherosclerotic plaques (Ross 1999).

Risk factors for atherosclerosis can be divided into those with a strong genetic component and environmental risk factors. Environmental risk factors have been estimated to account for approximately 50% of the disease burden, with estimates on the effect of the genetic component ranging from 20% to 60% (Kraus 2000, Smith et al. 2000a). In reality, hardly any risk factor is independent of genetic modulation. For example, the effect of smoking is modulated by genetic factors (Stephens and Humphries 2003). Traditional risk factors include hypertension, smoking, high LDL cholesterol levels, diabetes, obesity, physical inactivity, male sex and insulin resistance.

Genetic research has many approaches. Genome-wide linkage studies and, more recently, genome-wide association analyses have provided novel ways of exploring

candidate genes. However, candidate gene studies are still needed to validate preliminary results and to elaborate the possible mechanism by which specific genetic factors could exert their influence (Kronenberg 2008).

The present study is a candidate gene study. It was commenced to explore the atherogenic properties of the Interleukin (IL) 18 gene. IL-18 is a pleiotrophic and pro-inflammatory cytokine. It is produced mainly by macrophages. The main function of IL-18 is to stimulate of the expression of interferon gamma (IFN-γ) by T lymphocytes (Arend et al. 2008). The IL-18 gene was selected, because IL-18 has previously been linked with the development of atherosclerosis in murine models and it has also been associated with the development of unstable plaques (Elhage et al. 2003, Mallat et al. 2001a, Mallat et al. 2001b). Circulating IL-18 levels have also been found to predict the occurrence of acute coronary syndromes (Blankenberg et al. 2002, Espinola-Klein et al. 2007). Since the present study was commenced, a few studies have emerged on the relation between the variation of the IL-18 gene and the risk of MI as well as death due to cardiovascular causes (Bis et al. 2008, Kretowski and Kinalska 2003, Tiret et al. 2005).

The present study aimed to clarify whether the variation of the IL-18 gene would affect the expression of atherosclerosis in different phases of the disease and among different populations. This was accomplished by exploring the possible associations between IL-18 gene polymorphism and 1) the development of subclinical atherosclerosis among a healthy Caucasian population, 2) the expression of angiography-verified coronary artery disease and mortality among Finnish patient population, 3) the occurrence of sudden cardiac death among Finnish men, and, finally, 4) the risk factors as well as the interactions of genotype and risk factors affecting the risk of SCD.

REVIEW OF THE LITERATURE

1.1 The epidemiology of atherosclerotic diseases

Atherosclerosis is a disease that affects large arteries, and it is the underlying cause of approximately 50% of all deaths in the Western world (Lusis 2000). Atherosclerosis has many clinical manifestations ranging from mild ischemia of the lower limb to SCD and stroke. Ischemic heart disease is a major complication of atherosclerosis. According to the statistics of the World Health Organization, it was the most frequent individual disease as a cause of death globally, accounting for 12.7% of all deaths in the year 2002 (WHO 2002). The second most frequent cause of death was cerebrovascular disease (9.6%). In developed countries, where fatal communicable diseases are less frequent, the problem is more evident when compared to the global situation. In the year 2008, ischemic heart disease alone claimed 11,761 lives in Finland, which accounts for 24.0% of all deaths, thus making it a major problem (StatisticsFinland 2008).

1.2 Major atherosclerosis-related cardiovascular diseases

Cardiovascular disease (CVD) is a large entity that contains many diseases. Not all of them are directly due to atherosclerosis, but many of the major diseases are intimately linked with the condition.

As stated before, ischemic heart diseases are the leading entity in global mortality statistics. The condition is basically chronic, but it can have severe acute complications. Chronic ischemic heart diseases include conditions such as coronary artery disease [CAD] (usually diagnosed by an angiography-verified narrowing of at least one of the coronary arteries) and ischemic cardiomyopathy. Acute diseases include, for example, stress-related stable angina pectoris (AP), unstable AP, acute MI and the subsequent complications of MI and other types of acute ischemic heart disease that can lead to heart failure (Boersma et al. 2003, Fuster et al. 1992, WHO 2007).

Cerebrovascular disease is also a chronic condition that can have severe clinical implications. The acute complications related to atherosclerosis include cerebral infarction due to occlusion or thrombosis of the precerebral or cerebral arteries (e.g.

stroke). Transient ischemic attack can also be a manifestation on atherosclerotic cerebrovascular disease.

Atherosclerosis also affects all other arteries such as the aorta, sometimes leading to the formation of an aortic aneurysm. Furthermore, the complications of peripheral artery disease can vary from chronic disability to acute ischemia of the lower limb. Hypertension, diabetes and hypercholesterolemia are considered diseases that intimately mediate the development of atherosclerosis (Boersma et al. 2003, Fuster et al. 1992).

1.3 Risk factors for atherosclerosis

In a concise review published in Nature in 2000, the risk factors associated with atherosclerosis and coronary heart disease (CHD) were divided into those with a strong genetic component and environmental risk factors (Lusis 2000). However, this division is not strict and some risk factors are strongly influenced by lifestyle factors as well as genetics. Another classification approved by the American Heart Association has emerged dividing risk factors into five main categories (Smith et al. 2000b). The first category includes causative risk factors such as smoking, elevated blood pressure, elevated serum cholesterol (or LDL cholesterol or, alternatively, elevated apolipoprotein B [ApoB]), low high-density lipoprotein cholesterol (HDL) and diabetes mellitus. The second category includes conditional risk factors such as triglycerides, small LDL particles, lipoprotein (a), homocysteine, coagulation factors (plasminogen activating factor inhibitor-1 and fibrinogen) and elevated C-reactive protein levels (CRP). All of these factors, except for CRP, are considered conditional when serum levels are abnormally high. The third group is classified as predisposing risk factors such as overweight and obesity, physical inactivity, male sex, family history of premature CHD, socioeconomic factors, behavioural factors (e.g. mental depression) and insulin resistance. The fourth category contains risk factors that are related to the coronary plaque burden. This category includes age and nonspecific ST segment changes in the resting electrocardiogram. Age is therefore a definitive risk factor, but its effect is mainly conveyed through the accumulation of other risk factors (Pencina et al. 2009, Smith et al. 2000b). The last risk factor, defined as a separate susceptibility factor, is left ventricular hypertrophy of the heart.

As noted earlier, many risk factors have a strong genetic component, although

they are also regulated by lifestyle factors. A good example of this is LDL cholesterol, which is associated with lifestyle (diet) but also controlled by genetic factors (Kathiresan et al. 2008, Roy et al. 2009). Other similar risk factors include, for example, HDL cholesterol, lipoprotein levels, elevated blood pressure, elevated homocysteine levels and diabetes. The strongest risk factor that may be considered environmental is a high-fat diet. Naturally, cigarette smoking can be classified into this category as well (Lusis 2000).

It is difficult to estimate the contribution of genetic and environmental factors to the development of atherosclerosis, although a family history of premature CHD has been shown to be a very significant risk factor even after controlling for all other significant risk factors. Estimates of the overall effect of the genetic component in the development of CAD range from 20%–60%, and it is presumed to be more pronounced at a younger age (Kraus 2000). Supporting this, a large twin study conducted in Sweden revealed that the relative risk of a fatal CHD is markedly pronounced for a monozygotic twin after the other twin has died of CHD (the risk ratio for men whose twin had died under the age of 55 was 8.1 [2.7-24-5] and the risk ratio for women whose twin had died under the age 65 was correspondingly 15.0 [7.1-31.9]) (Marenberg et al. 1994). Furthermore, twin studies have revealed that the heritability (e.g. the statistical estimate of the degree to which genes contribute to a multifactorial trait) of an MI is 26% for males and 60% for females (Nordlie et al. 2005). As a reference, the major causal risk factors (smoking, elevated blood pressure, elevated serum total cholesterol [and LDL cholesterol in particular], low HDL cholesterol and diabetes) among asymptomatic high-risk patients account for approximately 50% of the variability in the risk of CHD (Smith et al. 2000a). In other populations, the explanatory value of major risk factors diminishes. For example, only 25% of the excess CHD risk for diabetic patients can be attributed to established risk factors (Pyorala et al. 1987). As both environmental and genetic risk factors fail to explain the whole disease process, it is obvious that the gene-environment interactions are also major players affecting the outcome of the disease. For example, smoking has been shown to interact with ApoE, lipoprotein lipase and IL-6 genotypes, affecting the risk of CHD (Stephens and Humphries 2003).

Currently, it is also possible to use risk factor data (age, blood pressure, smoking, HDL cholesterol and total cholesterol) in order to approximate the individual risk of CVD. This method is called SCORE (= Systematic Coronary Risk Evaluation),

and the algorithm is based on the results of a large general population-based study (Graham et al. 2007). The calculator for individual risk assessment is freely available on the internet. In Finland the National Institute for Health and Welfare has also published a calculator for the risk assessment of CVD. The algorithm is based on the data of the Finnish FINRISK study (Jousilahti et al. 2005, Vartiainen et al. 1994). In addition to including the data of the above-mentioned variables used in the SCORE calculator, the FINRISK calculator also incorporates family history of CVD to the algorithm. This calculator has also been published on the internet and it is freely available.

1.4 Manifestations of atherosclerosis

1.4.1 Subclinical atherosclerosis

Increased carotid artery intima-media thickness (IMT), carotid artery elasticity and flow-mediated dilatation (FMD) of the brachial artery are early subclinical markers of atherosclerosis and predict future coronary events (Lorenz et al. 2006, Schroeder et al. 1999, Simons et al. 1999). Carotid IMT can be measured easily and non-invasively using ultrasound, and it has been recognized to be a valid method for evaluating the development of atherosclerosis (Greenland et al. 2000). Increases in carotid IMT associate with a higher risk of future cardiovascular vascular events independently of traditional risk factors (Bots et al. 1997, O'Leary et al. 1999). Furthermore, patients with cardiovascular disease have higher carotid artery IMT when compared to healthy controls (Burke et al. 1995). In a very recent article by Franks et al. published in the New England Journal of Medicine, it was also shown for the first time that hypertension, obesity and impaired glucose tolerance predict premature death for endogenous causes (Franks et al.).

Carotid artery compliance (CAC) depicts the ability of the arteries to expand under the influence of pulse pressure. It can be evaluated by simultaneous ultrasonic measurement of carotid artery dilatation and measurement of pulse pressure from the brachial artery. Diminished arterial elasticity has been shown to be an independent predictor of cardiovascular events and mortality in high-risk individuals (Barenbrock et al. 2002, Blacher et al. 1998).

FMD of the brachial artery quantifies the amount of vasodilatation in response to endothelial activation by an increase in local blood flow (Celermajer et al. 1992). It

is evaluated by first inflating a pneumatic tourniquet, placed around the forearm, up to a pressure of 250 mmHg and over. After 5 minutes, the pressure is released and the responding dilatation of the brachial artery measured. The dilatation can be attributed mainly to the nitric oxide released by endothelial cells (ECs) (Mullen et al. 2001). Even before the anatomical evidence of atherosclerosis appears, FMD is impaired in young symptom-free subjects with vascular disease risk factors. Impaired brachial endothelial function also associates with the prevalence and extent of clinically determined and angiography-verified coronary artery disease (CAD) (Neunteufl et al. 1997).

1.4.2 Severe clinical manifestations of cardiovascular disease

1.4.2.1 Myocardial infarction

MI is a serious complication of coronary atherosclerosis. The term itself refers to death of cardiomyocytes due to prolonged ischemia (2000). This acute coronary syndrome is caused by a sudden decrease in blood flow due to an interruption in coronary artery circulation. This interruption is caused by intraluminal thrombi, inducing a total or severe occlusion of the artery, and the situation might be further complicated by vasoconstriction and microembolisation of the thrombi (Fuster et al. 1992). Sometimes an acute coronary event is provoked by a heavy stenosis without thrombi due to an advanced fibrocalcific plaque (Virmani et al. 2000a). If the occlusion of the artery lasts for more than 30 minutes, the myocardium can suffer irreversible damage (Hermens et al. 1992). If the occlusion persists longer, the damaged area increases in size, and after 6 hours the affected area of the myocardium becomes necrotic. This can lead to serious loss of function in the myocardium, and the most severe possible end point resulting from this is death (Boersma et al. 2003).

The cause of intraluminal coronary thrombi is the disruption of the vessel wall. In MIs resulting in death, the underlying cause is most often the disruption of a thin-cap fibroatheroma (50%–60% of all deaths). The other two causes with thrombotic aetiology are thrombosis due to erosion of the endothelium (20% of deaths) and thrombosis due to the protrusion of a calcified nodule into the arterial lumen (2% of deaths). As mentioned before, heavy coronary stenosis without thrombi, due to advanced fibrocalcific plaque formation, can also provoke an MI resulting in death (20%–30% of all deaths) (Insull 2009, Virmani et al. 2000a). However, not all plaque ruptures lead to acute coronary syndrome. A smaller plaque rupture can also happen

with no clinical manifestations (Falk et al. 1995). According to autopsy data, approximately 9% of healthy individuals have disrupted plaques in their coronaries, and this proportion can rise up to 22% among people with hypertension and diabetes (Davies et al. 1989). Smaller plaque ruptures with superimposed thrombus can be repaired by new formation of a fibrous cap, and this can also lead to increased narrowing of the lumen (Mann and Davies 1999). The results of an autopsy study by Burke et al. showed that among sudden cardiac death victims, 61% had healed ruptures in coronary arteries and that many of the deceased had multiple healed ruptures at the same sites. Furthermore, multiple healed ruptures were associated with increased coronary narrowing (Burke et al. 2001). According to an extensive review by Falk et al. in 1995, which summarized the results of four independent autopsy studies, the majority of culprit lesions are identified in sites with less than 50% stenosis, although increased narrowing may result in higher disability and certainly incurs a great risk for acute coronary syndrome. The authors concluded that this is due to the fact that the number of sites with less severe stenosis is overwhelmingly greater and the smaller plaques are probably more likely to lead to acute clinical events, because they are less frequently associated with protective collateral circulation (Falk et al. 1995).

According to the European Society of Cardiology and the American College of Cardiology (ESC and ACC), an MI can be diagnosed if the patient has ischemic symptoms (typical chest pain) or changes in the electrocardiogram indicative of myocardial infarction (ST elevation or depressions or pathological Q waves indicating of transmural myocardial infarction) and, at the same time, the patient presents with a typical rise in the troponin levels or creatine kinase-MB. If the patient has typical chest pain but no ST elevations or Q waves are found in the electrocardiogram, and the troponin levels or creatine kinase-MB levels in the serum do not demonstrate the typical rises, the condition is diagnosed as unstable angina pectoris (UAP)(ESC/ACC 2000)

1.4.2.2 Sudden cardiac death

Sudden cardiac death (SCD) is caused by cardiac arrest leading to the termination of effective cardiac function. According to the World Health Organization, SCD is defined as death from cardiac causes within 1 hour of symptom onset if the death is witnessed. If not witnessed, the patient has had to have be observed alive and asymptomatic within the previous 24 hours. Most often the events leading to SCD are ventricular

tachycardia accelerating into ventricular fibrillation and, ultimately, to asystole or pulseless electrical activity (Huikuri et al. 2001, Winslow et al. 2005). In rare cases, mechanical defects caused by a previous MI, such as ventricular or papillary muscle rupture, pericardial tamponade, septal defects and ischemic valvular dysfunction, may also lead to SCD in the absence of ventricular tachycardia (Bunch et al. 2007). The underlying cause of SCD is most often (~80%) coronary atherosclerosis. Hypertrophic and dilated cardiomyopathy account fo approximately 10%–15% of the cases. These conditions can be attributed to genetic predisposition combined with hypertension and infections as well as other yet unknown factors. The underlying cause for SCD can also be primary electrical ion channel abnormality and valvular or genital heart disease (<5%) (Huikuri et al. 2001). Men have a higher risk for SCD. Currently, 75% of all SCD victims are men (Zipes and Wellens 1998). A positive family history also clearly increases the risk of SCD independently of risk factors. The results of previous studies indicate that if one first-degree relative has died of SCD, the risk is increased by 50%–80% and a parent's SCD increases the risk 9-fold for the offspring (Friedlander et al. 1998, Jouven et al. 1999). In developed countries, SCD accounts for over 50% of all cardiac deaths (Zipes and Wellens 1998).

Coronary atherosclerosis is not only the biggest risk factor for SCD, but SCD is often the first manifestation of the disease. It is also the most common manifestation of the condition (Schatzkin et al. 1984). As mentioned before, SCD can be caused by acute MI resulting from the occlusion of the coronary artery by a thrombus. This is the case in more than half of the cases, but the estimate varies due to different study settings (Farb et al. 1995, Mehta et al. 1997). In the rest of the cases, no thrombosis can be found. The recent MI can also have been caused by progressive arterial narrowing, or SCD may be due to arrhythmias arising from old myocardial infarction scars in the absence of a recent MI (Farb et al. 1995, Mehta et al. 1997). This conclusion is backed up by the fact that old infarction scars of the myocardium, a known history of ischemic heart disease and three-vessel disease all inversely associate with the presence of coronary arterial thrombus at autopsy (Davies et al. 1989).

1.5 Normal arteries

The pathogenesis of atherosclerosis starts in childhood and the disease aetiology is complex (McGill et al. 2000, Ross 1999). It affects large arteries and its clinical manifestations are usually not seen until middle age. The normal artery has three layers (intima, media adventitia). In atherosclerosis, the mainly affected layer is **the intima** whose luminal side is normally covered by endothelial cells, thus forming an endothelial layer resting on a basement membrane. The internal elastic lamina, which is considered a part of the media, denotes the outside border between the intima and media. There are two main layers in the intima: the proteoglycan layer subjacent to the lumen (composed mainly of proteoglycan, sparse smooth muscle cells and isolated macrophages) and the musculoelastic layer (composed of smooth muscle cells [SMC], elastic fibres and collagen) (Stary et al. 1992). **The media** consists mainly of SMCs and extracellular matrix (elastic fibres and collagen). The SMCs of this layer are responsible for vasoconstriction and vasodilatation (Ross and Glomset 1976). The media is normally much thicker than the intima, but the ratio between these can vary anywhere between 0.1 and 1 (Stary et al. 1992). The media is separated from the adventitia by an external elastic lamina. **The adventitia** consists of fibroblasts (loose connective tissue), SMCs, mast cells, collagen fibres and proteoglycan as well as the vaso vasorum which is responsible for the blood supply to the adventitia and approximately 2/3 of the outer layer of the media. The oxygen supply to the intima and the inner third of the media is provided via diffusion from the luminal blood flow. Through the adventitia comes also lymphatic drainage and small nerves innervating the SMCs of the media, thus controlling the contractile function of the arteries. Lymphatic vessels do not reach the intima (Ross and Glomset 1976).

1.6 Classification of atherosclerotic lesions

Perhaps the most commonly used classification of atherosclerotic lesions is the one recommended and updated by the American Heart Association (Stary 2000, Stary et al. 1995, Stary et al. 1994). It classifies lesions into: initial change lesions (I), minimal change lesions (fatty streak) (II), intermediate lesion pre-atheromas (III), atheromas (IV), fibroatheromas (V), haemorrhagic/thrombotic lesions (VI), calcified lesions (VII) and fibrotic lesions (VIII). In the previous AHA classification, lesion types VII and

VIII were classified as subtypes of type V lesions (Vb and Vc correspondingly). The current AHA-recommended classification is presented in Table 1. An alternative classification of atherosclerotic plaques has been presented by Virmani et al., but although this classification is based on autopsy data and contains an extensive description of lesions prone to rupture, it has not been adopted and the Stary classification remains the standard (Virmani et al. 2000b). The classification by Virmani et al. is presented in Table 2.

Table 1. The classification of atherosclerotic lesions according to the American Heart Association.

Lesion type	**Cellular composition**
I Initial change	Isolated macrophage foam cells
II Minimal change	Multiple layers of foam cells
IIa: Progression-prone; Abundant SMCs	Few lymphocytes
IIb: Progression resistant; Few SMCs	Isolated mast cells
III Preatheroma	Isolated pools of densely packed extracellular lipids SMCs accumulate lipid droplets
IV Atheroma	Confluent core of extracellular lipids Increased number of lymphocytes SMCs decrease in numbers
V Fibroatheroma	Fibrous tissue and collagen added Intimal SMCs increase in number
VI Hemorrhagic/thrombotic lesion	Lesion becomes fissured and/or thrombotic
VII Calcified lesion (Previously type Vb)	Calcification predominates
VIII Fibrotic lesion (previously type Vc)	Fibrous tissue changes predominate Lipid core is nearly absent

Table 2. Classification of autopsy-verified atherosclerotic lesions by Virmani et al.

Lesion name	Lesion description by histopathology	Thrombosis
Non-atherosclerotic intimal lesions		
1. Intimal thickening	Normal accumulation of SMCs in the intima with the absence of lipid or macrophage foam cells	Thrombus is absent
2. Intimal xhantoma of fatty streaks	Subendothelial accumulation of foam cells in the intima with no necrotic core or fibrous cap; animal and human data show that such lesions usually regress	Thrombus is absent
Progressive lesions		
3a. Pathologic intimal thickening	SMCs in a proteoglycan-rich matrix with areas of extracellular lipid accumulation without necrosis	Thrombus is absent
3b. With erosion	Luminal thrombosis, plaque the same as above	Thrombus most often mural and infrequently occlusive
4a. Fibrous cap atheroma	Well-formed necrotic core with overlying fibrous cap	Thrombus is absent
4b. With erosion	Luminal thrombosis; plaque the same as above, no communication of thrombus with necrotic core	Thrombus most often mural and infrequently occlusive
5. TCFA	A thin fibrous cap infiltrated with macrophages and lymphocytes, rare SMCs, and an underlying necrotic core	Absent, with intraplaque haemorrhage/fibrin
a. With rupture	Fibroatheroma with cap disruption; luminal thrombus communicates with underlying necrotic core	Thrombus usually occlusive
6. Calcified nodule	Eruptive nodular calcification with underlying fibrocalcific plaque	Thrombus usually non-occlusive
7. Fibrocalcific plaque	Collagen-rich plaque usually with significant stenosis; contains large areas of calcification with few inflammatory cells; necrotic core may be present	Thrombus is absent

Abbreviations: SMC = smooth muscle cell; TCFA = thin-cap fibroatheroma (Virmani et al. 2000b)

1.7 The development of atherosclerosis

1.7.1 Initial change, minimal change lesions, pro-atheroma and atheroma

In the beginning of the process of atherosclerosis, LDL particles accumulate onto the intima. This early fatty streak development begins in childhood and adolescence (Enos et al. 1953, Insull 2009, McGill and McMahan 1998, Newman et al. 1986). The accumulation is positively correlated with circulating levels of LDL. The preferred sites for LDL accumulation are those were the blood flow is disturbed due to branching or curvature of the artery. In these sites the endothelium has an altered morphology (the cells are polygonal in shape and have no particular orientation) and thus have increased permeability for macromolecules such as LDL (Gimbrone 1999). Furthermore, the disturbed blood flow in these sites has been shown to reduce the activity of atheroprotective molecules such as nitric oxide and increase the production of vascular cell adhesion molecule 1 (VCAM-1) which enables the recruitment of monocytes and lymphocytes (Jongstra-Bilen et al. 2006, Packard and Libby 2008). The retention of LDL within the intima involves interaction between matrix proteoglycans and apoB located on the surface of lipoproteins (Boren et al. 1998a, Boren et al. 1998b). In addition, lipoprotein a (Lp(a)), which also contains apoB, can accumulate in the intima (Grainger et al. 1994). If the trapped lipoprotein particles are not processed from the intima rapidly, they undergo progressive oxidation and are subsequently internalized by phagocytes through the function of scavenger receptors (scavenger receptor A and CD36) (Hajjar and Gotto 2003, Moore and Freeman 2006). However, even before the LDL particles are oxidized sufficiently for recognition by scavenger receptors, they can have pro-inflammatory properties. This ‘minimally oxidized’ LDL can stimulate the ECs to produce adhesion molecules such as VCAM-1, chemotactic proteins such as monocyte chemotactic protein 1 (MCP-1), and growth factors such as the macrophage colony stimulating factor (M-CSF) (Lusis 2000).

Within the vessel wall, monocytes are transformed mainly into macrophages (but also to dendritic cells [DC]), and they engulf the oxidized LDL particles, forming foam cells. It is noteworthy that SMCs can also uptake oxidized LDL and thus form foam cells (Young et al. 2002). However, as noted earlier, the LDL cholesterol particles must first be oxidized. The enzymes involved in this process are reactive oxygen species (ROS) produced by macrophages and ECs in addition to other enzymes found

in atherosclerotic lesions, such as myeloperoxidase, sphingomyelinase, and secretory phosfolipase (Lamon and Hajjar 2008). The appearance of oxidized LDL within the intima further propagates the inflammatory response, as pro-inflammatory cytokines (such as IL-8 tumour necrosis factor α [TNF-α] and IL-1β) promote the attachment, rolling and infiltration of leukocytes to the site (Boudjeltia et al. 2006, Martin-Fuentes et al. 2007). During the chemotactic process, T cells are also accumulated onto the site. This creation of foam cells together with the accumulation of T cells stipulates the formation of a fatty streak.

In these early phases of atherosclerotic plaque development, medial SMCs begin to migrate to the scene (Bentzon et al. 2006). This is most likely due to direct mitogenic activities of cytokines or cytokine-induced production of mitogenic factors such as the platelet-derived growth factor (PDGF) (Myllarniemi et al. 1997). Meanwhile, the increasing amounts of foam cells within the plaque contribute to the process by secreting numerous cytokines (IL-1β, IL-6, IL-12, IL-18, TNF-α, TNF-β, IFN-β and IFN-γ), chemokines (MCP-1, IL-8), growth factors (PDGF and tumour growth factor β [TGF-β]), colony stimulating factors (M-CSF and GM-CSF), and proteolytic enzymes (matrix metalloproteinases [MMP] and cathepsins). As more foam cells form in response to the lipid accumulation, the plaque begins to grow, and this orchestra of different inflammatory factors begins to influence the form of the plaque. The accumulation of macrophages and foam cells denote the formation of an early fibroatheroma. This process begins in persons in their teens and early twenties (Enos et al. 1953, Insull 2009, McGill and McMahan 1998, Newman et al. 1986, Stary et al. 1994).

1.7.2 The formation of the atheroma and fibroatheroma

As noted earlier, PDGF is a strong stimulator of SMC migration. The importance of this factor is even more underlined by the fact that it has also been shown to inhibit SMC apoptosis (Newby and George 1996). The over-expression of factors such as IL-1, TNF-α and CD40L in the plaque are known to enhance the PDGF production by SMCs and ECs, allowing the indirect contribution of these cytokines to the migration of SMCs. The migration and proliferation of SMCs is also provoked by matrix metalloproteinase 9 (MMP-9)(Mason et al. 1999). At the site, SMCs can also produce many other pro-atherosclerotic cytokines (TGF-β, IFN-γ and MCP-1) (Doran et al. 2008). The increasing numbers of SMCs migrated from the media through the internal

lamina to the intima and subendothelial space begin to produce extracellular matrix (ECM), which marks the formation of a fibrous cap onto the plaque (Libby 1995, Ross 1999). The ECM in atherosclerotic plaques is formed mostly by proteoglycans with scattered type I collagen fibrils and fibronectin (Ross 1999). Furthermore, PDGF not only affects the migration and survival of SMCs but also augments, along with TGF-β, the production of the ECM. Interestingly, IFN-γ, produced mainly by T cells, has been shown to inhibit this basal IL-1β- and TGF-β-stimulated collagen synthesis (Amento et al. 1991b).

1.7.3 Formation of the necrotic and pro-thrombotic core of the fibroatheroma and haemorrhagic/thrombotic lesions

An atherosclerotic plaque may have numerous fates, and all depends on the amount and speed of the lipid accumulation and the nature of the cytokine environment produced by the cells within the plaque. If enough lipids are accumulated within the plaque and given the right cytokine environment, the foam cells inside the plaque may undergo apoptosis or oncosis, thus forming a necrotic and pro-thrombotic core within the plaque (Cai et al. 1997, Majno and Joris 1995). For example, cytokines such as IL-1β, IFN-γ and TNF-α can augment the production of ROS to the extent of causing apoptosis and oncosis (Geng et al. 1996, Li et al. 1999). In addition, macrophages may be sensitized by IFN-γ and TNF-α to die through apoptosis induced by Peroxisome-Proliferator-Activated Receptor (PPAR) α and γ ligands (Chinetti et al. 1998). The Fas ligand produced by T cells can also induce Fas-mediated apoptosis in SMCs and ECs (Fukuo et al. 1997).

1.7.4 The complicated vulnerable plaque, thin fibrous cap and plaque rupture

When the atherosclerotic plaque develops further, it begins to transform. Normally, the core of an atherosclerotic plaque is formed by foam cells and extra-cellular lipid droplets and surrounded by a cap of SMCs and ECM (Hansson 2005, Stary et al. 1995, Virmani et al. 2000b). However, the cap can also contain inflammatory cells which are most often macrophage foam cells. The thickness of the fibrotic cap is crucial for the development of the plaque. If the fibrous cap transforms into a thin cap, it becomes more prone to rupture, which can lead into either accelerated plaque development or an ischemic event (Falk 1985, Fuster 1995, Fuster et al. 1992). The thinning of the cap is most likely due to decreased ECM synthesis by the decreasing number of SMCs

residing in the cap and to matrix degradation by infiltrating macrophages (Thim et al. 2008). In fact, it has been shown that ruptured caps of human atherosclerotic plaques have increased macrophage and decreased SMC density when compared with intact caps (Davies et al. 1993). In the most rupture-prone shoulder regions of the plaques, T cells, mast cells and macrophages can be found abundant (Jonasson et al. 1986, Kovanen et al. 1995). Hence, the changing inflammatory environment in the shoulder regions can lead to plaque rupture (Packard and Libby 2008). It is believed that the increased inflammatory stimuli cause macrophages to excrete MMPs, which leads to the loss of ECM.

MMPs have been identified to cause matrix degradation through their proteolytic activity. There are 23 different types of MMPs, and collectively they can completely degrade collagen and other ECM components (Nagase et al. 2006). Besides macrophages, various other cells, such as ECs, SMCs and macrophages, can also produce MMPs. Agonists for MMP expression include cytokines such as IL-1β and TNF-α, extracellular MMP inducer and bacterial lipopolysaccharide (LPS). MMPs also have antagonists called tissue inhibitors of matrix metalloproteinases (TIMPs), and the activity of MMPs is therefore not directly related to their expression within the plaques (Newby 2008). Furthermore, IFN-γ derived mainly from T lymphocytes has been shown to greatly inhibit the ability of SCMs to synthesize new collagen, making the cap even more vulnerable (Amento et al. 1991a). Calcification and neovascularization also occur in complicated plaques, representing a further feature linked with plaque instability (Harvey and Ramji 2005). If the conditions within the plaque lead to rupture, the outcome is not always an ischemic event. A clinically silent rupture is healed by SMCs that immediately begin to secrete ECM with a high glycosaminoglycan content and collagen (Mann and Davies 1999). This can lead to rapid narrowing of the afflicted artery, as the residual thrombus is organized within the newly reforming plaque (Burke et al. 2001). Endothelial eruption can also lead to the formation of a thrombus and thus contributes to the further formation of an advanced plaque (Hansson 2005).

1.8 The role of T lymphocytes in atherosclerosis

Atherosclerotic plaques contain T lymphocytes, and the majority of them are CD4+ T lymphocytes. CD4+ lymphocytes are the main target of IL-18 within the plaques. The activation of T lymphocytes by IL-18 leads to the production of highly pro-atherosclerotic INF-γ (Okamura et al. 1995, Okamura et al. 1998). Other T cells that

can be found in atherosclerotic lesions are CD8+ cytolytic T cells and CD1+ Natural Killer T (NKT) cells (Jonasson et al. 1986, Tupin et al. 2004). The majority of the T cells are formed by memory T cells in late or chronic state of activation (Stemme et al. 1992), and most of them can be found in clusters in the shoulder regions of atherosclerotic plaques (Jonasson et al. 1986, van der Wal et al. 1989). Usually, they are surrounded by major histocompatibility (MCH) class II-expressing macrophages and dendritic cells (Bobryshev and Lord 1995, Jonasson et al. 1985, Kishikawa et al. 1993).

A large body of evidence suggests (presented below) that CD4+ T cells have a more prominent role in the disease process than CD8+ T cells and NKT cells. However, clear evidence also exists of the involvement of other T cells. For example, the presence of NKTs in human lesions has been proven (Melian et al. 1999), and the results of several studies conducted on mice have established the pro-atherosclerotic role of NKT cells possibly through the production of atherogenic IFN-γ (Aslanian et al. 2005, Major et al. 2004, Nakai et al. 2004, Tupin et al. 2004). Furthermore, activated CD8+ cells can kill neighbouring cells via cell–cell contact, and several mediators produced in atherosclerotic lesions can propagate CD8+ cells to kill SMCs and macrophages (Ludewig et al. 2000). The role of T lymphocytes in atherosclerosis in described in Figure 1.

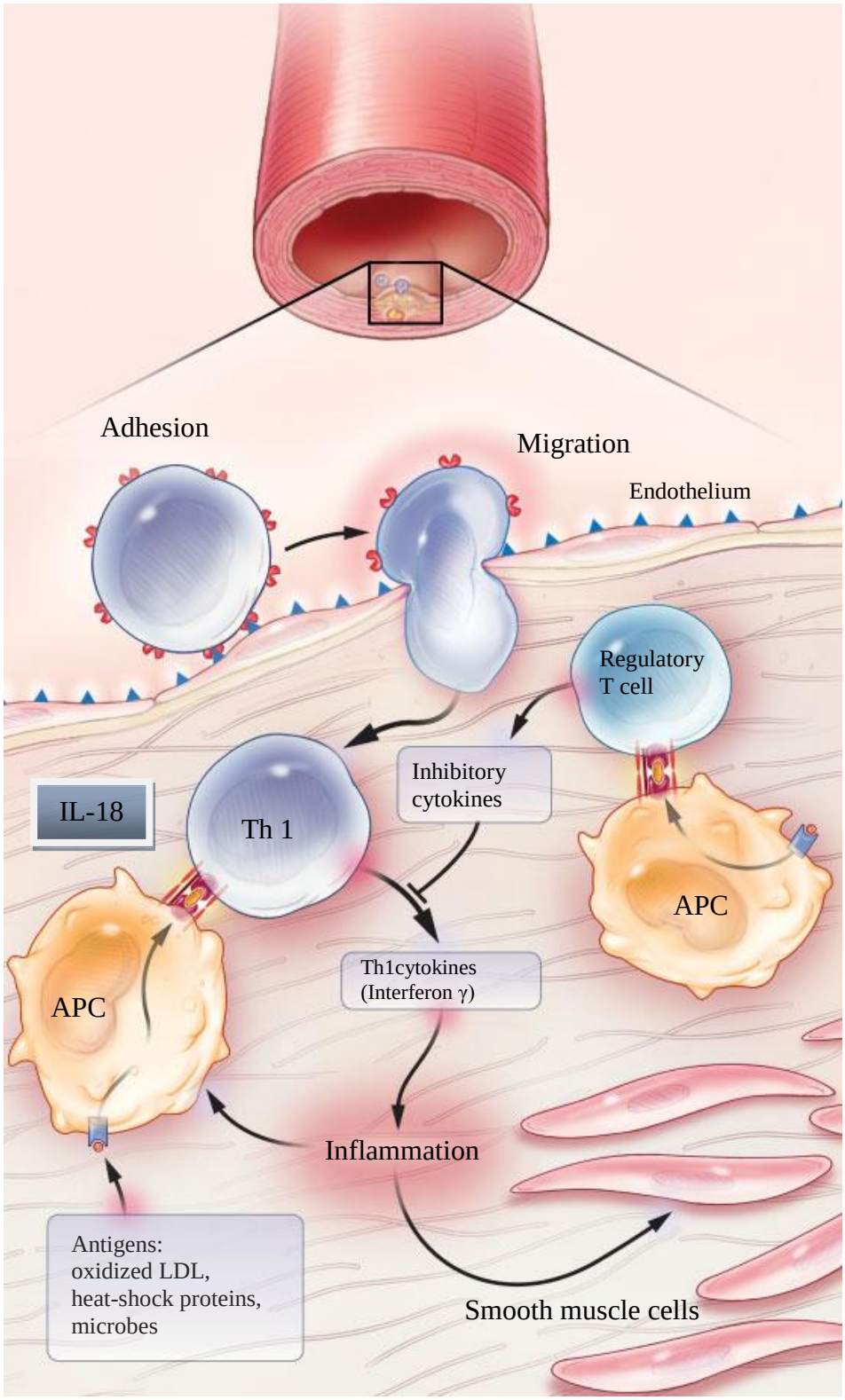


Figure 1. Main functions of T lymphocytes and antigen presenting cells (APCs) in atherosclerotic lesions. Antigens presented by macrophages and dendritic cells trigger the activation of antigen-specific T cells in the artery. Most of the activated T cells produce Th1 cytokines (e.g., interferon-γ) in response to concomitant stimulation with IL-18 and IL-12. This leads to the activation of macrophages and vascular cells, leading to inflammation. Regulatory T cells modulate the process by secreting anti-inflammatory cytokines (such as interleukin-10 and transforming growth factor β). Picture adopted from the article by Hansson et al. 2005; reproduced with permission of the publisher.

1.8.1 T lymphocytes, antigen presenting cells (macrophages) and adaptive immunity

The T cell response, e.g. adaptive immunity, is elicited when an antigen-presenting cell (APC) presents an antigenic peptide to a naïve T cell. Each naïve T cell is specific for a certain type of antigen, and the presented antigen must therefore be correct for the activation to occur (Huppa and Davis 2003). In atherosclerosis, the initial antigen presentation most likely happens in regional lymph nodes in the adventitia by DCs (APCs), and from there on the T cell homes to the site for secondary activation (e.g. plaque) (Bobryshev 2005, Galkina et al. 2006, Grabner and El-Matbouli 2009) (see Figure 2.). Within the plaque itself, many different types of cells aside from DCs, such as macrophages and possibly even ECs and SMCs, can act as APSs and be responsible for the secondary activation of T cells (Bobryshev and Lord 1998, Hansson et al. 1986, Jonasson et al. 1985, Murray et al. 1995). However, the most prominent agents for secondary activation of T cells are mature DCs. In fact, clusters of mature DCs and T cells have been identified in rupture-prone regions of the arteries, thus further linking T cell activation to unstable plaque formation (Bobryshev and Lord 1998, Yilmaz et al. 2004). Culprit plaques responsible for acute coronary syndromes have a high proportion of activated T cells (Hosono et al. 2003, van der Wal et al. 1994).

The CD4+ T lymphocytes recognize protein antigens presented by major-histocompatibility-complex (MHC) class II molecules. The CD4+ T cells that are found in the human atherosclerotic plaques can react to oxidized LDL, heat-shock protein 60, and Chlamydia pneumoniae elementary bodies (de Boer et al. 2000, Stemme et al. 1995, Xu 2002). The importance of T cells is underlined by the fact that approximately 10% of all T cells in human atherosclerotic plaques recognize oxidized LDL in MHC class II restricted manner (Stemme et al. 1995). Once the CD4+ T cell is activated, the inflammatory reaction in the cell is modulated either towards T helper (Th) 1 cell response or Th2 cell response. Within the human atherosclerotic plaque, the inflammatory environment usually promotes specialization to a Th1 response, but Th2 cells have also been identified to take part in the disease process (Frostegard et al. 1999, Uyemura et al. 1996). Recently, other types of $CD4^+$ T cells (T regulatory) have also been found within the plaques. These cells seem to have anti-atherogenic properties, possibly acting via the indoleamine 2,3-dioxygenase (IDO) or IDO-induced tryptophan degradation-dependent pathways which might suppress other T cells (Ait-

Oufella et al. 2009, Niinisalo et al. 2010).

The activation by antigens in itself is not sufficient to mount a strong T cell mediated immunity response. Cross-talk and co-stimulation between the players of the inflammatory cascade are also important, and in atherosclerosis they have been linked with primary as well as secondary activation of the T cells in the atherosclerotic lesions. As a result of co-stimulation, T cells can activate macrophages to produce more inflammatory cytokines, MMPs and tissue factor.

Despite the fact that only a small proportion (less than 10%) of the CD3+ T cells do express CD28+ in human atherosclerotic plaques, one of the co-stimulatory pairs could be the CD28+ expressed by T cells and the CD80/CD86 expressed by APCs (de Boer et al. 1997, de Boer et al. 1999). Similarly, at least in advanced plaques (Stary classes V-VI), the CD28+ surface markers have been shown to be clearly up-regulated (Niinisalo et al. 2010). However, the actual function of this pair in atherosclerosis is still unclear (Buono et al. 2004). CD4+ CD28null cells, on the other hand, are potent players, vividly expressing IFN-γ and TNF-α (Robertson and Hansson 2006). They are also believed to be able to lyse ECs and trigger apoptosis in SMCs (Nakajima et al. 2003, Pryshchep et al. 2006).

Another pair for co-stimulation is the one formed by CD40, which is expressed by APCs, and CD40L (CD154), expressed by CD4+ T cells. The activation of this pair leads to priming and expansion of antigen-specific CD4+ T cells (Xu and Song 2004). In mice, the interruption of CD40 signalling has been demonstrated to reduce atherosclerosis and promote the expression of a more stable plaque phenotype (Lutgens et al. 2000, Mach et al. 1998, Schonbeck et al. 2000). Ligation between CD40 and CD40L increases the expression of CD80 and CD86, and the stimulation of these two results in an increased expression of CD40L (Buono and Lichtman 2004, Xu and Song 2004).

The CD40/CD40L pair is also linked with the progression of atherosclerosis independently of leukocytes (Smook et al. 2005), and both CD40 and CD40L are expressed by many other cells, such as platelets, macrophages, SMCs and ECs, providing the pair with other evident pro-atherosclerotic mechanisms alongside the co-stimulation between leukocytes (Andersson et al. 2009, Schonbeck and Libby 2001). For example, EC ligation of the CD40/CD40L leads to increased expression of chemokines and adhesion molecules, thus provoking leukocyte recruitment (Karmann et al. 1995), and CD40L ligation has also been linked with platelet activation and

thrombosis (Henn et al. 1998, Inwald et al. 2003). All in all, the CD40/CD40L is associated with increased pro-inflammatory cytokines, chemokines, adhesion molecules, metalloproteinases and tissue factor, which is a pro-coagulant (Karmann et al. 1995, Schonbeck and Libby 2001).

Ox40, expressed by T cells, and its ligand OX40L, which in turn is expressed by activated APCs, form another pair for co-stimulation. The ligation to OX40 facilitates the survival of the immune response, and inhibits T regulatory cell development and function (anti-inflammatory). Alongside atherosclerosis, this pair has also been linked with other autoimmune diseases (Martin-Orozco et al. 2003, Ndhlovu et al. 2001, Wang et al. 2005). APCs of human atherosclerotic plaques have been shown to express Ox40, but most of the evidence linking Ox40 with the development of atherosclerosis on vessel wall level has arisen from experiments with mice (van Wanrooij et al. 2007, Wang et al. 2005).

Furthermore, other potential co-stimulatory pairs affecting the development of atherosclerosis have been identified (ICOS/ICOSL, CD137/CD137L, PD1/PD-L1 and PDL2), but as of yet, the data is sparse (Gotsman et al. 2007, Gotsman et al. 2006, Olofsson et al. 2008).

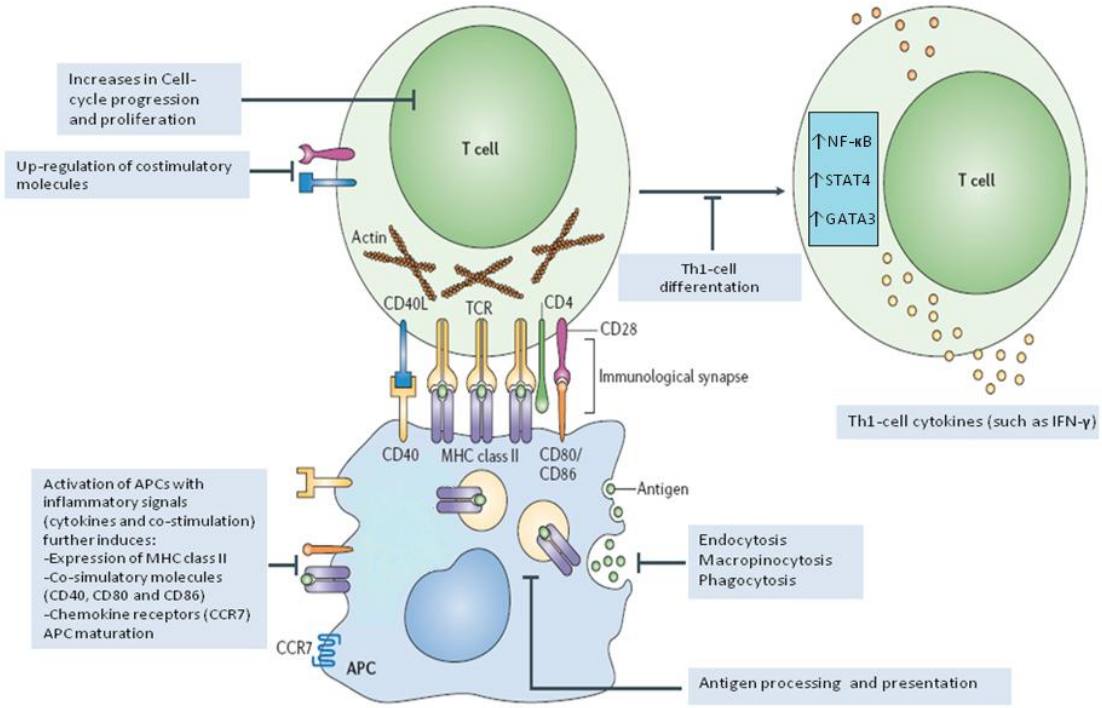


Figure 2. Antigen presentation in an inflammatory environment favourable for Th1 cell differentiation. Antigen presentation with co-stimulation further stimulates the expression of major histocompatibility class II molecules used in antigen presentation and co-stimulatory molecules. The activation of Th1 cells leads to the activation of transcription factors (NK-κB, STAT4, GATA3) resulting to the expression of Th1 cell cytokines such as IFN-γ. Picture adopted and modified from the article by Greenwood et al. 2006. Reproduced with permission of the publisher.

1.8.2 Th1 cell-related immunological responses in atherosclerosis

As mentioned before, most of the CD4+ T cells in atherosclerotic plaques are Th1 cells. These cells induce macrophage activation and produce IFN-γ and TNF-α which have many atherosclerotic properties (Hansson et al. 2006) (see Figure 1). To underline this fact, local expression of IFN-γ dominates over the expression of Th2-related cytokines (IL-4 and IL-5) in human lesions (Hansson et al. 2006). In fact, IFN-γ is produced by the majority of T cells in human atherosclerotic plaques (Hansson et al. 1989b, Stemme et al. 1995). IL-12 and IL-18 synergistically act to promote the Th1 response and IFN-γ production (Benagiano et al. 2003, Uyemura et al. 1996). Furthermore, in genetically altered mice, lineages with Th1 predominance have been associated with increased development of atherosclerosis when compared to lineages with Th2 predominance (Schulte et al. 2008).

IFN-γ is involved in the recruitment of T cells and macrophages, increased expression of MCH class II, increased macrophage lipid up-take to and therefore the formation of foam cells, in addition to increased activation of APCs and enhanced secretion of Th1-promoting cytokines (Leon and Zuckerman 2005). IFN-γ plays a major role in regulating macrophage involvement and, supporting this, roughly 25% of the macrophage transcriptome is regulated by IFN-γ (Ehrt et al. 2001). Murine studies have shown that IFN-γ is associated with increased atherosclerosis. IFN-γ has also been associated with the development of unstable plaques (Andersson et al. 2009).

IFN-γ is associated with the recruitment of inflammatory cells to the lesions. First of all, it induces the expression of chemokines, one of the most important of which is MCP-1, a potent chemotactic for monocytes and T-cells (Harvey and Ramji 2005, Valente et al. 1998). In addition, MCP-1 causes the adhesion of recruited cells to ECs and potentiates the polarization towards a Th1 response in T cells. IFN-γ also induces the expression of VCAM-1 and intracellular adhesion molecule (ICAM) -1 from ECs and SMCs (Chung et al. 2002, Li et al. 1993). It is recognized to promote both, the differentiation of monocytes to macrophages and causes the activation of both macrophages and T cells (Boehm et al. 1997, Schroder et al. 2004). Furthermore, lipid accumulation onto macrophages and the formation of foam cells are known to increase due to IFN-γ (Panousis and Zuckerman 2000, Reiss et al. 2004). As noted above, the apoptosis of the foam cells results in the formation of the necrotic core of the lesion and, consequently, IFN-γ has also been shown to promote apoptosis of macrophage-derived foam cells and vascular SMCs (Geng et al. 1996, Inagaki et al. 2002). Furthermore, IFN-γ is a very prominent factor causing plaque destabilization by inhibiting the expression of collagen genes (e.g. collagens I and III) and the proliferation of SMCs. These effects contribute to the formation of the ECM (discussed earlier). Not surprisingly, IFN-γ has also been directly linked with the weakening of the protective fibrous cap of the lesions (Buono et al. 2003, Gupta et al. 1997). Furthermore, IFN-γ has been found to reduce proliferation on ECs and SMCs and to reduce the differentiation and collagen synthesis of vascular SMCs (Amento et al. 1991a, Friesel et al. 1987, Hansson et al. 1989a).

In atherosclerotic lesions, IFN-γ can also be secreted by numerous other cell types, such as CD8+ lymphocytes, activated macrophages, NK cells, B cells and vascular SMCs (Bancroft et al. 1991, Flaishon et al. 2000, Gerdes et al. 2002a, Munder et al. 1998, Sad et al. 1995). NK T cells have also been shown to secrete high amounts

of IFN-γ (Aslanian et al. 2005, Tupin et al. 2004). Interestingly, despite the fact that IFN-γ is considered to associate with increased atherosclerosis, it has been related to some anti-atherogenic functions such as the inhibition of macrophage lipoprotein lipase (aids the up-take of modified LDL) and that of the oxidation of LDL (McLaren and Ramji 2009).

1.8.3 Other CD4+ T cells

Other CD4+ T cells identified in atherosclerotic lesions are Th2 cells, Th17 cells and T regulatory cells. The role of the Th2 cells is not clear. According to studies conducted with mice, cytokines related with Th2 response (IL-4 and IL-5) seem to have different effects. IL-4 would seem to have a pro-atherogenic influence, and IL-5 would appear to associate with decreased lesion formation (Binder et al. 2004, Davenport and Tipping 2003, King et al. 2002). T regulatory cells, which characteristically express transcription factors FoxP3 and CD25, are immunosuppressive cells, and their presence has been verified in atherosclerotic lesions of both mice and men (Heller et al. 2006, Niinisalo et al. 2010, Shevach et al. 2006, Veillard et al. 2004). T regulatory cells have been related to decreased lesion formation in murine models, and the suppressive effect is thought to be attributable at least partly to their expression of TGF-β and IL-10 (Andersson et al. 2009), and possible throughout the indoleamine 2,3-dioxygenase (IDO)-related tryptophan depletion-mediated T-cell depression (Niinisalo et al. 2010). Th17 cells are involved in the recruitment of neutrophils, and they elaborate IL-17, but their role in atherosclerosis remains unclear (Andersson et al. 2009).

1.9 Interleukin 18

IL-18 was initially identified as an IFN-γ-inducing factor, and it was observed to be involved in orchestrating the Th1 response (Okamura et al. 1995). It is a pleiotrophic cytokine influencing both the innate and the acquired immune system (Okamura et al. 1998). IL-18 is part of the IL-1 family (alongside with IL-1 and IL-33), which means that it has structural similarities with the other members of the family. The involvement of IL-18 has been identified in several inflammatory and autoimmune diseases such as multiple sclerosis, arthritis, inflammatory bowel diseases and psoriasis (Arend et al. 2008). It also seems to play an important role in atherosclerosis

1.9.1 The production and secretion of interleukin 18

IL-18 is produced mainly by monocytes and macrophages, but it can also be produced by many other cell types, such as DCs, Kupffer cells, keratinocytes, chondrocytes, synovial fibroblasts and osteoblasts (Gracie et al. 1999, Horwood et al. 1998, Olee et al. 1999, Zepter et al. 1997). IL-18 is initially produced as a 24 kDa precursor peptide, and it has to be cleaved to its mature form (18 kDa) before it can be secreted. The precursor mRNA has a stable structure and is constitutively and intracellulary stored (Gu et al. 1997). The cleavage of pro-IL-18 to its active form and its secretion is believed to be influenced strongly by the activity of the intracellular cysteine protease known as caspase-1 (Casp-1) (Arend et al. 2008). However, it has also been shown that ECs, for example, secrete mature IL-18 independently of Casp-1 activity (Kolinska et al. 2008). One possible alternative for IL-18 processing is the serine proteinase known as protease-3 (PR-3), which can cleave precursor IL-18 into active mature IL-18. PR-3 can be found on the surface membranes of macrophages, neutrophils and ECs, and it can also be released in response to inflammatory stimuli (Fantuzzi and Dinarello 1999). The caspase-3 enzyme can cleave precursor IL-18 and mature IL-18, but this leads to accumulation of biologically inactive products, suggesting a scavenger function for Casp-3 (Arend et al. 2008, Fantuzzi and Dinarello 1999).

The excretion of IL-18 is stimulated by several agonists that activate the pattern-recognition receptors (PPRs) of monocytes/macrophages. PPRs, such as Toll-like receptors (TLR) which are found mainly on cell membranes, are an important part of the innate immune system (Liew et al. 2005). At least two agonists for TLRs (lipopolysaccharide [LPS] and flagellin) have been identified to stimulate IL-18 expression (Bachmann et al. 2006, Grobmyer et al. 2000, Seki et al. 2001). Interestingly, also IFN-γ and TNF-α may provoke the expression of mature IL-18 in cultured epithelial cells (Kolinska et al. 2008). The fact that peripheral blood monocytes can also express IL-18 in response to stimulation by Chlamydia pneumonia (independently of TLR2 and TLR4) quite clearly links IL-18 with an innate immune response (Netea et al. 2004). C-reactive protein can also activate the release of IL-18 in human endothelial cells.

Furthermore, cardiomyocytes can express IL-18 in response to stimulation by TNF-α, aldosterone, endothelin-1 and angiotensin II. These responses have been attributed to the stimulation of nuclear factor-kappa B, which has a binding site in the

IL-18 gene (Chandrasekar et al. 2003, Doi et al. 2008).

1.9.2 IL-18 receptor (IL-18R) and IL-18 binding protein (IL-18BP)

The IL-18 receptor complex comprises two chains of IL-18 receptor (R) α and IL-18Rβ. IL-18Rα is a binding chain, and it is expressed on most of the key players associated with atherosclerosis (naïve T cells, mature Th1 lymphocytes, NK cells, macrophages, B cells, neutrophils, mast cells, ECs and SMCs) (Arend et al. 2008, Gerdes et al. 2002b, Nakamura et al. 2000, Yoshimoto et al. 1998). IL-18Rβ is an accessory protein, which binds with ligated IL-18Rα, resulting in the activation of the complex. This leads to the activation of intracellular adapter molecules (myeloid differentiation factor 88, IL-1R-associated kinase, TNF-α associated kinase 6), thus resulting in the activation of signal transduction pathways such as nuclear factor-kappa B, c-Jun N-terminal kinase and p38 mitogen-associated protein kinase (Thomassen et al. 1998). The expression of the IL-18 receptor complex, and especially that of the IL-18Rβ, are influenced by many cytokines, and the effects of IL-18 are therefore also regulated by the expression of its receptors in response to inflammatory stimuli (Arend et al. 2008). In addition, angiotensin II can enhance the expression of IL-18Rα on SMCs (Sahar et al. 2005).

IL-18BP is a soluble protein with four naturally occurring human isoforms, two of which have a high affinity for binding to mature IL-18 (Kim et al. 2000). The binding of IL-18BP to IL-18 results in the neutralization of IL-18 and therefore prevents its further interaction with IL-18R. The two isoforms can neutralize over 95% of IL-18 at a molar excess of 2 (Kim et al. 2000). IL-18BP is produced constitutively in the human spleen (Novick et al. 1999). In addition, monocytes/macrophages, ECs and epithelial cells are also known to express IL-18BP (Corbaz et al. 2002). The expression IL-18BP in endothelial cells is enhanced by the release of IFN-γ, thus suggesting a negative feedback loop for IL-18 signalling (Paulukat et al. 2001).

1.9.3 The biological functions of IL-18

One of the main functions of IL-18 is to induce IFN-γ production and promote the Th1 response (Okamura et al. 1995). However, despite the fact that IL-18 is a very potent inducer of IFN-γ production from Th1 cells and clearly promotes the Th1 response, synergy with IL-12 is important for these responses (Robinson et al. 1997, Takeda et al. 1998). Interestingly, IL-18 in synergy with IL-12 also promotes the expression of IFN-

γ in macrophages and SMCs (Darwich et al. 2009, Gerdes et al. 2002a) The interplay between IL-18 and IL-12 is clear, as IL-12 can induce the expression of IL-18Rβ (Neumann and Martin 2001). In fact, three different cytokines (IL-15, IL-21, and IL-23) have been identified to act in synergy with IL-18, inducing the production of IFN γ from T cells (Hoeve et al. 2003, Strengell et al. 2003, Strengell et al. 2002). IL-21 has also been demonstrated to augment the expression of the IL-18R gene (Strengell et al. 2002).

Alone, IL-18 stimulates the expression of IL-6 (pro-atherosclerotic cytokine), IL-8 (chemokine), ICAM-1 and MMPs (MMP-1/-9/-13) in cultured human SMCs, ECs and mononuclear phagocytes (Chandrasekar et al. 2006, Gerdes et al. 2002a). It also promotes human coronary SMC migration through the increased production of MMP9 (Chandrasekar et al. 2006). Interestingly, IL-18 can also act as a chemoattractant for T CD4+ cells (Komai-Koma et al. 2003) and enhance the IFN-γ production as well as the cytolytic capability and proliferation of CD8+ T cells (Okamoto et al. 1999, Tough et al. 2001). The expression of IFN-γ can also have a positive feedback on the production of IL-18. It was recently demonstrated that IL-18 is expressed by DCs upon antigen presentation to T cells, and this expression of IL-18 was stimulated by IFN-γ (Iwai et al. 2008). IL-18 can also increase the OX40 and OX40L expression on T cells and APCs, therefore further facilitating the interaction between these two cell types via co-stimulation (Maxwell et al. 2006). Furthermore, it has been shown that the priming of NK T cells by IL-18 leads to ligand-activated IFN-γ production (Uchida et al. 2007). Not surprisingly, when combined with IL-12, it leads to the secretion of IFN-γ (Fujibayashi et al. 2007).

IL-18 also seems to have a strong role in regulating the activity of NK cells. It was shown quite recently that priming of NK cells with IL-18 in vivo enhances the IL-12 induced translation of IFN-γ mRNA (Chaix et al. 2008). Previous studies have demonstrated that IL-18 augments the NK cell activity and, in combination with IL-15, NK cell proliferation and IFN-γ expression (French et al. 2006, Hyodo et al. 1999, Strengell et al. 2003, Takeda et al. 1998). IL-18 can also enhance the Fas ligand on NK cells (as well on T cells) and induce apoptosis in cells that express Fas (Faggioni et al. 2001).

Additionally, IL-18 yields diverse effects on monocytes and macrophages. Therefore, it also directly influences innate immunity. For example, IL-18 can cause peripheral blood mononuclear cells to express GM-CSF, TNF-α, IL-1β and IL-8, in

addition to other chemokines such as MCP-1(Puren et al. 1998). Preconditioning of macrophages with IL-18 and IL-12 increases the IFN-γ-dependent nitric oxide response and the TNF-α response to inflammatory stimuli in vivo (Bastos et al. 2007). In addition, the treatment of monocytes with IL-18 and IL-12 prevents spontaneous apoptosis and promotes differentiation to macrophages as well as the expression of chemokines (CXC chemokine ligands -8/-9/-10, which activate chemokine receptors on granulocytes, T cells and monocytes) (Coma et al. 2006, Moser et al. 2004, Nold et al. 2003). Human neutrophils also express IL-18R and can be activated by IL-18 (Leung et al. 2001).

Although IL-18 is a potent inducer of Th1 responses and has been related to them in atherosclerosis, it can also, under certain conditions, promote Th2 responses (Nakanishi et al. 2001). In combination with IL-2, it can stimulate the production of IL-13, a cytokine related to the Th2 response, by T cells and NK cells (Fujibayashi et al. 2007, Hoshino et al. 2001). Furthermore, IL-18 in synergy with IL-23 has been associated with the polarization and activation of Th17 cells, but as mentioned before, even the role of Th17 cells is unclear in atherosclerosis, rendering the importance of the link between these two factors in regard to atherosclerosis an issue to be clarified (Arend et al. 2008).

1.9.4 The role of IL-18 in atherosclerosis

According to several murine models, IL-18 clearly associates with increased atherosclerosis measured in plaque sizes (Elhage et al. 2003, Mallat et al. 2001b). IL-18 is also connected with plaque instability (less T cells, lipids, cell death, macrophages and increased collagen and SMC content) (Elhage et al. 2003, Mallat et al. 2001b) One of the models has also indicated that the pro-atherogenic effect of IL-18 is IFN-γ-dependent (Whitman et al. 2002). Interestingly, the IFN-dependency is not dependent on T cells, and the potent pro-atherosclerotic response can also be obtained from macrophages, NK cells and vascular cells (Tenger et al. 2005).

In humans IL-18 has also been associated with the development of atherosclerosis on the vessel wall level. In a study by Mallat et al., IL-18 and IL-18Rα were found to be highly expressed in atherosclerotic samples obtained from carotid arteries when compared to normal arteries. Furthermore, unstable plaques had higher levels of IL-18 mRNA when compared to stable plaques (Mallat et al. 2001a). Another study has also confirmed the higher expression of IL-18 in atherosclerotic plaques

when compared to normal arteries (Graebe et al. 2009).

Further evidence of the link between IL-18 and the development of atherosclerosis is presented in studies that depict the development of the disease using other end-points than the vessel wall level plaque development. One previous study associated plasma IL-18 levels with intima media thickness (IMT), a marker of subclinical atherosclerosis, among patients with obstructive sleep apnoea-hypopnoea syndrome (Li et al. 2009). Another study that associated higher IL-18 plasma levels with IMT revealed that circulating IL-18 levels also correlate with decreased carotid femoral pulse wave velocity (a marker depicting the stiffness of arteries) among men with no manifestations of cardiovascular disease (Vlachopoulos et al.). Two other studies have confirmed the finding concerning IMT among healthy populations, but whether this association is independent of traditional risk factors remains unclear (Chapman et al. 2006, Yamagami et al. 2005).

The relation between the polymorphism of the IL-18 gene and carotid IMT has been studied, but the results obtained were not clear. Although some major haplotypes of the IL-18 gene associated with circulating IL-18 levels, IL-18 gene polymorphism did not seem to affect carotid atherosclerosis. However, among a subpopulation of CHD patients, one major haplotype seemed to associated with a higher risk of developing a carotid artery plaque (Thompson et al. 2007a). Furthermore, circulating IL-18 levels have been associated with coronary plaque areas in patients suffering from MI as assessed by quantitative coronary angiography (Hulthe et al. 2006), and another study reported a positive association between IL-18 levels and the extent of coronary atherosclerosis among patients with unstable AP (Chen M. C. et al. 2007). In one previous study, the atherosclerotic burden was assessed using combined angiography measurements and ultrasound evaluation from the femoral and carotid artery. In this study setting, IL-18 levels were associated with the over-all atherosclerotic burden (Espinola-Klein et al. 2007).

Among healthy men, plasma levels of IL-18 also have a predictive value for acute coronary syndromes (Blankenberg et al. 2003). One later study confirmed that baseline IL-18 concentrations predict the occurrence of MI during follow-up, but this result did not hold after adjustment for risk factors (Thompson et al. 2007b). In line, patients with unstable angina pectoris and MI have higher plasma concentrations of IL-18 when compared to controls (Hulthe et al. 2006, Mallat et al. 2002, Rosso et al. 2005). In patients with CAD, serum levels of IL-18 predict the occurrence of

cardiovascular mortality independently of risk factors (Blankenberg et al. 2002, Espinola-Klein et al. 2007, Tiret et al. 2005).

The variation of the IL-18 gene has also been linked with a higher risk of MI and cardiovascular mortality. In 2005 Tiret et al. linked the polymorphism of the IL-18 gene with cardiovascular mortality among CAD patients (Tiret et al. 2005). A few years later, another study was published revealing that among a combined population of hypertensive patients and postmenopausal women, the risk for MI, but not for stroke, also associates with IL-18 gene polymorphism (Bis et al. 2008).

1.9.5 Polymorphism of the IL-18 gene

In 2005, Tiret et al. published a comprehensive analysis of the variability of the IL-18 gene in a Caucasian population, and they reported that the variation of the IL-18 gene can be covered with 6 haplotypes accounting for 99.0% of all chromosomes (Tiret et al. 2005). The gene was found to have three major haplotypes (minor allele frequencies approximately: 0.306, 0.286 and 0.247) and three minor haplotypes (minor allele frequencies approximately: 0.103, 0.027 and 0.024). These 6 haplotypes can be constructed using 5 tag single nucleotide polymorphisms (SNPs). Obviously, the whole IL-18 gene contains more SNPs, but they can be organized into five clusters or BINs because the majority of them are inherited in complete association (Tiret et al. 2005).

Few individual SNPs of the IL-18 gene have been shown to have functional properties. Giedraitis et al. showed that a haplotype carrying the minor allele of two promoter region SNPs (rs1946518, position -607[C/A], and rs187238, position -137 [G/C]) are associated with lower transcriptional activity of the gene in vitro using HeLa cells (Giedraitis et al. 2001). The latter (rs187238) also seemed to associate with the production of mature IL-18, and it was therefore speculated to be responsible for the observed significant change in the transcriptional activity. Subsequently, the same polymorphisms were associated again with similar patterns in transcriptional activity in cultured HepG2 cells (Liang et al. 2005). Further evidence of the functionality of the -137 (G/C) SNP has been presented in two independent studies, both showing that this particular SNP also associates with the production of mature IL-18 from monocytes constitutively as well as under stimulation, with the minor C allele associating with decreased transcriptional activity (Arimitsu et al. 2006, Khripko et al. 2008). The other study reported that the rs1946518 (-607 [C/A]) also associates with the transcriptional activity of the gene, but the effect seemed weaker compared to the

effect of -137 (G/C) (Khripko et al. 2008). Two other studies have also reported positive associations between two new SNPs (rs360716 and rs5744247) and the expression of IL-18 mRNA, but these results have not been confirmed in other studies (Harada et al. 2009, Sanchez et al. 2009). Furthermore, as the rs360716 is in complete association with the -137 (G/C) SNP, it is impossible to assume that the observed association would be independent. The minor C allele of the -137 (G/C) SNP has been associated with lower circulating levels of IL-18 (Liu et al. 2009)

Haplotype studies have also revealed interesting results of the functionality of the IL-18 gene polymorphism. The study by Tiret et al. demonstrated that two major haplotypes are associated with circulating IL-18 levels (Barbaux et al. 2007, Tiret et al. 2005). Both haplotypes associated with lower IL-18 concentrations in serum. The other haplotype is the only one carrying the minor C allele of the -137(G/C) SNP. The other major haplotype is the only one carrying the minor A allele of the rs5744292 SNP (+183[A/T]). In a further functional haplotype study using lymphoblastoid cell lines, the haplotype carrying this SNP (rs5744292) was shown to clearly associate with a lower expression level of the gene. The association between the haplotype carrying the -137 G/C SNP and expression levels was also significant but did not seem so evident (Barbaux et al. 2007). Subsequently, two other haplotype studies have further proved that these two major haplotypes associate with lower circulating IL-18 levels (Thompson et al. 2007a, Thompson et al. 2007b).

1.10 The methodology of genetic studies

Genetic studies can basically be divided into two bigger entities: candidate gene association studies and genetic linkage studies (genome-wide linkage studies, genome-wide association studies, GWAS). The present study utilizes the candidate gene approach, because IL-18 and the variation in the IL-18 have already been identified to affect the development of atherosclerosis.

Genome-wide linkage studies are used to find quantitative trait loci (QTL) within chromosomes that regulate atherogenesis. QTLs, however, can contain hundreds of genes. Therefore, even after one QTL has been identified repeatedly in independent populations as regulating atherogenesis, the testing of all of the genes in the specific chromosomal region is a daunting task. However, genome-wide linkage studies have been very efficient in finding genes with simple Mendelian inheritance (Chen Y. et al.

2007). Atherosclerosis is a common disease with a complex aetiology, and it is not easy to find common variants that would have a strong effect on disease progression using the QTL approach.

The completion of the human genome sequence and the international HapMap project a few years ago, in addition to the lowering costs of genotyping and sequencing, have lead to the submersion of a new method known as GWAS (Manolio et al. 2007). GWAS can be used to find SNPs or haplotypes associating strongly or moderately with common disease traits, such as those related to atherosclerosis. Over 20 million SNPs are available at www.ncbi.nlm.nih.gov/SNP. In 2007 the HapMap project had genotyped more than 3 million SNPs, and the sequence data has confirmed that the vast majority of the SNPs are strongly correlated with nearby proxies. Therefore, it is not generally required to genotype all SNPs in GWAS. In an African population, where the genetic diversity is broad, approximately 1 million SNPs suffice, whereas in non-African populations, 500.000 SNPs guarantee excellent power to determine more than 90% of the common SNP variation (2005, Altshuler et al. 2008). Despite the high costs, GWAS seems to be a good way of establishing new associations between common polymorphisms and disease traits. Nevertheless, the power for detecting associations has thus far been relatively low. Detecting a common allele with a frequency of 20% and a factor of 1.2 for the effect requires 8,600 samples. This is due to the fact that a genome-wide scan usually means testing approximately 1 million independent hypotheses, and in order to correct for type I error, a very stringent significance level must be assumed ($P=5 \times 10^{-8}$) (Altshuler et al. 2008). GWAS reports of significant associations with CAD, coronary disease, MI (also early onset), and CVD are listed in Table 3, and for other atherosclerosis-related traits, such as lipids, CRP and diabetes, lists can be found in the Catalog of Published Genome-wide Association Studies (www.genome.gov/gwastudies)

Table 3. Genome-wide association studies presently reporting significant associations with CAD, coronary disease, MI (also early onset) and CVD. The Odds Ratios (ORs) for each risk factor range from 1.08 to 1.91 and the reported P-values for the ORs from 7 $x10^{-7}$ to 3 $x10^{-44}$. Data was extracted from Catalog of Published Genome-Wide Association Studies. Available at: www.genome.gov/gwastudies

Publication	Disease/Trait	Gene	Region	Platform [SNPs]
Erdmann et al.	CAD	*MRAS*	3q22.3	Affymetrix [567,119]
08 February 2009		*HNF1A,C12orf43*	12q24.31	
Nat Genet				
Kathiresan et al.	MI (Early onset)	*CDKN2A, CDKN2B*	9p21.3,	Affymetrix [~2.500.000] (imputed)
08 February 2009		*CELSR2, PSRC1, SORT*	1p13.3	
Nat Genet		*SLC5A3, MRPS6, KCNE2*	21q22.11	
		MIA3	1q41	
		PHACTR1	6p24.1	
		LDLR	19p13.2	
		CXCL12	10q11.21	
		PCSK9	1p32.3	
Tregouet et al.	CAD	*Two Four SNP haplotypes formed by*	6q25.3	Affymetrix [~500,000]
08 February 2009		*SLC22A3, LPAL2 and LPA*		
*N*at Genet				
Larson et al.	Major CVD	*Intergenic*	6p24.1	Affymetrix [70,897]
19 September 2007				
BMC Med Genet				
Samani et al.	Coronary disease	*intergenic*	9p21.3	Affymetrix [377,857]
18 July 2007		*PSRC1*	1p13.3	
N Engl J Med		*MTHFD1L*	6q25.1	
		CXCL12	10q11.21	
		SMAD	15q22.33	
		pseudogene	2q36.3	
WTCCC	Coronary disease	*CDKN2A,CDKN2B*	9p21.3	Affymetrix [469,557]
07 June 2007		*Intergenic*	1q43	
Nature		*Intergenic*	22q12.1	
		Intergenic	16q23.3	
Helgadottir et al.,	MI	*CDKN2A,CDKN2B*	9p21.3	Illumina [305,953]
03 May 2007				
Science				

AIMS OF THE STUDY

The pro-atherogenic and plaque-destabilizing role of IL-18 has been established with murine models, and some evidence also exists that it has the same properties in human atherosclerotic plaque development. Circulating levels of IL-18 seem to be associated with the development of subclinical atherosclerosis and the occurrence of acute coronary syndromes. Although the IL-18 gene has functional SNPs affecting IL-18 expression, the role of the variation in the IL-18 gene in the development of subclinical atherosclerosis is not clear due to a lack of studies and conflicting results. Furthermore, only one previous study has associated the polymorphism of the IL-18 gene with cardiovascular mortality among patients with CAD, and the role of IL-18 gene variation in the development of different stages of atherosclerosis and with SCD remains unclear.

The present study focuses on the possible role of IL-18 gene variation in the development of different stages of atherosclerosis by using autopsy-verified atherosclerotic lesions and the manifestation of atherosclerosis over the course of life in different populations.

The specific aims of the present study are:

1. To study the possible association between the polymorphism of the IL-18 gene and the development of sub-clinical atherosclerosis, as measured by carotid artery intima media thickness, carotid arterial elasticity and endothelial function, among young asymptomatic and healthy Caucasian adults (Study I)
2. To study whether IL-18 gene polymorphisms or their haplotypes have any effect on cardiovascular mortality among a Finnish patient population at an intermediate risk of death due to cardiovascular causes; and to investigate the possible effect of IL-18 gene polymorphism on the occurrence of angiography-verified coronary artery disease (Study II).
3. To explore whether a functional IL-18 promoter polymorphism associates with the risk of SCD due to coronary heart disease and SCD caused by non-coronary diseases among Caucasian men (Study III).
4. To explore the possible interactions between traditional risk factors and IL-

18 gene functional SNPs affecting the risk of SCD and the expression of autopsy-verified coronary artery disease. We also wanted to study the possible effect of these interactions on the expression of IL-18 as well as IFN-γ mRNA in atherosclerotic samples obtained from live patients subjected to vascular surgical procedures (Study IV).

MATERIALS AND METHODS

1.1 Clinical series

1.1.1 The Cardiovascular Risk in Young Finns Study

1.1.1.1 Subjects

The Cardiovascular Risk in Young Finns Study is an ongoing prospective multicentre cohort study, with a study population of 2,282 young adults. Details of the cohort have been published previously (Åkerblom et al. 1985). The study began in 1980. The 21-year follow-up was carried out in 2001. All of the data used in the present study was collected in the year 2001, and participants with type 1 diabetes were excluded from further analyses. The Ethical Review Committee of Turku University Hospital approved the research plan, and the study was conducted according to the tenets of the Declaration of Helsinki. Patients gave an informed consent before entering the study.

1.1.1.2 Clinical and biochemical characteristics

A standardized questionnaire was used to assess cardiovascular risk factors (smoking, alcohol consumption, geographical origin and familial history of coronary heart disease). The classification of these variables has been described earlier in more detail (Hernesniemi et al. 2008). The study subjects' height and weight were used to calculate their body mass index (BMI = weight, kg/(height, m)^2), and their blood pressure (BP) was recorded. Fasting venous blood samples were used to determine C-reactive protein (CRP), insulin, glucose, serum lipids, apolipoprotein A-1 (ApoA-1), apolipoprotein B (ApoB), and homocysteine concentrations. Total cholesterol, high-density lipoproteins (HDL) and triglycerides were determined enzymatically. Low-density lipoprotein (LDL) was calculated with the Friedewald formula. Standardized methods were employed in all determinations (Juonala et al. 2004a, Raitakari et al. 2003). Information on geographical origin was also recorded, because the Eastern Finnish population is genetically more predisposed to the development of atherosclerosis than the population of Western Finland (Juonala et al. 2005).

1.1.1.3 Measurements of subclinical markers of atherosclerosis

Carotid artery IMT was measured by ultrasound, and in order to determine CAC, the concomitant brachial blood pressure was also monitored. The brachial artery FMD was assessed by measuring the left brachial artery diameter both at rest and during reactive hyperaemia (after five minutes of comprimation). Ultrasound examinations were performed using Sequia512 mainframes (Acuson, Mountain View, California) with a 13.0 MHz linear array transducer. The procedures have been discussed earlier in more detail (Raitakari et al. 2003).

To determine the intra-individual reproducibility of the measurements, the ultrasound measurements were replicated for a small random sample of participants (n=57, 2,5%) 3 months after the initial visit. The between-visit coefficient of variation was 6.4% for IMT, 16.3% for CAC and 26% for FMD measurements.

1.1.2 The Finnish Cardiovascular Study

1.1.2.1 Study cohort

FINCAVAS is a cohort study with the purpose of constructing a risk profile to identify individuals at a high or low risk of cardiovascular diseases, events and deaths by using genetic, hemodynamic and electrocardiographic (ECG) markers (Nieminen et al. 2006). The participant pool consists of patients (n=2152) who underwent an exercise stress test at Tampere University Hospital between October 2001 and December 2004 and were willing to participate in the study. Indications for the exercise test were: suspicion of coronary heart disease (CHD, n=959), testing vulnerability to arrhythmia during exercise (n=465), evaluation of working capacity (n=387) and adequacy of drug therapy (n=330), as well as obtaining an exercise profile prior to surgery (n=284) and post-MI (n=171). In some cases, the patients had more than one indication. Angiography was performed on a subsample of patients for interventional and diagnostic indications (n=461). The coronary angiographies were performed before June 2005. The study protocol was approved by the Ethical Committee of the Hospital District of Pirkanmaa, Finland, and all patients gave informed consent prior to study initiation, according to the Declaration of Helsinki.

1.1.2.2 Collection of risk factor data

Data on demographics, classical cardiovascular risk factors, lifestyles, medications and medical history were gathered using a computer-based questionnaire before the exercise stress test was performed. Blood samples were drawn for DNA analyses after the exercise test. The definition of risk factor data has been described previously in detail (Fan et al. 2006).

1.1.2.3 Angiography-verified CAD

The same dedicated cardiologist analysed all coronary angiographies, and the percentages of stenosis in the different parts of the coronary arteries were registered. Angiography was performed on 461 patients. Based on the angiographies, 255 (34%) patients had main branch CAD (defined as over 50% stenosis in at least one of the major coronary arteries, including the left main, left anterior descending, right, and left circumflex coronary artery). In addition, 37 (8%) patients had a side branch stenosis of 50% or more, while none of the patients had concomitant main branch disease.

1.1.2.4 Mortality data collection

Death certificates were received from the Causes of Death Register, maintained by Statistics Finland, in September 2009; this source has been proven reliable (Pajunen et al. 2005). The certificates included causes of death according to the tenth revision of the International Classification of Diseases (ICD-10). Diagnosis numbers and certificate texts were used to determine if the diseased had died of cardiovascular causes or due to other causes. The autopsy rate was 40% for all deaths and 60% for patients with SCD.

1.1.3 The Tampere Vascular Study

1.1.3.1 Samples

The Tampere Vascular Study (TVS) material comprises arterial samples of atherosclerotic lesions (types IV–VI) (Stary et al. 1995) from the carotid arteries (n=9), femoral arteries (n=4) and aortas (n=7) of 20 patients subjected to vascular surgical procedures, in addition to healthy control samples of the left interior thoracic artery and the left interior mammary artery of patients (n=6) undergoing coronary artery bypass surgery at the Divisions of Vascular and Cardiothoracic Surgery, Tampere University

Hospital. The details of this study have been described previously in more detail (Oksala et al. 2009). Genome-wide expression analysis (GWEA) was performed on all samples. The expression levels of IL-18 receptor α (IL-18Rα), IFN-γ, and IL-12 have been previously measured using real-time polymerase chain reaction (PCR) for a separate publication, and these measurements were incorporated into the data to obtain more accurate results (Oksala et al. 2009).

1.1.3.2 RNA isolation and genome-wide expression analysis (GWEA)

The fresh tissue samples (n=26) were immediately soaked in RNALater solution (Ambion Inc., Austin,TX, USA), and total-RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and the RNAEasy Kit (Qiagen, Valencia, CA, USA). The GWEA Microarray experiments were performed by using Sentrix® Human-8 Expression BeadChips analyzing over 23,000 known genes and gene candidates (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. BeadChips were scanned with the Illumina BeadArray Reader. The method has been more accurately described previously (Oksala et al. 2009). The accuracy of the Illumina Sentrix® Human-8 Expression BeadChips microarray methodology in measuring gene expression was verified by real-time quantitative TaqMan PCR through quantifying the expression of 20 genes with both methods. (Laaksonen et al. 2006)

1.2 Autopsy series

1.2.1 The Helsinki Sudden Death Study

1.2.1.1 Subjects

The Helsinki Sudden Death study (HSDS) is based on the autopsy data of 700 Finnish Caucasian men who had died suddenly out of hospital in the area of Helsinki (Mean age 53 years, range 33 to 70 years). The material includes two independent autopsy series. They were collected in 1981–1982 (A series, n=400) and 1991–1992 (B series, n=300). All the men were subjected to a medicolegal autopsy, because of their unexpected sudden death occurring outside a hospital and often unwitnessed, unless the deceased had suffered from a clinically diagnosed condition with a high probability of causing an untimely and sudden demise, such as severe chronic heart failure. The

HSDS material covers 25.3% of all the 2,850 deaths of 33–70-year-old males during the study period, covering 35.0% of all deaths which occurred due to ischemic heart disease in the area of Helsinki. The leading cause of death within this study population was cardiac causes (41%, n=288). Other causes of death were verified as other diseases (20%, n=140) and unnatural deaths resulting from accidents or suicides (39%, n=272). All medicolegal autopsies were performed according to the same protocol at the Department of Forensic Medicine at the University of Helsinki, and the study was approved by the Ethics Committee of the department/university.

1.2.1.2 Post-mortem verification and classification of varying causes underlying SCD and MI

The deaths caused by cardiac diseases were divided into two main categories by the autopsy findings – SCDs caused by CHD (80%, n = 220) and SCDs caused by non-coronary diseases in the absence of CHD (20%, n = 55). Most of the men with CHD died of SCD due to an MI (n = 154) (death due to acute M [n = 101], or/and arrhythmias associated with the scar of a prior MI [n = 53]), or CHD with no acute/prior MI (n = 64). Non-coronary SCDs were due to cardiomyopathy, hypertrophy or dilatation of the heart, or valvular diseases. MI was verified by a macroscopic and histological examination of the myocardium. Fibrous scar tissue of the myocardium was considered the diagnostic criterion for old MI and the presence of neutrophil granulocytes for an acute MI. When coronary arteries were opened longitudinally, the presence of a coronary thrombus was recorded.

1.2.1.3 Autopsy measurement of coronary stenosis and atherosclerosis

A silicon rubber cast was made from the three main epicardial coronary arteries (the left anterior descending, left circumflex and right coronary artery). The degree of coronary stenosis was determined from these rubber models. The cut-off value for the classification of CAD was over 50% stenosis in any part of one or several main coronary arteries. In order to analyze the areas of different types of atherosclerotic lesions and overall atherosclerosis, the coronary arteries were fixed in 10% buffered formalin and stained for fat by the Sudan IV staining method. The methods of these measurements have been described previously. (Mikkelsson et al. 2001)

1.2.1.4 Collection of risk factor data in the HSDS

A close friend, spouse or relative of the deceased was interviewed by means of a questionnaire to obtain risk factor data. Complete interview data on risk factors was available in 402 (60.6%) of the 663 cases whose IL-18 genotype was successfully determined. (Mikkelsson et al. 2001) Risk factor data included the following variables: hypertensive (yes/no), diabetic (yes/no), smoker (yes/no; smokers and ex-smokers were combined in to the category of smokers for the statistical analysis), daily alcohol consumption and body mass index (BMI). BMI was calculated from the autopsy data. The victim was determined hypertensive if his hypertension had been diagnosed clinically by a physician and/or he had been medically treated for hypertension, or if it was known that hypertensive blood pressure values had been measured from the subject prior to his death.

1.3 DNA isolation and genotyping methods

1.3.1 DNA isolation

In the clinical series, genomic DNA was extracted from peripheral blood leukocytes by using the QIAamp®DNA Blood Minikit and automated biorobot M48 extraction (Qiagen, Hilden, Germany). In the HSDS, the cardiac muscle tissue samples collected for the A series were stored as paraffin-embedded blocks, and the samples from the B series were stored frozen. The DNA extraction method from these samples has been described earlier (Isola et al. 1994).

1.3.2 Genotyping methods

Within a Caucasian population, 99% of the genetic variation of the IL-18 gene is covered by six haplotypes formed by five common tag SNPs (rs1946519, rs360717, rs549908, rs4937100 and rs5744292), or SNPs existing within the same bin with these five common haplotypes (SNPs in nearly complete association with each other) (Tiret et al. 2005). In the Cardiovascular Risk in Young Finns Study, and in the Finnish Cardiovascular Study, we genotyped the same polymorphisms. Genotyping of four SNPs (rs4937100, rs549908, rs360717, and rs1946519) was performed using Taqman® SNP Genotyping assays, as well as the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). For the SNP rs5744292, the genotyping was performed using a custom Taqman assay designed according to the Custom

TaqMan® Assays instructions and manufactured by Applied Biosystem's Custom TaqMan® Assays Service. In the HSDS and TVS, the promoter region SNP (rs187238) was genotyped using the same protocol, and the nucleotide sequences of the primers and the fluorogenic allele-specific oligonucleotide probes used in PCR were deduced from published sequences in the GenBank database; they were chosen and synthesized in co-operation with Applied Biosystems.

PCR reaction containing genomic DNA, 2 × TaqMan Universal PCR Master Mix, 900 nM of each primer, and 200 nM of each probe was performed in 384-well plates according to a standard protocol in a total volume of 5 μl. Water controls and random duplicates were used as a quality control. Parallel samples were genotyped to monitor genotyping errors. No errors were detected. A description of the principles of the 5'nuclease assay method can be found in a previous publication by Livak et al. (1999) (Livak 1999).

In the Cardiovascular Risk in Young Finns Study, the genotyping was successful in 94.8%–98.8% of the cases depending on genotype. Genotyping of the IL-18 SNP (rs187238) was successful for 663 (94.7%) of the 700 tissue samples (A series, 382 cases; B series, 281 cases) in the HSDS and 25 (96.2%) of the 26 blood samples in the TVS.

1.4 Statistical Analyses and methods

Haplotype analyses. The haplotype reconstructions were performed by PHASE software (Version 2.0.2) (Stephens and Donnelly 2003, Stephens et al. 2001) in studies x-y. The THESIAS software was used to analyze the risk factor-adjusted global association between the variation of the IL-18 gene and cardiovascular mortality as well as the occurrence of main branch CAD and all CAD in the Finnish Cardiovascular Risk Study (Tregouet and Garelle 2007). A p-value of < 0.05 was considered statistically significant. The computations were carried out with SPSS for Windows software (Version 14.0, SPSS Inc, USA). Values of significance on all non-parametric tests are presented as asymptomatic and 2-tailed. In all study settings (I–IV), analysis of variance (ANOVA) was used to evaluate the possible association between risk factors and the polymorphism of the IL-18 gene. Non-parametric tests such as the Mann-Whitney U and Kruskall-Wallis H test were applied if the continuous variable was not normally distributed. Categorical variables and Hardy Weinberg equilibrium were tested using the χ^2-test.

In study I (the Cardiovascular Risk in Young Finns Study), in order to investigate the possible association between IL-18 gene polymorphism and the subclinical markers of atherosclerosis, we applied adjusted analysis of variance (ANCOVA). Only significant covariates were accepted into the models (the procedure has been previously described in more detail) (Hernesniemi et al. 2008). First we studied the possible factor-by-sex interactions. If the interaction between the haplotype or SNP and sex was found significant, we proceeded to analyze the effect of the haplotype or SNP among men and women separately, and the results from the level of the whole study population were not interpreted. To study the effect of haplotypes, we divided the population into carriers and non-carriers. The effects of individual genotypes were studied without pooling the genotypes. Previously, pregnancy has been associated with changes in serum values of IL-18, and this tendency has been found to be modulated by IL-18 genotype (Ida et al. 2000, Kashef et al. 2008). For this, the analyses were performed before and after the exclusion of the 61 women who were pregnant during the time of the study. However, the results remained almost unchanged, and we therefore only present the results obtained from the whole study population. Variables with skewed distributions (insulin, glucose and CRP) were $\log_{10}$-transformed for the analyses.

The Li & Ji method was applied in order to correct for multiple testing (Li and Ji 2005). This method is the improved version of the method originally introduced by Nyholt et al. for adjusting for multiple testing in multilocus analyses (Nyholt 2004). This correction method was used because of the high linkage disequilibrium between the SNPs.

In study II (the Finnish Cardiovascular Study), Cox regression analysis was used to study mortality among different genotypes and haplotypes over the follow-up period. The analysis was adjusted with age, sex, use of beta-blockers, percentage of age-adjusted maximal heart rate reached as well as prior diagnoses of CHD, MI and diabetes. All covariates fulfilled the proportionality assumption based on correlations of survival rankings with Schoenfeld residuals. When studying the possible association between IL-18 gene variation and the expression of CAD, we used binary regression. We first studied whether the effect of specific genotypes would be sex-dependent. If a significant interaction was observed, the study population was stratified by sex. Otherwise, no stratification by sex was performed. The risk factors preliminarily

considered as covariates in each regression model were age, sex, diabetes, smoking, resting systolic blood pressure and hypercholesterolaemia. In all adjusted analyses, only significant covariates were accepted in the final models, as insignificant covariates were filtered out by backward elimination.

One of the IL-18 genotype distributions (533T>C, rs4937100) was not in Hardy Weinberg equilibrium in the study population of the Cardiovascular Risk in Young Finns Study ($p<0.001$). Nevertheless, we included it into our haplotype reconstruction in order to verify whether our haplotype frequencies were in line with those observed in previous studies (Tiret et al. 2005). This SNP was responsible for dividing one common haplotype (haplotype aCTA with a frequency of 0.173) into two smaller haplotypes (aCTAT, frequency 0.132 and aCTAc, frequency of 0.041) (See Table III below). Because the genotype distributions of the 533T>C SNP were not in Hardy Weinberg equilibrium, this SNP was excluded from further analyses, and only the effect of the combined aCTA haplotype was studied.

In the HSDS **(Studies III and IV)**, due to the small number of CC homozygotes (n = 43, 6.5%), they were combined with the GC heterozygotes to form a group of C allele carriers for statistical analyses. However, the analyses were also repeated without pooling the genotypes.

Logistic regression analysis was used to calculate the odds ratios (OR) for dependent variables according to genotype groups. Analyses were carried out with and without adjustment for autopsy data (body mass index [BMI] and age) and interview data (daily alcohol consumption, smoking, diabetes and hypertension). The control group consisted of men who had died of other diseases and men who had died of unnatural causes. This was included because the IL-18 genotype group did not associate with the rate of deaths caused by non-cardiac-related diseases or unnatural causes.

Binary logistic regression analysis was used to calculate the IL-18 genotype-by-risk-factor interactions. Analyses for each genotype group by risk factor interaction were carried out with and without adjustment for autopsy data (BMI and age) and interview data (alcohol consumption, smoking, diabetes and hypertension). If the interaction between IL-18 genotype group and a risk factor was found significant, the effect of the risk factor on the occurrence of SCD was studied separately using unadjusted and adjusted binary logistic regression analysis, stratifying the population by IL-18 genotypes. In all analyses, covariates were included in the model in a

stepwise manner. Only statistically significant ($p < 0.05$) covariates were accepted in the final adjusted model

In study IV (the Tampere Vascular study), the patients were divided into two groups: GG homozygotes and C allele carriers. This was done because only one patient carried two copies of the minor C allele of the promoter region -137 G/C SNP. The expression levels of IL-18 mRNA and IFN-γ mRNA were compared over IL-18 genotype groups, and patients with or without hypertension using ANCOVA. The study population was divided into two groups by median systolic arterial pressure (140 mmHg) in order to study the effect of hypertension on the dependent variables. All ANOVAs were adjusted with the mRNA expression of surface structures expressed by APCs (CD80 and CD86 transcript variant 1 [v1] and CD86 transcript variant 2[v2]) and by T cells (CD4, CD28, and CTLA-4) (Sansom 2000). This allowed us to compare the expression of IL-18 and IFN-γ between samples with different inflammatory background, and it was necessary because the current material consist of atherosclerotic samples from different vascular beds with variation in inflammatory background. The expression of the mRNA of these factors is subject to inflammatory stimuli (Sansom 2000). Therefore, they do not directly represent the quantity of the T cells or APCs in the samples. However, at the same time, they provide some adjustment on the inflammatory activity within the samples. These covariates were selected because macrophages, which also act as APCs, are major producers of IL-18, and T cells are major producers of IFN-γ (Okamura et al. 1995). Only significant covariates were selected into the model by means of a backward elimination procedure. All analyses were repeated with log-transformation of the continuous variables, but this did not improve the predictive value of the analyses (measured by pseudo R^2-value), which is why the results of the analyses performed with crude values are reported.

RESULTS

1.1 Genotype distributions of the IL-18 gene in different study populations

In the Finnish Cardiovascular Risk Study and the Cardiovascular Risk in Young Finns Study, we determined 5 tag SNPs (rs1946519, rs549908, rs360717, rs5744292, rs4937100. All of the genotype distributions were in accordance with the Hardy-Weinberg equilibrium, except for the rs4937100 SNP which, in both series, showed a significant deviation from the equilibrium ($p<0.0001$). Nevertheless, it was included it in the haplotype reconstruction in order to verify whether our haplotype frequencies were in line with those observed in previous studies, but it was excluded from further analyses. This SNP was responsible for dividing one common haplotype (haplotype ACTA with a frequency of 0.17, see table 5.) into two smaller haplotypes (see original publication I for more details). The haplotypes constructed from these tag SNPs as well as their frequencies were in accordance with the results of previous studies (Barbaux et al. 2007, Tiret et al. 2005). In brief, we found that the IL-18 gene has 5 common haplotypes (CTCA, AGTA, CTCG, ATCA and CGCA [underlined letters denote minor alleles of the specific SNP]). The haplotype AGTA was the only one carrying the minor T allele of the +127 (C/T) polymorphism (rs36017), and only the CTCG haplotype carried the minor G allele of the +415 (A/G) polymorphism (rs5744292).

In the HSDS, only the promoter region -137 (G/C) (rs187238) SNPs were genotyped due to the quality and quantity of the tissue samples. This polymorphism is in complete association with the +127 (C/T) polymorphism (rs360717), which was genotyped in the clinical series. Of all the men, 359 (54.1%) had the wild-type GG genotype, 261 (39.4%) were heterozygotes (CG), and 43 (6.5%) were CC homozygotes. The allelic frequency of the C allele was 0.262 and of the G allele 0.738. The genotype distributions in the TVS were: 15 (60%) GG homozygotes, 9 (36%) GC heterozygotes and 1 (14%) CC homozygote. In both studies, the genotype distribution was in line with the Hardy-Weinberg equilibrium.

Table 4. The genotype distributions among the study populations of the Cardiovascular Risk In Young Finns Study (I) and The Finnish Cardiovascular Risk Study (II). The topmost line of each genotype denotes the percentage of the population (number of subjects in brackets) with two copies of the wild-type allele. The following line denotes heterozygotes and the lowest line homozygotes carrying two mutated copies of the gene.

Study	Single Nucleotide Polymorphism									
	rs1946519 -656 (C/A)*		rs549908 +35 (T/G)		rs360717 +127 (C/T)*		rs5744292 +415 (A/G)		rs4937100 +533 (T/C)**	
I	30.0%	(673)	47.2%	(1062)	50.8%	(1125)	57.6%	(1204)	53.4%	(1195)
	47.1%	(1065)	42.9%	(966)	40.6%	(899)	36.6%	(766)	31.4%	(704)
	22.5%	(506)	9.9%	(223)	8.5%	(189)	5.8%	(121)	15.2%	(340)
II	32.1%	(683)	50.1%	(1058)	54.2%	(1151)	54.6%	(1156)	52.6%	(1076)
	48.7%	(1037)	41.1%	(868)	38.7%	(821)	38.8%	(823)	31.3%	(640)
	19.2%	(410)	8.8%	(185)	7.1%	(150)	6.6%	(140)	16.0%	(328)

*Genotype distribution significantly different ($p<0.05$) between the two study populations. **Genotype distributions not in Hardy-Weinberg equilibrium.

Table 5. The IL-18 gene haplotypes and their observed frequencies among the study populations of the Cardiovascular Risk in Young Finns Study (I) and The Finnish Cardiovascular Risk Study (II). Underlined letters denote the minor allele of the individual SNPs.

	Frequency	
	Study I	Study II
CTCA-haplotype	0.270	0.275
AGTA- haplotype	0.288	0.266
CTCG-haplotype	0.244	0.261
ATCA-haplotype	0.173	0.171
CGCA-haplotype	0.024	0.027

1.2 IL-18 gene polymorphism and subclinical markers of atherosclerosis (Study I)

According to adjusted ANOVA, there was a significant difference between men and women in the ways in which the CCTG haplotype associated with carotid artery IMT (p=0.011 after correcting for multiple testing). This analysis was adjusted with sex, BMI, age, smoking and geographical origin. The population was therefore stratified by sex. Among men, carriers of the CCTG had a significantly lower mean IMT when compared to the non-carriers (adjusted difference in means: -0.016mm 95% CI from -0.028 to -0.004, p=0.014 after correcting for multiple testing). The adjusted mean values by haplotype group were: 0.601mm (standard error [S.E.] 0.004mm) for non-carriers, 0.585mm (S.E. 0.005mm) for heterozygous carriers and 0.596mm (S.E. 0.014mm) for homozygous carriers of the CCTG haplotype. The difference in mean IMT between heterozygous and homozygous male carriers of this haplotype was not significant (corrected p=1.00). As the carriers of the CTCG haplotype are the only ones carrying the minor G allele of the +415 C/g SNP (rs5744292), it is clear that this allele is responsible for the significant difference between the haplotype groups. Among women we did not observe any significant differences (0.005 mm, 95% CI from -0.004 to 0.014, corrected p=0.498) between carriers and non-carriers of the CTCG haplotype.

None of the other haplotypes or SNPs associated significantly with IMT among the whole study population, and no other significant haplotype-by-sex-interactions were seen. None of the haplotypes or studied SNPs associated significantly with CAC or brachial artery FMD in the whole study population, and we did not observe any significant haplotype-by-sex-interactions.

We also found that the frequencies of the minor alleles of the +35 (T/g) SNP (p=0.005) and +127 (C/t) SNP (p=0.040) were higher among subjects with Eastern Finnish origin when compared to those originating from Western Finland. As geographical origin in Finland has been previously associated with the development of atherosclerosis, we also repeated all the analyses after excluding geographical origin form the covariates. The exclusion did not alter the results, and the observed associations remained significant.

1.3 IL-18 gene polymorphism and the expression of angiography-verified CAD (Study II)

According to the adjusted global association analysis, the genetic variation did not associate significantly with the occurrence of main branch CAD, when all five of the most common haplotypes were considered in the analysis (p=0.772), or with CAD defined as over 50% stenosis in any part of the coronary arteries (p=0.847). However, according to the interaction analyses executed with the binary regression method, one haplotype seemed to associate differently among men and women in the occurrence of main branch CAD (adjusted p=0.033 for the interaction). Among men, the carriers of the AGTA haplotype had a lower risk for main branch CAD (adjusted OR 0.576, 95% CI 0.340–0.978, p=0.041). Among women no significant difference was observed in the occurrence of main branch CAD (adjusted OR 1.562, 95% CI from 0.669–3.648, p=0.303). Other significant interactions were not found, and none of the haplotypes or SNPs were found to associate significantly with main branch CAD or with CAD in any part of the coronary arteries in the whole study population.

1.4 IL-18 gene polymorphism and cardiovascular mortality in a Finnish patient population (Study II)

According to the global association analysis, mortality from cardiovascular causes during the follow-up was not associated with IL-18 gene polymorphism, when all the five most common haplotypes were accounted for (p=0.344). Consistently, none of the studied SNPs or haplotypes associated individually with mortality during the follow-up according to Cox-regression analysis. The hazard ratios corresponding to the carriage of the haplotypes were (in comparison to the non-carriers): HR 1.122 for CTCA (p=0.588), HR 0.806 for AGTA (p=0.307), HR 1.355 for CTCG (p=0.144), HR 0.803 for ATCA (p=0.353), and HR 0.928 for CGCA (p=0.924).

1.5 IL-18 gene polymorphism and the occurrence of SCD (Study III)

According to the results of the autopsy series, the C allele carriers had a lower risk of SCD than GG homozygotes (50.3% of the men in the control population where C allele carriers, whereas the corresponding proportion among SCD victims was 36.9%; crude OR 0.65; 95% CI 0.48–0.89, p=0.007; and adjusted OR 0.49; 95% CI 0.31–0.77; p = 0.002). Furthermore, the C allele carriers had a significantly lower risk of both SCD due to CHD (adjusted odds ratio, 0.51; 95% CI 0.32–0.82; p = 0.005) and SCD caused by non-coronary heart diseases (adjusted odds ratio, 0.34; 95% CI 0.13–0.90, p = 0.030) when compared to the GG homozygotes. The results persisted when the analysis was limited to different sub-types of SCD caused by coronary atherosclerosis (Table 6).

In order to test whether the risk of SCD was age-dependent, we used regression analysis to analyze the possible age-by-genotype interactions affecting the risk of different subtypes of SCD. Most of these associations were independent of age (data not shown). However, there was a significant age-by-genotype interaction with the risk of SCD due to MI (crude and adjusted interactions p = 0.028/0.037). Therefore, we formed two categories of equal size by dividing the overall study population by the mean age (53 years). In the younger age group (age < 53 years), the C allele was strongly protective against SCD due to MI: of the 35 died men, 26 (74.3%) were GG homozygotes and only 9 were (25.7%) C allele carriers (crude odds ratio, 0.34; 95% CI 0.14–0.68; p = 0.004; and adjusted odds ratio, 0.16; 95% CI 0.05–0.51; p = 0.002). In the older age group, 68 (57.1%) of the 119 men who had suffered an SCD due to MI were GG homozygous and 51 (42.9%) were C allele carriers. This difference was not statistically significant.

Table 6. IL-18 -137 G/C (rs187238) genotype group frequencies among men who had suffered different subtypes of SCD or died of other causes, with corresponding adjusted odds ratios (OR)

	n	CG/CC	GG	Adjusted OR (95% CI)***	p Value
Controls*	388	50.3%	49.7%		
SCD not due to CHD**	55	32.7%	67.3%	0.34 (0.13-0.90)	0.030
CHD	220	41.4%	58.6%	0.51 (0.32-0.82)	0.005
MI aetiology	154	39.0%	61.0%	0.48 (0.28-0.81)	0.006
AMI	101	36.6%	63.4%	0.50 (0.28-0.90)	0.021
AMI with thrombus	58	32.8%	67.2%	0.40 (0.18-0.87)	0.021

*Death due to non-cardiac diseases or unnatural deaths.

**Death due to cardiomyopathy, cardiac hypertrophy or dilatation, or to valvular disease in the absence of CHD.

***model adjusted with age, body mass index, smoking, hypertension, diabetes and daily alcohol consumption

Abbreviations: IL-18, interleukin 18; CHD, coronary heart disease; SCD, sudden cardiac death; (A)MI, (Acute)myocardial infarction.

To verify our results, we studied the association between IL-18 genotype and SCD separately in both of the independent autopsy series which formed the entire study population. These autopsy series were collected ten years apart (please see the Material and Methods section for the description of the autopsy series). The C allele carriers seemed to be underrepresented among SCD victims in both independent autopsy series. In the A series, the difference was statistically significant ($p = 0.039$ by two-sided exact Pearson's chi-square test). In the B series, there was a similar but not significant trend ($p = 0.085$ by two-sided exact Pearson's chi-square test) (Figure 3.). IL-18 genotype associated with the occurrence of various phenotypes of SCD similarly in both series, but the associations were not significant due to smaller sample sizes. The major conclusions are therefore based on combined data from series A and B.

1.6 The interactions between risk factors and IL-18 gene polymorphism on the risk of SCD and CAD (Study IV)

According to unadjusted binary logistic regression analysis, IL-18 genotype group interacted significantly with hypertension (crude p = 0.009 and adjusted p = 0.011) as regards the effect on the risk of SCD, and this association persisted even after applying the Bonferroni correction (crude p = 0.045 and adjusted p = 0.055). When the IL-18 genotype information was entered in the analysis without pooling CG heterozygotes and CC homozygotes, the result remained significant (crude p = 0.022 and adjusted p = 0.029). Interactions with daily alcohol consumption (p = 0.600), BMI (p = 0.075), smoking (p = 0.998), or diabetes (p = 0.943) were not statistically significant.

Similarly, in unadjusted binary regression analysis of the occurrence of CAD, there was a statistically significant interaction between IL-18 genotype group and hypertension (p = 0.020), which remained significant after adjustment for other risk factors (p = 0.026). After Bonferroni correction, the interaction was no longer significant (crude p = 0.100 and adjusted p = 0.130). Interactions with daily alcohol consumption (p = 0.701), BMI (p = 0.833), smoking (p = 0.805), and diabetes (p = 0.316) were not statistically significant.

Because of the statistically significant genotype-group-by-hypertension interaction, we divided the study population according to IL-18 genotype in order to study the effect of hypertension separately by IL-18 genotype.

1.7 The effect of hypertension on the expression coronary atherosclerosis among IL-18 genotypes (Study IV)

In order to study how hypertension affects atherosclerotic plaque formation and composition among IL-18 genotype groups, we focused on the control group. This was done to avoid the evident selection bias presented by the significantly different mortality due to CHD-related SCD among the different IL-18 genotype groups. Among GG homozygotes, according to ANOVA adjusted with autopsy data (BMI and age), the coronary arteries of hypertensive men were more afflicted by overall atherosclerosis, and the relative surface areas of both fatty streaks and fibrotic plaques were larger when compared to normotensive men (Table 7). According to adjusted regression analysis, hypertension was also a significant risk factor for the occurrence of complicated plaques (OR 8.38 with 95% CI 2.39–29.33, p < 0.001; covariates: BMI

and age). Among C allele carriers, hypertension was not associated with the overall atherosclerotic burden (Table 7). In addition, when the groups of GC heterozygotes and CC homozygotes where analyzed separately, hypertension did not associate significantly with plaque areas or the occurrence of complicated plaques.

Furthermore, hypertension was a major risk factor of CAD among GG homozygotes (adjusted OR 3.08; 95% CI 1.48–6.41, $p < 0.003$) but not among the combined group of C allele carriers (OR 0.78; 95% CI 0.32–1.89, $p = 0.581$), nor among GC heterozygotes (OR 0.83; 95% CI 0.32-2.11, p=0.687) or CC homozygotes (p=1.00 evaluated by Fisher's exact χ^2-test because only 5 men had suffered from hypertension, and only two of them had CAD).

Table 7. The effect of hypertension on the relative surface area of the coronary arteries covered by atherosclerotic lesions among different IL-18 genotype groups. P-values were determined by using analysis of variance adjusted with age and body mass index.

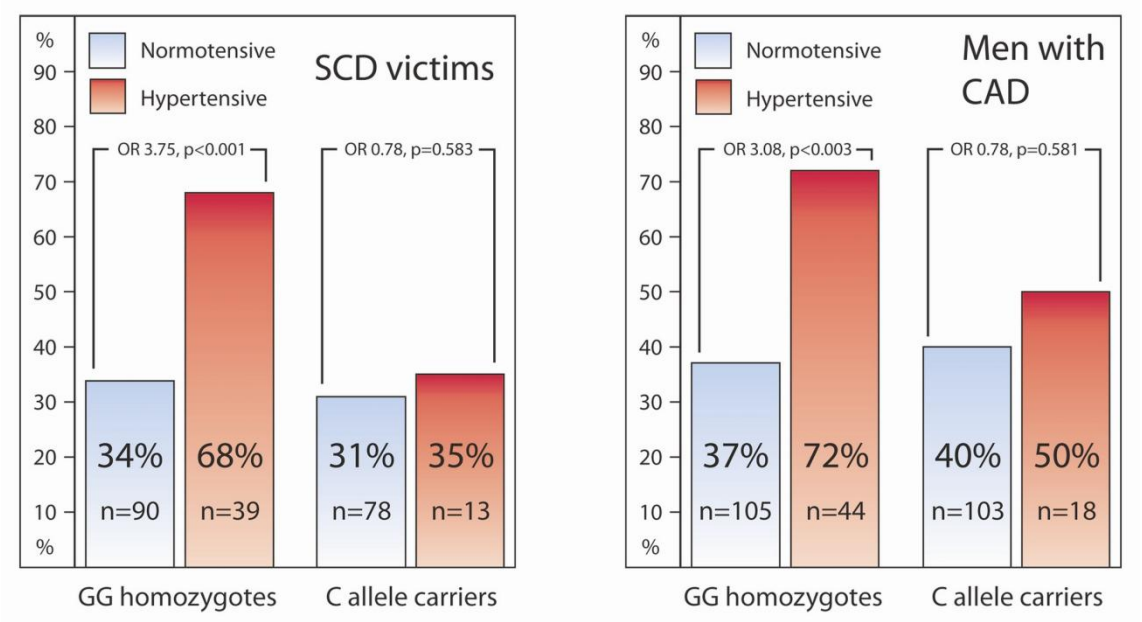
	GG homozygotes		p-value	C allele carriers		p-value
	SBP <140	SBP <140		SBP <140	SBP >140	
Fatty streak	7.1% (1.3)	4.2% (0.4)	0.026	6.8% (1.0)	5.2% (0.5)	0.268
Fibrotic plaque	4.6% (0.7)	2.0% (0.2)	<0.001	2.5% (0.6)	3.2% (0.4)	0.268
Overall lesion area	11.7% (1.7)	6.2% (0.5)	0.002	9.3% (1.1)	8.4% (0.7)	0.701

Abbreviations: SBP, systolic blood pressure.

1.8 The effect of hypertension on the risk of SCD among IL-18 genotypes (Study IV)

Hypertension was a major risk factor for SCD and CAD among GG homozygotes, but not among the combined group of C allele carriers, nor among GC heterozygotes or CC homozygotes, when the genotypes were analyzed separately. Among GG homozygotes, hypertension associated significantly with SCD due to CHD (adjusted OR 3.75; 95% CI 1.78–7.91, p = 0.0005), SCD due to old or acute MIs (sudden cardiac death due to arrhythmias caused by an old MI scar and/or AMI) (adjusted OR 4.56; 95% CI 2.05–10.11, p = 0.0002), and with fatal acute MI (adjusted OR 4.69; 95% CI 2.01–10.95, p = 0.0004). Among C allele carriers, hypertension was not associated with higher risk of SCD due to CHD (adjusted OR 0.78; 95% CI 0.32–191, p = 0.583), SCD due to old or acute MIs (sudden cardiac death due to arrhythmias caused by an old MI scar and/or AMI) (adjusted OR 0.88; 95% CI 0.32–2.40 p = 0.805), and fatal acute MI (adjusted OR 1.14; 95% CI 0.39–3.37, p = 0.814).

Figure 3. The effect of hypertension on the risk of Sudden Cardiac Death (SCD) and Coronary Artery Disease (CAD) among GG homozygotes and C allele carriers of the IL-18 gene -137G/C polymorphism. Odds ratios (OR) are derived by logistic regression adjusted with traditional risk factors for CAD.



1.9 The effect of IL-18 genotype and hypertension among IL-18 genotype groups on the expression of IL-18 and IFN-γ mRNA in atherosclerotic arterial samples (Study IV)

According to adjusted ANOVA, the effect of hypertension on the expressions of IL-18 mRNA and IFN- γ mRNA were modulated by the IL-18 genotype ($p = 0.030$ [IL-18] and $p = 0.004$ [IFN- γ] for the interactions). Among GG homozygotes, hypertension did not associate with the expression level of intracellular precursor IL-18 mRNA (0.89 fold increase, $p = 0.217$), whereas among C allele carriers hypertension augmented the expression of intracellular precursor IL-18 mRNA (1.31 fold increase, $p = 0.001$). Almost inversely, among GG homozygotes, hypertension associated with a higher expression level of pro-atherosclerotic IFN-γ (1.58 fold increase, $p = 0.006$), whereas hypertension in C allele carriers seemed to associate with a lower expression level of IFN-γ (0.58 fold increase, $p = 0.047$).

In the atherosclerotic samples obtained from GG homozygotes, the expression levels of the intracellular precursor IL-18 mRNA were lower (0.68 fold increase, $p < 0.001$), but the expression levels of IFN-γ mRNA were higher (1.85 fold increase, $p < 0.001$), when compared to the atherosclerotic samples of the C allele carriers. Furthermore, according to the unadjusted Mann-Whitney U-test, the expression levels of IL-18 mRNA seemed to be significantly higher in the healthy control samples when compared to the atherosclerotic samples (1.33 fold increase, $p = 0.052$).

Significant covariates associating with the expression of IL-18 mRNA were: CD80 ($p = 0.006$), CD86v1 ($p = 0.012$), CD4 ($p = 0.013$), CD86v2 ($p = 0.010$), Caspase-1 variant α ($p = 0.023$), and Caspase-1 variant ε ($p = 0.009$). The expression levels of Caspase-1 (Casp-1) variants were also introduced to this model, because Casp-1 cleaves the IL-18 precursor protein into biologically active IL-18, resulting in the secretion of mature IL-18 protein. This intrinsic processing is likely to affect the amount of intracellular IL-18 precursor mRNA, which has a stable mRNA structure and is constitutively and intracellularly stored (Nakanishi et al. 2001).

Significant covariates associating with the expression of IFN-γ mRNA were: CD80 ($p < 0.001$), CD86v1 ($p < 0.001$), CD4 ($p = 0.014$), CTLA-4 ($p = 0.010$), IL-12 ($p = 0.004$), and IL-18Rα ($p = 0.003$). The expression levels of IL-12 and IL-18Rα were also included in this model, because IL-12 in synergy with IL-18 augments the production of IFN-γ, and IL-18Rα plays an important role in IL-18 signalling

(Nakanishi et al. 2001). The expressions of IL-18 binding protein, Casp-1vα and Casp-1vε were also added to the analysis, but these covariates were not significant in the model and were not used for further adjustment.

DISCUSSION

1.1 Study populations

1.1.1 The Cardiovascular Risk in Young Finns Study

In the Cardiovascular Risk in Young Finns Study, the original population was randomly selected from the national population register to include different parts of Finland (from urban areas as well as from rural areas) and equal numbers of participants from both sexes. The initial sampling consisted of 4,320 subjects aged 3, 6, 9, 12, 15 and 18. A total of 3,596 subjects (83.2%) participated. In 2001, data was collected for a 21-year follow-up. A total of 2,283 subjects participated (63.5% of the original study cohort). Both the sample of participants in 1980 and the follow-up sample of 2001 were concluded to be representative of the original randomly selected population because of the high participation rate, and because there did not seem to be any systematic reason of for non-participation in 1980 and, finally, because there was no difference in the baseline risk factors (measured in 1980) between dropouts and participants (Akerblom et al. 1985, Juonala et al. 2004b).

Based on the fact that the study population can be considered a good representative of a population of Caucasian healthy adults, this multicentre study provides a good population for genetic studies. Also, the relatively large study population (n=2,283) secures enough power for the study. We can also presume that the genotypic distributions obtained from this population represent the genotypic distribution of the general population in Finland. Furthermore, within this population, the effect of other risk factors has been very extensively studied, and it is therefore easy to size the associations between the genetic risk factors and the end-points.

1.1.2 The Finnish Cardiovascular Study

In the Finnish Cardiovascular Study, the population comprises patients (men and women) who have undergone a clinical exercise stress test for diagnostic indications at Tampere University Hospital. This ongoing study is designed to construct a risk profile of individuals at high risk of cardiovascular diseases, events and deaths. The results of the present study are based on the data collected between October 2001 and December 2004. By the end of 2004, 2,152 patients had been included in the study population. The study population in itself is fairly heterogeneous due to the selection criteria. The

indications for the clinical exercise stress test were diagnosis of CHD (frequency 44.6%), testing vulnerability to arrhythmia during exercise (21.6%), evaluation of working capacity (n=18.0%) and adequacy of drug therapy (15.3%), as well as obtaining an exercise profile prior to surgery (13.2%) and post MI (n=7.9%). Some of the patients had multiple indications. Furthermore, the patients in the study population come from different backgrounds with varying lifestyles. They also have different medical histories and the age spectrum is wide. For this reason, the study setting is challenging for the study of associations between individual genetic factors and mortality to cardiovascular causes. A genetic factor may be a clear risk factor for one set of patients but not for others. Unfortunately, the study population is still relatively small for division into subpopulations. However, as the study is ongoing, more patients are being recruited and the follow-up time is increasing. Eventually, the study will provide a clear setting for genetic risk stratification also in sub-populations. In the present study population, the mortality rate was also not very high, weakening the power of the study (during a follow-up of 6.3 years, 5.3% of the patients had died of cardiovascular causes).

Coronary angiography was performed on a subset of patients (21.4%) for clinical and interventional indications. This patient population is highly selected, and the expression of CAD is thus likely to be similar despite the differences in the genetic background of the patients. However, as the number of patients in this sub-population increases, significant genetic factors will most likely become evident, and the risk factor profile should be different between patients with different backgrounds.

Despite these limitations, the Finnish Cardiovascular Study is unique, because it incorporates extensive exercise data with other clinical variables and genetic markers. It includes extensive data on the demographics (i.e. age, sex, body mass index), lifestyle factors and, most importantly, the exposure to classical cardiovascular risk factors, such as smoking, hypertension, diabetes, occurrence of hyperlipidemia and family history. Medications and prior diagnoses of CHD and MIs are also recorded. In conclusion, the study setting allows adjustment of all the analyses with extensive risk factor data.

1.1.3 The TVS

The TVS comprises atherosclerotic samples from different arteries (carotid arteries, femoral arteries and the aorta). The healthy control samples were obtained from

different arteries (interior mammary arteries and interior thoracic arteries) during coronary artery bypass surgeries. The study is limited due to the fact that the patients were of different sexes and had very different backgrounds and medications. Furthermore, the vascular samples were obtained from different arteries, which limits the power of the study not only because the hemodynamic environments are different, thus possibly modifying the atherosclerotic process, but also because it is clear that lesions in different sites may have different properties. In fact, within the samples of the present study, the expression levels of the mRNA of factors expressed on T cells and APCs were very different (data not shown). However, we were able mitigate this problem by adjusting the statistical analyses with these factors, allowing us to provide some adjustment of the inflammatory activity in the samples. Currently, this study material is being expanded to include more samples, and in the future the diversity of the samples will become an advantage, as it will be possible to explore the expression patterns of candidate pathways in different vascular beds. The fact that all atherosclerotic samples were of advanced plaques with similar histology (Classes IV and V according to the AHA/Stary classification) is another advantage. Furthermore, it is important to bear in mind that the study data allows us to observe the expression patterns directly on the human artery wall level.

1.1.4 The Helsinki Sudden Death Study

The HSDS provides a good material for studying the possible associations between genetic and environmental factors and the occurrence of SCD among men, as well associations with autopsy-verified atherosclerotic lesion areas in different arterial beds (coronaries, aorta). This is due to the fact that this study includes nearly all SCDs which occurred in the Helsinki area during the time of the autopsy series and, therefore, the study population is quite representative of the population at risk of SCD. Also, the proportion of SCD among the entire study population is extremely high (41%), thus making the study sensitive for candidate gene studies. The study setting also provides an opportunity to study the effects of different factors on the development of coronary atherosclerosis in a population with no survival bias. Furthermore, by studying the development of coronary atherosclerosis among the control group (men who had died of unnatural causes or diseases unrelated to cardiac causes), we can circumvent the obvious selection bias if the suspected risk factor has also affected the occurrence of SCD. As the study population only comprises men, the results are not

directly applicable to women.

One obvious limitation, besides the fact that there are no measurements of the cholesterol levels of the deceased, is that we were unable to obtain interview data on all subjects. The risk data concerning diabetes, hypertension, smoking and alcohol consumption therefore did not cover the entire population. We also lack data on possible pre-mortem medications of the deceased. Furthermore, the validity of the risk factor data derived by interviewing a close friend, spouse or relative cannot be considered as high as that of clinical measurements or an interview of the subject himself. Fortunately, though, the validity of interview data concerning, for example, hypertension is high (Wu et al. 2000).

Although the study setting is challenging, its strength lies in the unambiguous post-mortem verification of the cause death and the accuracy of the data concerning the development of coronary atherosclerosis on the vessel wall level. By using vulcanized rubber models of the arteries, we were able to determine the degree of stenosis accurately. The presence of thrombus in the coronary arteries was also verified, and we were able to ascertain the presence of old infarction scars and the presence of AMI reliably by examining the myocardium.

1.2 IL-18 gene variation and subclinical atherosclerosis

The genetic variation of the IL-18 gene was not found to associate with CAC or FMD in a population of young healthy adults. The present study is the first one to explore the possible connection. One recent study linked circulating IL-18 levels inversely with carotid femoral pulse wave velocity but not with FMD among men. The observed association was independent of risk factors (Vlachopoulos et al.). We did not perform any sex stratification, because we did not observe any significant interactions with IL-18 genotype and sex influencing CAC or FMD. Furthermore, the mean age of the previous study population was significantly higher (58 years). If, indeed, there were any connection between IL-18 gene polymorphism and CAC, it could perhaps present itself in a risk population with higher sub-clinical atherosclerotic burden.

Interestingly, we did find that one major haplotype of the IL-18 gene (also the only one carrying the minor G allele of the +415 C/G SNP) associated with lower IMT values. The scale of the difference was comparable or even more evident than that of many traditional risk factors (such as the 1 standard deviation [14.4mmHg] in systolic

blood pressure, sex and smoking). Among women, we observed no significant association. Although many studies have already linked circulating IL-18 levels with IMT, only one has examined the possible association between IL-18 gene polymorphism and carotid atherosclerosis (Chapman et al. 2006, Li et al. 2009, Thompson et al. 2007a, Vlachopoulos et al., Yamagami et al. 2005). In a recent report by Thompson et al., the authors suggested that haplotypes of the IL-18 gene did not associate with carotid IMT, but they did find that one haplotype, which also associated with higher IL-18 levels among men with CHD, seemed to associate with a higher risk of the presence of a carotid artery plaque in ultrasound (Thompson et al. 2007a). Based on the carriage of SNPs inherited in the same BINs, and on the frequency of this haplotype, we can deduct that it most likely corresponds with the ATCA haplotype (frequency 0.13 in Study I and 10% in the study by Thompson et al.). We did not observe any significant findings related to this haplotype. However, the observed association by Thompson et al. was of borderline significance, and even they did not find this haplotype to associate significantly with circulating levels of IL-18 among healthy adults.

1.3 IL-18 gene polymorphism and mortality from cardiovascular causes

In the present study, we did not observe any significant association between cardiovascular mortality and IL-18 gene polymorphism among a Finnish patient population. One previous study has linked IL-18 gene polymorphism with the CV mortality among CAD patients and the occurrence of MI among a combined group of hypertensive patients and postmenopausal women (Bis et al. 2008, Tiret et al. 2005). The haplotype linked with lower risk for cardiovascular mortality by Tiret et al. is the only one that carried the minor G allele of the +183 A/G SNP. However, our results are not necessarily contradictory. Unfortunately, our study lacks the power to conclude that there is no association between IL-18 gene polymorphism and cardiovascular mortality among a Finnish patient population. The whole study population is very heterogeneous comprising patients with different indications for exercise test, and many of the patients in our study population were not high-risk individuals. The overall mortality from cardiovascular causes was therefore not high. This reduces the power of the current study, and we cannot conclude that the variation in the IL-18 gene indeed does not associate with cardiovascular mortality. For example, in comparison to our study,

the mortality for cardiovascular causes was clearly higher in the study by Tiret et al. They followed 1,299 CAD patients for a median follow-up period of 5.9 years. During that time, the mortality rate was 11% (Tiret et al. 2005). Our follow-up time was similar, but the mortality rate reached only 5%.

1.4 IL-18 polymorphisms and the expression of angiography-verified CAD

In Study II we found that the AGTA haplotype associated, independently of risk factors, with lower risk of angiography-verified main branch disease among men. This haplotype is the only one carrying the minor T allele of the +127 C/T polymorphism. Furthermore, it is the only one carrying the minor C allele of the -137 G/C SNP, because these two are inherited simultaneously in the same haplotype. Previous studies have repeatedly shown that the -137 G/C SNP is functional. Therefore, it is most likely that the observed significant association between the AGTA haplotype is due to the promoter region -137 G/C polymorphism. As the C allele is associated with diminished transcriptional activity of the gene and diminished production of mature IL-18, it is plausible to suggest that the carriers of the C allele are less prone to develop occlusions in the main branches of their coronary arteries. However, we did not find that the variation in the IL-18 gene is associated with the occurrence of angiography-verified CAD. This is due to the fact that none of the haplotypes showed significant deviation from the occurrence of CAD in reference to the most common haplotype among the entire study population. Even if the variation in the IL-18 gene did affect the development of CAD, the effects would have to be very specific in order for them to be seen among angiography patients who have been selected to the procedure according to a strict diagnostic evaluation. In a sub-population such as this one, the effect expression of CAD is likely to be similar, despite the differences in the genetic background of the patients.

1.5 IL-18 gene promoter region functional -137 G/C SNP and the risk of SCD

In Study III we found that a functional IL-18 gene promoter region -137 (G/C) SNP (rs187238) associates with the occurrence of sudden cardiac death among men. The minor C allele of the SNP associated with lower risk of SCD due to CHD and with SCD due to other causes in the absence of CHD. A large haplotype study Tiret et al.

showed that the variation in the IL-18 gene associates with CV mortality during follow-up (Tiret et al. 2005). Our findings support the result. However, in the study by Tiret et al. found that only one haplotype associates independently with CV mortality. The same haplotype also associated with decreased circulating IL-18 levels. This haplotype is the only one carrying the minor G allele of the +183A/G polymorphism. The authors hypothesized that this polymorphism, located in the 3'untranslated region, potentially affected mRNA stability, thus exerting its effect. In the present study, we did not genotype this SNP and are therefore unable to ascertain whether it is related to the occurrence of SCD. What we do know is that the minor G allele of the SNP does not co-exist in the same major haplotype with the minor C allele of the -137 polymorphism. Therefore, as the haplotype carrying the minor G allele of the +183 (A/G) SNP is also associated with attenuated production of IL-18, it only emphasizes our results because among GG homozygotes of the -137G/C polymorphism, there are more G allele carriers of the +183A/G polymorphism than among the C allele carriers (Barbaux et al. 2007, Tiret et al. 2005).

The association between the -137 (G/C) SNP and the risk of SCD due to causes not related to CHD (cardiomyopathy, hypertrophy, or dilatation of the heart) is first of its kind to be reported. Although there is no prior evidence of the involvement of IL-18 genetics in this field, it has been previously shown that patients with congestive heart failure have higher circulating IL-18 levels when compared to controls, and that higher IL-18 levels associate with the clinical outcome of heart failure (Mallat et al. 2004, Naito et al. 2002, Seta et al. 2000). One possible source for IL-18 in the myocardium alongside macrophages is cardiomyocytes. Cardiomyocytes can produce IL-18 in response to stimulation by TNF-α, aldosterone, endothelin-1 and angiotensin II (Chandrasekar et al. 2003, Doi et al. 2008). In the myocardium, IL-18 has many possible ways for aggravating the situation. IL-18 induces the production of anti-natriuretic peptide from myocytes and can thus also stimulate hypertrophy of the myocardium (Chandrasekar et al. 2005, Seta et al. 2000).

1.6 The interplay between IL-18 gene polymorphism and hypertension

In Study IV we showed that the promoter region -137 G/C SNP significantly modulates the effect of hypertension on the risk of SCD due to CHD. The study aimed to clarify the possible mechanism by which this promoter region polymorphism affects the risk

of SCD as observed in our previous study. Other risk factors were not seen to interact with IL-18 polymorphism, which is why further investigation was focused on the effect of hypertension in different IL-18 genotype groups. As we divided the study population into wild-type GG homozygotes and carriers of the C allele, we found that hypertension was a major risk factor among GG homozygotes for SCD due to CHD, as well as for CAD and the development of coronary atherosclerosis. Among C allele carriers, hypertension did not associate with any of these end-points. In publication IV, we have already discussed the possible mechanisms by which this promoter region polymorphism interacts with hypertension. In brief, many factors intimately associated with hypertension, such as β2-receptor activation, aldosterone and angiotensin II, may induce IL-18 expression and modulate the whole IL-18 pathway (causing changes in IL-18mRNA stability and IL-18 receptor expression) (Chandrasekar et al. 2004a, Chandrasekar et al. 2004b, Doi et al. 2008, Sahar et al. 2005). It is also possible that the simple overall pro-atherogenic properties of hypertension have less severe effects on those with impaired IL-18 response (e.g. C allele carriers of the -137 G/C SNP) (Ross 1999).

We also observed, in an independent material, that hypertension has very different effects on the expression of IL-18 and IFN-γ mRNA in human atherosclerotic vascular samples when comparing GG homozygotes to C allele carriers. Among GG homozygotes, hypertension associated significantly with higher expression of IFN-γ mRNA, whereas among C allele carriers the effect seemed contrary. Interestingly, in the samples obtained from hypertensive GG homozygotes, the expression levels of IL-18 did not significantly differ from those of the corresponding samples obtained from GG homozygous subjects with no hypertension. Among C allele carriers, hypertension associated with higher expression of IL-18. Based on this data, it is impossible to deduct a causative mechanism between these two findings. IL-18 mRNA must be processed before it is secreted in an active form, and although we controlled for Caps-1 mRNA expression levels, the results remained the same. However, Casp-1 must also be cleaved into active form before it becomes biologically active, and this adjustment does not guarantee that this confounding factor is completely controlled for. Protease 3 can also cleave IL-18 into active form, which was unfortunately not accounted for in our analyses. Nevertheless, the whole pathway leading to the secretion of mature IL-18 is still unknown, making it impossible to take all factors into account. Although our results leave room for further elaboration, they do show that hypertension affects, in an

IL-18 genotype-dependent manner, the inflammatory environment as regards IL-18 and IFN-γ mRNA expression in human atherosclerotic plaques.

The combined results derived from the Helsinki Sudden Death Study and Tampere Vascular Study provide strong evidence of the link between IL-18 gene polymorphism and hypertension in the development and manifestation of atherosclerosis. The fact that we observed the same interplay affecting the occurrence of SCD in the whole study population, and in the expression of coronary atherosclerosis among men who had died of other causes (e.g. controls), adds further weight to this finding. By limiting the analysis to the control population, we were able to circumvent the possible confounding effect of selection bias. Furthermore, the results of an independent study material supported the findings.

Although our study is the first one to present a significant interaction between hypertension and IL-18 genotype affecting the risk of a major clinical end-point, one previous study has reported a significant interaction between IL-18 gene polymorphism and smoking impacting on the risk of CVD. Recently, Grisoni et al. reported, in a very accomplished paper, that the IL-18 gene -105 C/T SNP (rs360719), and a major haplotype carrying the minor T allele, interacts significantly with smoking, thus modulating the risk of CVD disease. Similar analyses were performed treating CHD stroke events separately and excluding cases with CVD at baseline. The interaction was confirmed in all of the analyses. The study was based on combined study populations of five separate sub-cohorts from Finland, Sweden, Ireland and France, and the combined number of subjects was 2,271.

Two facts make the results of the study very interesting from the point of view of our study. Firstly, the -105 C/T SNP is in nearly complete association with the promoter region -137 G/C SNP (e.g. inherited in the same BIN). Secondly, Grisoni et al. observed that while none of the SNPs genotyped in the study, nor the variation of the IL-18 gene globally, associated with the occurrence of CVD, IL-18 gene variation and especially the -105 C/T genotype among smokers did. The authors observed that in smokers the carriage of the minor T allele was significantly higher among cases than among controls. They also found that among non-smokers, this association was inverse but not as strong. When they studied the effect of smoking on the risk of CVD among IL-18 genotype groups, they found that smoking was associated with an almost two times higher risk among T allele carriers. The significance of this finding was evident ($p=3.5 \times 10^{-8}$). Among CC homozygotes, the effect of smoking on the risk of CVD

was not as strong (OR 1.25, p=0.05). Grisoni et al. concluded that the minor T allele, or at least some related factor (e.g. another SNP), was associated with increased risk of CVD in smokers. This conclusion is hardly wrong but could certainly be elaborated further.

In the combined study population of the MORGAM project (on which the study by Grisoni et al. is based), the frequencies of the minor T allele (of the -105 C/T SNP) among non-smoking controls and cases were 0.29 and 0.27, respectively. This difference between the groups reflects the previously hypothesized protective property associated with the T allele. Nevertheless, these frequencies are not extremely different from the 0.28 observed previously in a large study population of Caucasian CAD patients. In the study population of the Finnish Cardiovascular Risk Study, formed by patients selected for a clinical exercise test, the frequency of the minor T allele of the +127 C/T SNP (inherited in almost complete association) was 0.27. The corresponding frequency in the Cardiovascular Risk in Young Finns study population, which represents the normal Finnish population, was 0.29. However, in the MORGAM project, the corresponding frequencies for smoking controls and cases were 0.24 and 0.28, respectively. If we assume that the T allele does not provide any protection against CVD, the frequency of the T allele carriers among smoking CVD cases should be clearly higher than their frequency within the normal population. This should then also apply to the frequency of CC homozygotes, but the difference would not be as dramatic. The frequencies presented in the MORGAM study reveal that this is not the case. An alternative hypothesis is that the T allele is associated with a protective effect but that the effect is efficiently abolished by smoking. This would explain the fact that although smoking is associated with a higher risk of CVD among T allele carriers than CC homozygotes, the frequency of T allele carriers among smoking patients with CVD is lower than the corresponding frequency of CC homozygotes.

1.7 Methodological considerations and limitations of the study

We did not measure circulating IL-18 concentrations for any of the study populations of the present study. This is a clear limitation. Although previous studies have already reported some evidence on the matter, it could have provided us additional information on the association between IL-18 gene polymorphism and circulating IL-18 levels. Given the robust study setting, the Cardiovascular Risk in Young Finns Study would

probably have provided us valid information on the connection between these two. Most haplotype studies that have connected IL-18 gene polymorphism and circulating levels of IL-18 have shown that the variation of the gene explains 1.7%–5.2% of the variation in the circulating IL-18 levels, independently of risk factors (Thompson et al. 2007a, Thompson et al. 2007b, Tiret et al. 2005).

However, some separate haplotypes have been identified with more dramatic changes in circulating IL-18 levels. Tiret et al. found that among CAD patients, two major haplotypes corresponding to the haplotypes AGTA and CTCG identified in our study associated with significant decreases in circulating IL-18 levels. The haplotype corresponding to the CTCG haplotype had the most dramatic effect, lowering the IL-18 levels by up to 9% in reference to the most frequent haplotype (Tiret et al. 2005). Another study also found that a haplotype related to CTCG was associated with 15% lower IL-18 levels among subjects carrying 2 copies of the haplotype when compared to non-carriers (Thompson et al. 2007a). The study population was based on adults without CHD, and the effect of the haplotype seemed to be linear (i.e. IL-18 values of heterozygotes fell between the values of the other two genotypes). In a population of men with premature CHD, the same haplotype associated with decreases of 12% and 22% among heterozygotes and homozygotes carrying two copies in reference to non-carriers (Thompson et al. 2007a). Interestingly, one smaller haplotype with a haplotypic frequency of 0.10 (corresponds to the ATCA haplotype in the present study) was associated with a corresponding increases of 15% and 55% when compared to non-carriers. Furthermore, a third haplotype study showed that among patients selected for coronary artery bypass surgery, baseline levels of IL-18 were approximately 25%–30% lower among carriers of haplotypes corresponding to the haplotypes AGTA and CTCG identified in our study. It seems that study populations with heavier disease burden have a greater IL-18 genotype-dependent response with regard to the circulating IL-18 levels. This suggests that inflammatory stress augments the IL-18 genotype-dependent systemic IL-18 response.

Another confounding factor is that IL-18 levels seem to be very positively and strongly correlated with risk factors such as age, metabolic syndrome traits (BMI, waist circumference, triglyceride concentrations, HDL concentrations [inverse correlation], fasting glucose levels, insulin levels and hyperglycaemia) and blood pressure (Esposito et al. 2002, Evans et al. 2007, Hung et al. 2005, Thompson et al. 2007c). Fortunately, we were able to adjust the analyses in the present study in reference to most major risk

factors. Furthermore, it is not clear that circulating IL-18 levels is responsible for conveying the risk of atherosclerosis and its end-points. Circulating levels of IL-18 might only reflect the overall inflammatory status or the burden of the disease, rendering the observed association a result of reverse causality. The local production of IL-18 in atherosclerotic plaques modulated by genetic factors is also a very probable factor causing aggravation of the disease.

The results of the present study rely on the candidate gene approach. The obvious pitfall of many candidate gene approaches has been their poor reproducibility of the results (Plump and Lum 2009). This is often due to the fact that the candidate genes have not been selected with enough scrutiny or the associations have been tested in very heterogeneous populations. Atherosclerosis is a multifactorial disease, and it is therefore very unlikely that a single genetic factor would have dramatic effects on major end points. The candidate gene (IL-18) selected for the present study is very promising, and this is emphasized by the fact that IL-18 has been previously linked with the development of atherosclerosis on the vessel wall level as well as with major clinical end points (see the Review of the literature). Furthermore, previous association studies have shown a connection between IL-18 gene variation and severe clinical end points. Therefore, our study aimed to clarify the possible effects of IL-18 gene variation on different end points of atherosclerosis among different populations. We also wanted to explore whether IL-18 interacts with some of the known risk factors. This was done to elucidate the possible mechanisms behind the associations.

GWAS is another possible approach for genetic research. The technological improvements made during the past few years have made GWAS a reality. However, while GWAS might be a powerful tool for recognizing possible loci associating with major clinical end points, the approach only provides a starting point for the search of functional SNPs and genes (Plump and Lum 2009). Basically, GWAS provides a hypothesis-free approach for exploring candidate genes. The subsequent validation and replication of results must be carried out by means of association studies (Kronenberg 2008).

1.8 Future Prospects

The need for more specific studies concerning gene-environment interactions is evident. In a multifactorial disease such as atherosclerosis, it is very unlikely that one

specific common mutation would have a dramatic response with regard to clinically significant end points. This fact is underlined by the vast number of genetic association studies failing to replicate the borderline results of previous studies. Genetic research of common variants in common diseases is not likely to produce much direct clinical value in the sense of risk prediction. This is emphasized by the fact that, as most common variants hardly ever greatly augment the risk of any trait and as the development of diseases such as atherosclerosis is dependent on multiple factors, it is very unlikely that any given individual would bear the brunt of more than the average number of such genetic risk factors.

Even if risk prediction were not the primary aim, genetic research is required in clarifying the mechanisms of the diseases. The IL-18 pathway should be studied further on a vessel wall level. The results of the present and previous studies have shown that IL-18 is most likely associated with the expression of the unstable plaque phenotype and thus also the occurrence of severe complications. Genome-wide expression studies are most likely to clarify this issue further. They can provide interesting data directly from the vessel wall level concerning the development of atherosclerosis, facilitating further study of candidate pathways.

Identification of strong gene-environment interactions would also be useful to identify specific populations for targeted drug prevention. More studies are therefore needed, as much is still unknown. In the present study, we have shown that IL-18 gene polymorphism interacts with hypertension. This could be an interesting prospect for targeted treatment of hypertension. However, this interaction still requires further study, and the results must be replicated. Investigations of gene-gene interactions (e.g. epistasis) are also likely to provide much needed insight into the pathogenesis of common but deadly diseases such as atherosclerosis.

In conclusion, as the technology for genome-wide association and expression studies advances and the availability of such technologies imroves, it is highly likely that more interesting interacting pathways are discovered and the study of the present candidate pathways, such as the IL-18 pathway, is facilitated.

SUMMARY AND CONCLUSIONS

This candidate gene study explored the possible effects of IL-18 gene polymorphism on the expression of atherosclerosis in different study settings. We also searched for possible interactions between the variation of the IL-18 gene and risk factors. The main findings and conclusions were as follows:

1. The minor T allele of the +415 A/G SNP and also the only major haplotype carrying the G allele associated significantly with lower IMT among young healthy Caucasian men. Among women there was no association to be seen. This interaction was significant. Other polymorphisms or haplotypes were not observed to have any effect on IMT values (Study I).

2. The variation in the IL-18 gene was not seen to associate significantly with CV mortality among a Finnish patient population. Among patients who had coronary angiography, one major haplotype had a different sex-dependent impact on the expression of CAD. This major haplotype is also the only one carrying the minor T allele of the +127 C/T SNP and the minor C allele of the functional promoter region -137 G/C SNP. Among men, the carriers of this AGTA haplotype had a lower occurrence of severe CAD, whereas among women the haplotype was not seen to associate with the expression of CAD (Study II).

3. In an autopsy study of Caucasian men who had died suddenly out of hospital, the functional promoter region -137 G/C SNP was seen to associate with the occurrence of SCD due to CHD and due to other causes in the absence of CHD. The minor C allele was associated with a lower risk of both causes of death (Study III).

4. Hypertension interacts with the -137 G/C SNP, affecting the risk of SCD due to CHD and the development of coronary atherosclerosis. Hypertension was a major risk factor for both the risk for SCD and the development of coronary atherosclerosis among GG homozygotes. Among C allele carriers, we did not see any significant effect. Hypertension also affected the expression of IL-18 and IFN-γ mRNA in human atherosclerotic plaques obtained from live patients. This effect was also seen to be modulated by IL-18 genotype. (Study IV)

In conclusion, IL-18 gene variation seemed to associate with the development of subclinical atherosclerosis and the expression of CAD among men but not among women. Among men, IL-18 associates with the risk of SCD. Hypertension seems to affect the risk for SCD due to CHD in an IL-18 genotype dependent manner.

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Interleukin 18 gene polymorphism and markers of subclinical atherosclerosis. The Cardiovascular Risk in Young Finns Study

Running title: IL-18 gene polymorphism and subclinical atherosclerosis

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Abstract

Background and Aim. Interleukin 18 (IL-18) is a pro-atherosclerotic cytokine. We wanted to evaluate whether IL-18 gene polymorphism associates independently of risk factors, with early subclinical markers of atherosclerosis (intima media thickness [IMT], coronary artery compliance [CAC] and flow mediated dilatation [FMD]) in a population of young healthy Caucasian adults.

Methods. This study was based on the ongoing Cardiovascular Risk in Young Finns Study consisting of 2260 young adults, mean age being 31.7 (range 24-39years) (1247 women and 1013 men).

Results. Five studied tagSNPs formed six major haplotypes, which accounted for 99.9% of all variation of the IL-18 gene. According to adjusted analysis of variance the IL-18 gene polymorphism didn't associate with subclinical atherosclerosis in the whole study population. However, one major haplotype associated differently among men and women with IMT (p=0.011). Male carriers of a major **CCTgT** haplotype (n=441) seemed to have a lower IMT when compared to the non-carriers (-0.016mm, 95% CI -0.028 to - 0.004, p=0.014). Among women no significant association were observed.

Conclusions. Among all study subjects, the polymorphism of the IL-18 gene is not associated with subclinical markers of atherosclerosis. However, among men one major IL-18 haplotype seemed to associate with substantially lower IMT values.

Key words: arterial elasticity, intima media thickness, genetics, atherosclerosis, inflammation

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Key messages:

Interleukin 18 (IL-18) gene polymorphism has been previously associated with severe clinical end-points such as sudden cardiac death and the occurrence of myocardial infarctions.

According to the results of the present haplotype study, the IL-18 gene polymorphism doesn’t associate significantly with subclinical markers of atherosclerosis among a population of healthy young adults.

However, among men one major haplotype seems to associate significantly with lower intima media thickness of the carotid artery.

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Abbreviations:

1.	**IL-18**	**=**	**Interleukin 18**
2.	**MI**	**=**	**Myocardial infarction**
3.	**CAD**	**=**	**Coronary artery disease**
4.	**SCD**	**=**	**Sudden cardiac death**
5.	**SNP**	**=**	**Single nucleotide polymorphism**
6.	**IMT**	**=**	**Intima media thickness**
7.	**FMD**	**=**	**Flow mediated dilatation**
8.	**CAC**	**=**	**Carotid artery compliance**
9.	**BMI**	**=**	**Body mass index**
10.	**BP**	**=**	**Blood pressure**
11.	**CRP**	**=**	**C-reactive protein**
12.	**ApoA-1**	**=**	**Apolipoprotein A-1**
13.	**ApoB**	**=**	**Apolipoprotein B**
14.	**HDL**	**=**	**High density lipoprotein**
15.	**LDL**	**=**	**Low density lipoprotein**
16.	**ANOVA**	**=**	**Analysis of variance**

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Introduction

Atherosclerosis is an inflammatory disease which progresses through decades (1, 2). Interleukin 18 (IL-18), a pro-inflammatory and pro-atherosclerotic cytokine is produced mainly by monocytes and macrophages (3, 4). It seems to play a crucial role in the development of more vulnerable atherosclerotic plaques (5, 6) through inducing the production of interferon-γ (7).

In-vitro models have shown that the genetic variation of the IL-18 gene affects the monocyte´s production of IL-18 (8-11). Likewise, the expression of IL-18 in humans is regulated by the genetic variability of the IL-18 gene and according to several studies IL-18 gene polymorphism associates with circulating IL-18 levels (12-15).

Previously IL-18 levels have been shown to correlate with the extent of coronary atherosclerosis among patients with previous myocardial infarction (MI) and with unstable angina (16, 17) and also predict the mortality in patients with coronary artery disease (CAD) (18, 19). Genetic studies have revealed similar results: Polymorphism of IL-18 gene is associated with cardiovascular mortality among CAD patients and MI risk among hypertensive patients and postmenopausal women (12, 20).

Increased carotid artery intima-media thickness (IMT), elasticity and flow-mediated dilatation (FMD) are early subclinical markers of atherosclerosis and predict future coronary events (21-23). Carotid artery compliance (CAC) depicts the ability of the arteries to expand under the influence of pulse pressure. Diminished arterial elasticity has been shown to be an independent predictor of cardiovascular events in high-risk individuals (24, 25). FMD of the brachial artery quantifies the amount of vasodilatation

in response to endothelial activation by an increase in local blood flow (26). Even before anatomical evidence of atherosclerosis appears, FMD is impaired in young symptom-free subjects with risk factors for vascular disease (27). Carotid IMT has been showed to independently predict future vascular events especially among young subjects (21).

The increased circulating levels of IL-18 seem to be associated with greater carotid artery IMT (28, 29). Whether this association is independent of traditional risk factors is still unclear (28, 29). To the best of our knowledge the relation of FMD and CAC to IL-18 concentrations or IL-18 genotypes have not been previously studied.

Lately the reports have concentrated on studying the IL-18 levels/polymorphism and their association to the advanced atherosclerotic end-points e.g., acute MI, CAD. Thus we wanted to study, whether known IL-18 haplotypes or single-locus tagSNP polymorphisms affect, independently of risk factors, the early (subclinical) markers of atherosclerosis (IMT, CAC and FMD) in a population of young healthy Caucasian adults.

Materials and methods

Subjects

The Cardiovascular Risk in Young Finns Study is an ongoing prospective multicentre cohort study, which provided us the study population of 2282 young adults. Details of the cohort have been published previously (30-32). The study began 1980 and the 21 year follow-up was carried out in 2001. All of the data used in the present study was collected in the year 2001. Participants with type 1 diabetes were excluded from further analyses. The Ethical Review Committee of Turku University Hospital approved the research plan, and the study followed the tenets of the Declaration of Helsinki. Patients gave an informed consent before entering to the study.

Clinical and biochemical characteristic

Standardized questionnaire was used to assess cardiovascular risk factors (smoking, alcohol consumption, geographical origin and familial history of coronary heart disease). The classification of these variables has been described earlier in more detail (33). Study subjects height and weight were used to calculate their body mass index (BMI=weight, kg/(height, m)^2) and their blood pressure (BP) was recorded. Fasting venous blood samples were used to determine C-reactive protein (CRP), insulin, glucose, serum lipids, apolipoprotein A-1 (ApoA-1), apolipoprotein B (ApoB), and homocysteine concentrations. Total cholesterol, high-density lipoprotein (HDL) and triglycerides were

determined enzymatically. Low-density lipoprotein (LDL) was calculated by Friedewald formula. Standardized methods were used in all determinations (31, 32). The information of geographical origin was included as a covariate because the population originating from Eastern Finland is genetically more predisposed to the development of atherosclerosis than the population of Western Finland (34).

DNA isolation and genotyping of the IL-18 polymorphism

Genomic DNA was isolated from peripheral blood leukocytes by using QIAamp®DNA Blood Minikit and automated Biorobot M48 (Qiagen, Hilden, Germany) extraction. The five IL-18 SNPs (rs1946519, rs360717, rs549908, rs5744292, rs4937100) and haplotypes were genotyped by using the 5´ nuclease assay for allelic discrimination and fluorogenic TagMan MGB probes with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Forster City, CA, USA) (35). The nucleotide sequences of the primers and fluorogenic allele-specific oligonucleotide probes used in PCR were deduced from published sequences in the Gene Bank database. They were chosen and synthesized in co-operation with Applied Biosystems. PCR reaction containing genomic DNA, 2 × TaqMan Universal PCR Master Mix, 900 nM of each primer, and 200 nM of each probe was performed in 384-well plates according to standard protocol in a total volume of 5 μl. Water controls and random duplicates were used as a quality control.

Measurements of subclinical markers of atherosclerosis (IMT, CAC and FMD)

Carotid artery IMT was measured by ultrasound and for determining CAC the concomitant brachial blood pressure was also monitored. The brachial artery FMD was assessed by measuring the left brachial artery diameter both at rest and during reactive hyperemia. Ultrasound studies were performed using Sequia512 mainframes (Acuson, Mountain View, California) with 13.0 MHz linear array transducer. The procedures have been earlier discussed in more details (32).

To determine the intra-individual reproducibility of the measurements, the ultrasound measurements were replicated for small random sample of the participants (n=57, 2,5%) 3 months after the initial visit. The between-visit coefficient of variation was 6.4% for IMT, 16.3% for CAC and 26% for FMD measurements.

Statistical analyses

The statistical analysis was performed using the SPSS 14.0 for Windows (SPSS Inc., Chicago, IL, USA). In order to study the possible association between IL-18 gene polymorphism and the subclinical markers of atherosclerosis, we applied adjusted analysis of variance (ANOVA). Only significant covariates were accepted into the models (the selection criteria and procedure has been previously described in more detail) (33). First we studied the possible factor-by-sex interactions. If the interaction between the haplotype or SNP and sex was found significant we proceeded to analyze the effect of the haplotype or SNP among men and women separately and the results from the level of

the whole study population were not interpreted. To study the effect of haplotypes, we divided the population into carriers and non-carriers. The effects of individual genotypes were studied without pooling the genotypes. Previously pregnancy has been associated with changes in serum values of IL-18 and this tendency has been found to be modulated by IL-18 genotype (36, 37). For this, the analyses were performed before and after the exclusion of the 61 women who were pregnant during the time of the study. However, the results remained almost unchanged and thus we only present the results obtained from the whole study population.

ANOVA was used to evaluate the possible association between risk factors and the polymorphism of the IL-18 gene. Variables with skewed distributions (insulin, glucose and CRP) were $\log_{10}$-transformed for the analyses. Categorical variables and Hardy Weinberg equilibrium were tested using the χ^2-test.

A value of $P < 0.05$ was considered statistically significant. In order to correct for multiple testing we applied the Li & Ji method (38). This method is the improved version of the method originally introduced by Nyholt et al. for adjusting for multiple-testing in multilocus analyses (39). This correction method was used because of the high linkage disequilibrium between the SNPs. Frequencies of the most common haplotypes and the most probable haplotypes for each study subjects were determined using the PHASE program (Version 2.0.2) (40).

One of the IL-18 genotype distributions (**533T>C, rs4937100**) was not in Hardy Weinberg equilibrium in our study population ($p<0.001$). Nevertheless, we included it into our haplotype reconstruction in order to verify whether our haplotype frequencies would be in line with those observed in previous studies (12). This SNP was responsible

for dividing one common haplotype (haplotype **aCTA** with a frequency of 0.173) into two smaller haplotypes (**aCTAT**, frequency 0.132 and **aCTAc**, frequency of 0.041) (See Table III below). Because the genotype distributions of the **533T>C** SNP was not in Hardy Weinberg equilibrium, this SNP was excluded from further analyses and only the effect of the combined **aCTA** haplotype was studied.

Results

General Characteristics

The mean age of the study population was 31.7±5.0 (S.D) years and there were more women than men (n=1247 vs. n=1013, respectively). The average BMI of the study population was 24.9 ±4.2 S.D. Other general characteristics are presented in Table I. Genotyping was successfully performed in 92.6% to 99.6% of the cases depending on the genotype. Genotype distributions are presented in Table II.

Haplotype analyses

The five studied IL-18 SNPs (rs1946519, rs360717, rs549908, rs5744292, rs4937100) formed six major haplotypes: **CCTAc**, **atgAT**, **CCTgT**, **aCTAT**, **CCgAT** and **aCTAc**. The haplotype frequencies, presented in table III, were in line with the results of the earlier haplotype study by Tiret et al.(12). These haplotypes accounted for 99.9% of all variation in the IL-18 gene. The **CCTgT** haplotype was the only haplotype carrying the

G allele of the 415A>G polymorphism (rs5744292) and the **atgAT** haplotype was the only one to carry the T allele of the 127C>T polymorphism (rs360717).

IL-18 gene polymorphism and cardiovascular risk factors

None of the studied SNPs (Table II) or haplotypes (Table III) of the IL-18 gene associated with the clinical or biochemical risk factors of atherosclerosis measured in our study (variables in Table I) after correcting the analysis for multiple testing. Some significant differences were observed between the populations originating from Eastern and Western Finland before correcting for the number of tests performed within the group of risk factors. The frequencies of the minor alleles of the +35 (T/g) SNP (p=0.005) and +127 (C/t) SNP (p=0.040) were higher among population with Eastern Finnish origin when comparing to population originating from Western Finland.

IL-18 polymorphisms and markers of subclinical atherosclerosis

According to ANOVA adjusted with sex, BMI, age, smoking and geographical origin, the **CCTgT** haplotype associated with carotid artery IMT significantly differently among men and women (p=0.011 after correcting for multiple testing). Among men, the carriers of the **CCTgT** haplotype had a significantly lower IMT when compared to the non-carriers (adjusted difference in means: -0.016mm 95% CI -0.028 to -0.004, p=0.014 after correcting for multiple testing). The adjusted mean values by haplotype groups were: 0.601mm (standard error [S.E.] of 0.004mm) for non-carriers, 0.585mm (S.E. 0.005mm)

for heterozygous carriers and 0.596mm (S.E. 0.014mm) for homozygous carriers of the **CCTgT** haplotype. There difference in IMT between heterozygous and homozygous male carriers of this haplotype was not significant (corrected p=1.00). As the carriers of this CTCgT haplotype are the only ones carrying the minor g allele of the +415 C/g SNP (rs5744292), it is clear that the significant difference between the haplotype groups is caused by the minor g allele of the +415 C/g SNP. Among women no significant difference was observed (0.005 mm, 95% -0.004 to 0.014, corrected p=0.498). None of the other haplotypes or SNPs associated significantly with IMT among the whole study population and no other significant haplotype-by-sex-interactions were seen. None of the haplotypes or studied SNPs associated significantly with CAC or brachial artery FMD in the whole study population and we didn't observe any significant haplotype-by-sex-interactions. The exclusion of geographical origin from covariates left the results unchanged and thus also the observed associations remained significant.

Discussion

According to the novel results of the present haplotype study, the variation of the IL-18 gene is not associated with subclinical markers of atherosclerosis among population based sample of healthy young adults. However among men, the carriership of a major haplotype (**CCTgT**) of the IL-18 gene appears to associate with lower IMT independently of risk factors.

Thus far there are only contradictory results of the possible link between IL-18 and IMT. In 2005, Yamagami et al. reported that higher circulating IL-18 levels

associated independently with greater carotid IMT among a population without a history of cardiovascular accidents (28). A later study by Chapman et al., conducted with a younger study population with lower overall atherosclerotic burden, was unable to replicate this finding (29). The fact that the present study lacks the data of circulating IL-18 levels is a weakness. However, according to prior evidence, the genetic variation of the IL-18 gene affects the circulating levels of IL-18 (12-15). In fact, the very same haplotype that associated significantly with lower IMT among men in our study was also found to associate with lower IL-18 levels and cardiovascular mortality in a large haplotype study by Tiret et al.(12). It is also a possibility that the local production of IL-18 within the atherosclerotic plaque might affect independently the overall development of the disease. Supporting the hypothesis, we have demonstrated that the mRNA expression of IL-18 in atherosclerotic plaques is dependent of IL-18 gene polymorphism (41). One IL-18 gene promoter region SNP (-137G/c, rs187238) was seen to associate with the expression of both IL-18 and IFN-γ mRNA. This polymorphism, as well as one other SNP (rs360719), associating with the mRNA expression of IL-18 in peripheral blood monocytes, is in nearly complete concordance with the one of the SNPs studied in the present work (rs360717) (8, 12). The minor allele of the -137 G to C polymorphism (rs187238) is also linked with lower risk for sudden cardiac death (SCD) among men (41). Despite of these findings, according to the present study, this polymorphism doesn't associates with the early stages of the disease.

In fact, most of the positive results linking IL-18 to atherosclerosis are derived from older and more risk factor burdened patients (mean ages of patient groups ranging from 54 to 69 years) with more severe atherosclerotic disease end-points e.g., acute MI,

CAD and SCD (12, 16-20). Thus it seems, that IL-18 has a significant role in the development of atherosclerosis rather in the later than in the early stage of the disease. Atherosclerosis also develops faster in men and this could at least partly explain why the association between the **CCTgT** haplotype and IMT was seen among men but not among women. The male carriers of this **CCTgT** haplotype (also the only one carrying the g allele of the +415 C/g SNP) had 0.016 mm lower IMT compared to the male non-carriers. Clinically, this difference in IMT was greater in comparison to the effects of some more well known classical risk factors such as smoking (change of 0.011 mm), male sex (change of 0.010 mm) and BMI (0.011 mm change for 1 SD change in BMI [=4.2kg/m^2]) and systolic blood pressure (0.010 mm change of 1 SD change in BP[=14.4mmHg]) previously demonstrated in the same material (32). As men tend to suffer more frequently from cardiovascular diseases (42), and taking into consideration the size of the effect, it would be feasible to suggest that this finding could have clinical significance. However, this association should be replicated in independent populations before it can be considered significant.

In addition to the lack of measurements of the circulating IL-18 levels, another limitation of our study is that the blood pressure values that were used to derive the carotid artery elasticity were measured from the brachial artery. This limitation has been discussed earlier in more detail (43). An obvious strength of our study is the study design. Our results are based on a multi-centre study with a randomly selected study population and the risk factor data of this study is extensive and thus provides a good background for a genetic association study. Furthermore, with the 5 tagSNP genotyped in the present

study we were able to cover more than 99% of the variation of the IL-18 gene and thus the associative data is comprehensive.

In summary, according to the present haplotype study the polymorphism of the IL-18 gene is not associated with the development of subclinical atherosclerosis (measured by intima media thickness, carotid arterial elasticity or with endothelial function measured by flow mediated dilatation) among all young Caucasian adults. However, among men one major haplotype (**CCTgT**) seems to associate with substantially lower carotid artery IMT values.

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Conflicts of interests: None declared

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Table I. Characteristics of the study population.

Variable	All (n=2260)	Male (n=1013)	Female (n=1247)	P-value*
Age (years)	31.7 ±5.0	31.7 ±5.0	31.7 ±5.0	0.933
Body mass index (kg/m^2)	24.91 ±4.16	25.69 ±3.79	24.27 ±4.33	<0.001
Ever smokers, yes (%)**	959 (42.4)	493 (48.7)	466 (37.4)	<0.001
Systolic BP (mmHg)	122.0 ±14.4	129.2 ±13.5	116.2 ±12.4	<0.001
Diastolic BP (mmHg)	73.16 ±9.0	74.99 ±9.1	71.67 ±8.7	<0.001
Cholesterol (mmol/l)	5.16 ±0.98	5.26 ±1.03	5.09 ±0.93	<0.001
HDL cholesterol (mmol/l)	1.29 ±0.32	1.16 ±0.28	1.40 ±0.31	<0.001
LDL cholesterol (mmol/l)	3.27 ±0.85	3.42 ±0.92	3.16 ±0.78	<0.001
Triglycerides (mmol/l)	1.34 ±0.85	1.53 ±0.99	1.18 ±0.68	<0.001
Apolipoprotein A-I (g/l)	1.50 ±0.26	1.40 ±0.21	1.57 ±0.27	<0.001
Apolipoprotein B (g/dl)	1.06 ±0.26	1.13 ±0.27	1.00 ±0.24	<0.001
Insulin (mU/l)	7.63 ±5.21	7.52 ±5.19	7.72 ±5.22	0.382
Glucose (mmol/l)	5.00 ±0.45	5.15 ±0.42	4.87 ±0.44	<0.001
C-reactive protein (mg/l)	1.89 ±3.85	1.46 ±3.27	2.23 ±4.23	<0.001
Homocysteine (µmol/l)	9.82 ±3.81	10.86 ±4.18	8.97 ±3.24	<0.001

Values of continuous variables are expressed as mean ± standard deviation. Abbreviations: BP, Blood pressure; HDL, high density lipoprotein; LDL, low density lipoprotein.

*Significance calculated using one-way ANOVA for continuous variables and Pearsons χ^2-test for categorical variables.

**This category includes daily smokers, ex-daily smokers and occasional smokers.

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Table II. Interleukin -18 (IL-18) genotype distributions.

SNP, rs number	IL-18 Genotype, n (%)		
	Major Homozygote	Heterozygote	Minor homozygotes
-656 (C/a), rs1946519	673 (30.0%)	1065 (47.1 %)	506 (22.5%)
+127 (C/t), rs360717	1125 (50.8%)	899 (40.6%)	189 (8.5%)
+35 (T/g), rs549908	1062 (47.2%)	966 (42.9%)	223 (9.9%)
+415 (A/g), rs5744292	1204 (57.6%)	766 (36.6%)	121 (5.8%)
+533 (T/c)*, rs4937100	1195 (53.4%)	704 (31.4%)	340 (15.2%)

*Genotype distribution not in Hardy Weinberg equilibrium

Table III. Interleukin-18 (IL-18) haplotypes.

	IL-18 SNPs						
	-656(C/a) rs1946519	+127(C/t) rs360717	+35(T/g) rs549908	+415(A/g) rs5744292	+533(T/c) rs4937100	Haplotype Frequency	(SE)
Haplotype	C	C	T	A	c	0.270	(0.0005)
	a	t	g	A	T	0.288	(0.0003)
	C	C	T	g	T	0.244	(0.0003)
	a	C	T	A	T	0.132	(0.0005)
	C	C	g	A	T	0.024	(0.0001)
	a	C	T	A	c	0.041	(0.0004)
Minor allele Frequency	0.462	0.288	0.313	0.240	0.312		

Abbreviations: SNPs, single nucleotide polymorphisms

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Interleukin-18 promoter polymorphism associates with the occurrence of sudden cardiac death among Caucasian males: The Helsinki Sudden Death Study

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Abstract

Objective: The increased plasma concentrations of pro-atherogenic and cardiomyocyte hypertrophic cytokine interleukin 18 (IL-18) predict mortality in patients with coronary heart disease (CHD) in addition to predicting the outcome of heart failure. The IL-18 gene has a functional −137 G/C polymorphism (rs187238) in the promoter region. The C allele carriage is associated with attenuated IL-18 production. The effect of IL-18 genotype on SCD is unknown. We studied the association of the IL-18 gene −137 G/C polymorphism with the occurrence of sudden cardiac death (SCD).

Methods: Using the TaqMan 5′ nuclease assay, we genotyped two independent consecutive and prospective autopsy series which were included in the Helsinki Sudden Death Study.

Results: Of the 663 men, 359 (54.1%) had the wild-type GG-genotype, 261 (39.4%) were heterozygotes (CG) and 43 (6.5%) were CC homozygotes. Compared to the GG homozygotes, the C allele carriers (i.e. subjects having CC or CG genotypes) had a lower adjusted risk for SCD from any cause (odds ratio [OR] 0.49; 95% confidence interval [CI], 0.31–0.77, $p = 0.002$), for SCD due to CHD (OR 0.51; 95% CI, 0.32–0.82, $p = 0.005$), and for SCD caused by non-coronary heart diseases (OR 0.34; 95% CI 0.13–0.90, $p = 0.030$).

Conclusion: IL-18 promoter −137 G/C polymorphism, which regulates the expression of IL-18, is an important predictor of SCD from any cause as well as SCD in patients with and without underlying CHD.

Keywords: Myocardial infarction; Sudden cardiac death; Polymorphism; Genetics; Coronary heart disease

1. Introduction

Sudden cardiac death (SCD) leads to the loss of 300,000–400,000 lives a year in the United States [1]. Pre-existing coronary heart disease (CHD) and its consequences, e.g., acute coronary syndrome, myocardial scarring due to a previous infarction, and heart failure are manifested in 80% of all SCD victims [1]. The second largest group of diseases concurrent with SCD includes dilated or hypertrophic cardiomyopathy, both of which can develop in the absence of CHD [1]. The other cardiac disorders, e.g., the known genetically determined ion channel abnor-

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malities and congenital heart defects account for 5–10% of SCDs [1].

A family history of myocardial infarction (MI) [2] and sudden death [3] are both associated with the risk of SCD, but the specific genetic risk factors of SCD involving fibrinogen receptor and alpha(2B) adrenoceptor gene variations are poorly known [4–6].

One interesting candidate gene for SCD might be interleukin-18 (IL-18), formerly known as the interferon-γ (IFN-γ) inducing factor [7]. The increased plasma concentrations of this pro-atherogenic and cardiomyocyte hypertrophic cytokine have previously been associated with the occurrence of stable and unstable angina pectoris, MI, and acute fatal coronary events [8–13]. However, in one previous study, no association was found between IL-18 plasma concentrations and coronary events [14]. IL-18 is a pleiotropic pro-inflammatory cytokine, affecting both innate and acquired inflammatory response [15,16], and it has been associated with the development of atherosclerosis by stimulating the production of atherogenic IFN-γ [17]. Furthermore, the development of more vulnerable atherosclerotic plaques, usually associated with increased risk of acute coronary events, seems to be instigated by the expression of IL-18 [18]. IL-18 also appears to be involved in the development of congestive heart failure due to cardiomyopathy, and patients with congestive heart failure have higher circulating IL-18 levels than controls with no signs of chronic heart failure [19–21]. Moreover, IL-18 levels seem to predict mortality in patients with congestive heart failure [21].

The promoter region of the human IL-18 gene has a common single nucleotide polymorphism (SNP) at position −137 that changes guanine to cytosine (G/C) (rs187238). This SNP is in complete linkage disequilibrium with two other SNPs in the IL-18 gene (located at positions +113 and +127) [22]. This polymorphism has been shown to regulate the IL-18 production of peripheral blood monocytes and associated with transcriptional activity of the IL-18 gene [22–24].

To date, the association of IL-18 polymorphism with SCD has remained unknown. Therefore, and based on previous data, we tested the hypothesis whether the IL-18 −137 G/C polymorphism could be a risk factor for SCD and its subtypes with and without underlying CHD, using extensive autopsy material from two independent autopsy series which included a total of 700 men and were carried out in the Helsinki Sudden Death study. Furthermore, we investigated the effect of age on this possible association, because the genetic susceptibility to death caused by CHD and to atherosclerosis has been shown to decrease with advancing age [25,26].

2. Material and methods

2.1. Subjects

The Helsinki Sudden Death study is based on the autopsy material of 700 Caucasian Finnish men who had died suddenly out of hospital in the area of Helsinki (Mean age 53 years, range 33–70 years). The material includes two independent autopsy series collected in 1981–1982 (A series, $n = 400$) and 1991–1992 (B series, $n = 300$). Because of their unexpected sudden death occurring outside a hospital and often unwitnessed, all the men were subjected to a medicolegal autopsy, unless the deceased had suffered a clinically diagnosed condition with a high probability of causing an untimely and sudden demise, such as severe chronic heart failure. This autopsy study covers 25.3% of all the 2850 deaths of 33–70-year-old males during the study period, covering 35.0% of all deaths which occurred due to ischemic heart disease in the area of Helsinki. The leading cause of death within this study population was cardiac causes (41%, $n = 288$). Other causes of death were verified as other diseases (20%, $n = 140$) and unnatural deaths resulting from accidents or suicides (39%, $n = 272$). All medicolegal autopsies were performed according to the same protocol at the Department of Forensic Medicine at the University of Helsinki, and the study was approved by the Ethics Committee of the department/university.

2.2. Genotyping of the IL-18 polymorphism

The cardiac muscle tissue samples collected for the A series were stored as paraffin-embedded blocks, and the samples from the B series were stored frozen. The DNA was extracted from these samples using a method described by Isola et al. [27]. The IL-18 SNP (rs187238) was genotyped by using the 5′ nuclease assay for allelic discrimination with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) [28]. The nucleotide sequences of the primers and the fluorogenic allele-specific oligonucleotide probes used in PCR were deduced from published sequences in the GenBank database, and they were chosen and synthesized in co-operation with Applied Biosystems. PCR reaction was performed according to standard protocol for TaqMan MGB probes in a total volume of 5 μl. After cycling, end-point fluorescence was measured and the allelic discrimination analysis module carried out the genotype calling. Genotyping was successful for 663 of the 700 tissue samples (A series, 382 cases; B series, 281 cases). Water controls and known control samples were run in parallel with unknown samples.

2.3. Post-mortem verification of old and acute myocardial infarction

MI was verified by a macroscopic and histological examination of the myocardium. Fibrous scar tissue of the myocardium was considered the diagnostic criterion for old MI and the presence of neutrophil granulocytes for an acute MI. The presence of a coronary thrombus was recorded during autopsy when coronary arteries were opened longitudinally.

Table 1
General characteristics of the study subjects

	Valid *n*	All	IL-18 genotype groups		*p*-value
			CG/CC (*n* = 304)	GG (*n* = 359)	
Age (years)[a]	663	53.2 ± 9.5	52.9 ± 9.5	53.4 ± 9.5	0.56
Body mass index, (kg/m^2)[a]	663	24.7 ± 4.8	24.3 ± 4.9	25.0 ± 4.7	0.13
Alcohol use, (<60 g/d / ≥60 g/d)	432	215/217	97/97	118/120	1.00
Hypertensive, *n* (%)	663	101	39 (38.6%)	62 (61.4%)	0.13
Diabetic, *n* (%)	459	107	53 (49.5%)	54 (50.5%)	0.32
Smokers, *n* (%)	473	390	174 (44.6%)	216 (55.4%)	0.72
Coronary artery disease, *n* (%)	639	271	121 (44.6%)	150 (55.4%)	0.75

Statistics: *p*-values have been derived with analysis of variance (normally distributed continuous variables), Mann–Whitney *U*-test (non-normally distributed continuous variables), and chi-square test for categorical variables.
[a] Mean ± standard deviation.

2.4. Varying causes underlying SCD

The deaths caused by cardiac diseases were divided into two categories by the autopsy findings—SCDs caused by CHD (80%, *n* = 220) and SCDs caused by non-coronary diseases in the absence of CHD (20%, *n* = 55). Most of the men with CHD died of SCD due to an MI (*n* = 154) [death due to acute myocardial infarct (AMI) (*n* = 101) or/and arrhythmias associated with the scar of a prior MI (*n* = 53)], or CHD without AMI/prior MI (*n* = 64). Non-coronary SCDs were due to cardiomyopathy, hypertrophy or dilatation of the hearth, or valvular diseases.

2.5. Collection of risk factor data

The information on common risk factors was obtained by interviewing a close friend, spouse, or relative of the deceased by means of a questionnaire. Complete interview data was available in 402 (60.6%) of the 663 cases whose genotype was successfully determined. The interviewee was asked whether the deceased had suffered from arterial hypertension or diabetes prior to his death. Smoking habits and the quantity/frequency of the average daily alcohol consumption were also inquired about. Subjects were categorized as smokers and non-smokers. The ex-smokers were included in the category of smokers for the statistical analysis. The reliability of the risk factor data has been discussed earlier in more detail [29].

2.6. Statistical analyses

The small group of CC homozygotes in our study (*n* = 43, 6.5%) was combined with the GC heterozygotes for statistical analyses, because the risk for SCD was not significantly different between these two groups.

Logistic regression analysis was used to calculate the odds ratios for dependent variables according to genotype groups. Analyses were carried out with and without adjustment for autopsy data [body mass index (BMI) and age] and interview data (daily alcohol consumption, smoking, diabetes and hypertension). Covariates were included in the model in a stepwise manner if they were found statistically significant. Pearson's χ^2-test was also used.

The combined group of men who had died of other diseases and those who had died of unnatural causes was used as a control group. This was done because the IL-18 genotype group did not associate with the rate of deaths caused by non-cardiac-related diseases or unnatural causes (Of the 130 men who had died of other diseases, 48.5% (*n* = 63) were C-allele carriers and of the 258 men who had died of unnat-

Table 2
The number of men who had died of SCD or other causes and the percentages of C allele carries of the IL-18 gene −137 G/C polymorphism (rs187238) with the corresponding unadjusted and adjusted odds ratios (OR)

	n	CG/CC (%)	Crude OR (95% CI)	*p*-value	Adjusted OR (95% CI)[a]	*p*-value
Controls[b]	388	50.3				
SCD	275	39.6	0.65 (0.48–0.89)	0.007	0.49 (0.31–0.77)	0.002
Non-CHD[c]	55	32.7	0.48 (0.27–0.88)	0.016	0.34 (0.13–0.90)	0.030
CHD	220	41.4	0.70 (0.50–0.98)	0.035	0.51 (0.32–0.82)	0.005
MI	154	39.0	0.63 (0.43–0.92)	0.018	0.48 (0.28–0.81)	0.006
AMI	101	36.6	0.57 (0.36–0.90)	0.015	0.50 (0.28–0.90)	0.021
AMI with thrombus	58	32.8	0.48 (0.27–0.86)	0.014	0.40 (0.18–0.87)	0.021
AMI without thrombus	43	41.9	0.71 (0.38–1.35)	0.298	0.62 (0.29–1.33)	0.220

Abbreviations: IL-18, interleukin 18; CHD, coronary hearth disease; SCD, sudden cardiac death; (A)MI, (acute)myocardial infarction.
[a] Model adjusted with age, body mass index, smoking, hypertension, diabetes, and daily alcohol consumption.
[b] Death due to non-cardiac diseases or unnatural deaths.
[c] Death due to cardiomyopathy, cardiac hypertrophy or dilatation, or to valvular disease in the absence of CHD.

ural causes, 51.8% ($n = 132$) were C allele carriers). A value of $P < 0.05$ was considered statistically significant. The computations were carried out with SPSS for Windows software (Version 13.0, SPSS Inc., USA).

3. Results

3.1. Characteristics of the study subjects

The demographic characteristics of the entire study population are presented in Table 1. The mean age of the study population at the time of death was 53.2 ± 9.5 years (range 33–70 years). Of the 663 men, 359 (54.1%) had the wild-type GG-genotype, 261 (39.4%) were heterozygotes (CG), and 43 (6.5%) were CC-homozygotes. The allelic frequencies of the C-allele and G-allele were 0.262 and 0.738, respectively. The genotype distribution was in line with the Hardy–Weinberg equilibrium. The genotype groups did not have any significant associations with any common CAD risk factors or with the occurrence of CAD within the whole study population or among different age groups, nor were any significant differences observed between subjects with or without interview data available.

3.2. IL-18 polymorphism and the occurrence of SCD from any cause

In the entire study population, the C allele carriers had a lower occurrence rate for SCD than GG homozygotes [crude odds ratio, 0.65; 95% confidence interval (CI) 0.48–0.89; $p = 0.007$]. This association became more significant when the analysis was adjusted for the interview data (adjusted odds ratio 0.49; 95% CI 0.31–0.77; $p = 0.002$).

3.3. IL-18 polymorphism and SCD in subjects with or without underlying CHD

The odds ratios between the IL-18 genotype groups for different phenotypes of SCD are presented in Table 2. The C allele carriers had a significantly lower risk for SCD due to CHD (adjusted odds ratio, 0.51; 95% CI 0.32–0.82; $p = 0.005$) and for SCD caused by non-coronary heart diseases (adjusted odds ratio, 0.34; 95% CI 0.13–0.90, $p = 0.030$) when compared to the GG homozygotes. The association between IL-18 genotype group and SCD due to CHD remained significant when the control population was limited to men with autopsy verified CAD ($n = 92$) (adjusted OR, 0.45; 95% CI 0.24–0.85; $p = 0.014$)

Similarly, the C allele carriers had a lower occurrence rate for SCD due to MI (adjusted odds ratio, 0.48; 95% CI 0.28–0.81; $p = 0.006$). They were also at a significantly lower risk for AMI (adjusted odds ratio, 0.50; 95% CI 0.28–0.90; $p = 0.021$). This difference became even more pronounced when the analysis was limited to the AMI victims who had suffered an autopsy-verified coronary thrombus (adjusted odds ratio, 0.40; 95% CI 0.18–0.87; $p = 0.021$).

3.4. IL-18 polymorphism and the age-dependency of SCD

In logistic regression analyses, the risk of the C allele carriers for SCD from any cause, and for almost all other subtypes (see Section 3.3 and Table 2) of SCD, was lower than that of the GG homozygotes. These associations were independent of age (data not shown). However, there was a significant genotype-by-age interaction with the risk of SCD due to MI (crude age-by-genotype group interaction $p = 0.028$ and corresponding adjusted interaction $p = 0.037$ by logistic regression analysis). Therefore, we formed two categories of equal size by dividing the overall study population by the mean age (53 years). In the younger age group (age <53 years), the C allele was strongly protective against SCD due to MI: of the 35 younger men, 26 (74.3%) were GG homozygotes and only 9 were (25.7%) C allele carriers (crude odds ratio, 0.34; 95% CI 0.14–0.68; $p = 0.004$ and adjusted odds ratio, 0.16; 95% CI 0.05–0.51; $p = 0.002$). In the older age group, 68 (57.1%) of the 119 men who had died of SCD due to MI were GG homozygous and 51 (42.9%) were C allele carriers. This difference was not statistically significant.

3.5. IL-18 and SCD in separate and independent autopsy series

The C allele carriers seemed to be underrepresented among SCD victims in both independent autopsy series collected 10 years apart (A and B, please see Section 2 for the description of the autopsy series. In the A series, the difference was statistically significant ($p = 0.039$ by two sided exact Pearson's χ^2-test). In the B series, there was a similar but not significant trend ($p = 0.085$ by two sided exact Pearson's χ^2-test) (Fig. 1). IL-18 genotype associated with the occurrence of various phenotypes of SCD similarly in both series, but the associations were not significant due to smaller sample sizes. The major conclusions are therefore based on combined data from series A and B.

4. Discussion

The major result of the present study was that a promoter region SNP (G-to-C) of the IL-18 gene at position −137 (rs187238) associates with the occurrence (rate) of SCD. The C allele carriers had a lower occurrence rate for SCD when compared to the GG homozygotes. As a confirmation of this result, the same trend was evident in two independent autopsy series collected 10 years apart, which were combined to form the present study population. The C allele carriers remained at a lower risk for SCD caused by CHD and by non-coronary heart diseases even after stratification.

One reason for the selection of the IL-18 gene as a candidate gene for SCD was its clearly demonstrated functionality. Previously, this polymorphism has been shown to regulate the production of IL-18 from peripheral blood monocytes [23].

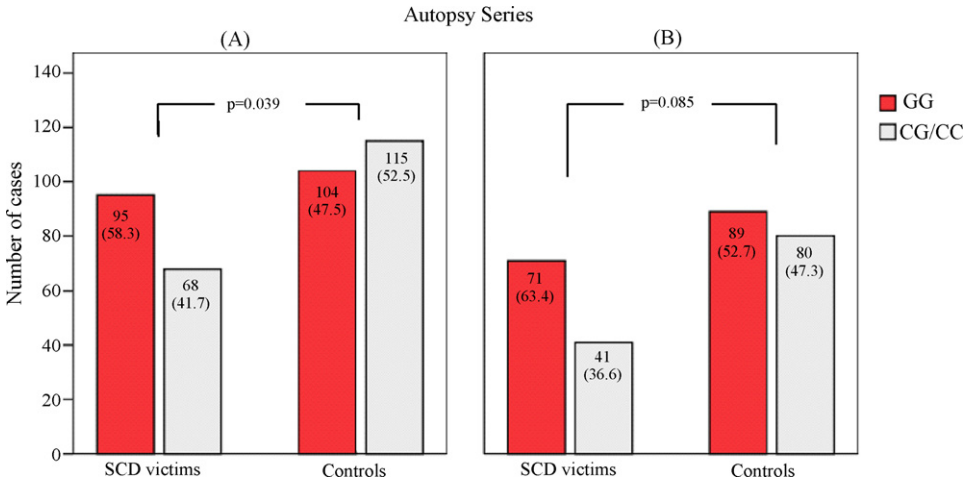

Fig. 1. The number of sudden cardiac death (SCD) victims and those who had died from other causes such as other diseases or unnatural death (Controls) by IL-18 genotype group in two consecutive and prospective autopsy series (A series 1981–1982 and B series 1991–1992). *p*-values for both series were obtained by employing the two-sided exact Pearson chi-square test. Numbers in parentheses are %.

Furthermore, Liang et al. [22] found this polymorphism to associate with decreased transcriptional activity of the IL-18 gene. The results of a previous haplotype study by Giedraitis et al. [24] are also in line with this finding, in addition to demonstrating that a change at position −137 from G to C changes the H4TF-1 nuclear factor binding site for an unknown factor found in the GM-CSF promoter. The same haplotype study also showed that the mRNA expression of atherogenic IFN-γ correlated significantly with the expression of IL-18 mRNA in GG homozygous patients but failed to correlate with patients with the C allele [24].

IL-18 is a pro-atherogenic cytokine associated with the occurrence of various cardiac complications [8–11,30]. In light of these previous findings, we can suggest that the decreased occurrence of SCD in the C allele carriers in our study is most likely associated with their impaired IL-18 production. Previously high IL-18 levels have been associated with the occurrence angina pectoris, MI, and fatal coronary events [9–13]. Furthermore, in a haplotype study by Tiret et al. [12], genetic variability of the IL-18 gene was shown to associate with serum IL-18 levels and cardiovascular mortality in CAD patients. In this earlier study, one of the haplotypes which were in almost complete linkage with the SNP studied in the present study was not significantly associated with either serum IL-18 levels or cardiovascular mortality. However, a similar tendency towards a lower hazard ratio for cardiovascular mortality was observed. Only one previous study has failed to find any association between plasma IL-18 levels and coronary events [14].

IL-18 has been associated with the many factors promoting the instability of atherosclerotic plaques – e.g., secretion of IFN-γ and matrix metalloproteinases [31,32] – in addition to being directly associated with the development of unstable plaques [18]. The instability of atherosclerotic plaques may result in an accelerated development of coronary stenosis [33,34], but it also makes the patients prone to SCD [35].

In present study, we observed that the lower risk of the C allele carriers for SCD due to MI was age dependent. After age stratification we found that younger (<53 years) middle-aged C allele carriers had significantly lower occurrence rate for SCD due to MI when compared to the GG homozygotes. This effect was no longer seen among older men. Previous studies have shown that genetic susceptibility to atherosclerosis and to death caused by CHD decreases with advancing age [25,26]. Most likely, the burden of accumulating environmental risk factor becomes more dominant with age than the effect of genetic factors.

In our study, the majority of the non-coronary SCDs were due to cardiomyopathy, hypertrophy, or dilatation of the heart. Interestingly, IL-18 has previously been connected with the pathology of all of these diseases. The literature has reported heart failure patients to have higher circulating IL-18 levels when compared to controls [19,20]. Moreover, IL-18 plasma levels have been associated with the severity of heart failure and its clinical outcome [21]. In a study by Chandrasekar et al. [36], IL-18 was proven to induce cardiomyocyte hypertrophy via increased transcription and expression of atrial natriuretic peptide (ANP). Prior to this, Seta et al. [20] had also reported a positive dose-dependent correlation between IL-18 stimulation and ANP mRNA production. Despite its role in ANP production, IL-18 may increase the risk of heart failure and SCD by increasing the pro-inflammatory response within the myocardium as a result of enhancing the expression of endothelial cell adhesion molecules and the production of such pro-inflammatory mediators as IL-1β, IL-8, tumor necrosis factor α (TNFα), and inducible nitric oxide [21]. Of these mediators, nitric oxide and TNFα are associated with the modulation of contractile function and the loss of cardiomyocytes [21]. Moreover, it has been demonstrated that IL-18 up-regulates membrane Fas ligand expression and may contribute to Fas-mediated apoptosis of Fas-expressing cardiomyocytes [21]. Interestingly, this pathway has been related

to cardiomyocyte apoptosis [37] and even to cardiomyocyte arrythmogenicity [38]. In line with these earlier findings, we observed a clear association between IL-18 polymorphism and the occurrence of SCDs which had not been induced by a predisposing CHD.

The obvious limitation of our study is the absence of information about serum IL-18, cholesterol and other lipid values of the deceased men. Furthermore, we were unable to obtain complete interview data on all subjects due to the fact that our subjects were victims of sudden, unexpected out-of-hospital deaths. All of the subjects were Caucasian men, and these results are thus not directly applicable to women and all ethnic groups as such. The possible confounding effect of medical therapies, for example the use of statins on the results of the present study, is also unclear, because in the present study reliable medical history of the study subjects was not obtained. On the other hand, this study was based on a large number of autopsies with reliable post-mortem verification of various causes of death, coronary narrowing, myocardial infarctions and thrombi. This study includes nearly all SCDs which occurred in the Helsinki area during the time of the autopsy series and, therefore, is quite representative of the population at risk of SCD.

It is generally required that genetic association results be repeatable in two independent samples. One strength of the present study is that it is based on two independent autopsy series collected 10 years apart. The major results were therefore also analyzed separately for these independent series. There was no contradiction in the results of the two separate autopsy series. The same association between IL-18 genotype group and the occurrence of SCD persisted in both independent series, albeit it was not always as significant as it was in the analysis conducted for the entire study population. This difference was due to smaller sample sizes and, therefore, lowers statistical power of the separate series. This led us to base the major conclusions of the study on the combined data from series A and B.

In the present study, the C allele of the IL-18 gene promoter region SNP (−137C/G, rs187238), previously associated with decreased production of IL-18 under inflammatory stimulation, also associates with a lower risk of SCD. This finding supports the hypothesis that inflammatory mechanisms are involved in the development of atherosclerosis and the instigation of fatal cardiac complications and SCD.

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Interleukin 18 gene promoter polymorphism: a link between hypertension and pre-hospital sudden cardiac death: the Helsinki Sudden Death Study

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Aims The interleukin 18 (IL-18) gene has a single nucleotide promoter region (−137) G-to-C polymorphism (rs187238) which leads to attenuated transcriptional activity of the gene and to lower production of pro-atherogenic IL-18. The C allele of this polymorphism is associated with a lower risk of sudden cardiac death (SCD). We examined the process by which this polymorphism alters the risk of SCD and coronary artery disease (CAD) by analysing the interactions between this polymorphism and environmental factors.

Methods and results TaqMan 5′ nuclease assay was used to genotype the study population of the Helsinki Sudden Death Study, comprising medicolegal autopsies of 700 men. According to adjusted logistic regression analysis, there was a significant interaction between IL-18 genotype and hypertension impacting on the risk of SCD due to coronary heart disease (CHD) ($P = 0.011$) and the severity of autopsy-verified CAD ($P = 0.026$). Among GG homozygotes, hypertension was a major risk factor for SCD due to CHD [adjusted odds ratio (OR) 3.75 with 95% CI 1.78–7.91, $P < 0.001$] and hypertension also associated with larger coronary atherosclerotic plaque areas ($P = 0.002$) and the occurrence of complicated plaques (adjusted OR 8.38 with 95% CI 2.39–29.33, $P < 0.001$). Among C allele carriers, hypertension was not a significant risk factor for CHD-related SCD or CAD and did not associate with the development of coronary atherosclerotic plaques. According to gene expression analysis of atherosclerotic tissue samples obtained from live patients, hypertension also interacted significantly with IL-18 genotype affecting the expression of IL-18 ($P = 0.030$) mRNA and interferon-γ mRNA ($P = 0.004$).

Conclusion Hypertension interacts with IL-18 gene promoter −137 G/C polymorphism, affecting the risk of SCD and the development of coronary atherosclerosis.

Keywords Genetics • Sudden cardiac death • Hypertension • Inflammation • Polymorphism • Coronary heart disease

Introduction

Sudden cardiac death (SCD) is a major killer in the developed nations.[1–3] For example, according to a report by the US Center of Disease Control (CDC), a total of 462 340 SCDs occurred in the USA in 1999, and 341 780 (74%) of these cases were classified as out-of-hospital deaths.[1] The underlying cause of most SCDs (80%) is pre-existing coronary heart disease (CHD),

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expressed as acute coronary syndrome, myocardial scarring due to a previous infarction or heart failure.[3]

The major risk factors for CHD include hypertension, smoking, male sex, obesity, and diabetes.[4] Many of the same factors, such as smoking, hypertension, and sex, also increase the risk of SCD, but on an individual scale the risk of SCD is hard to predict.[5–7] Even less is known about the specific genetic factors associated with SCD, although family history of SCD, primary cardiac arrest, and myocardial infarction (MI) are known to increase the risk.[7–9] The problem most likely lies in the complex aetiology of CHD and different phenotypes of SCD.[10] The interactions between traditional risk factors (e.g. hypertension and smoking) and genetic variation may explain individual differences in the predisposition for coronary artery disease (CAD) and SCD.

The human interleukin 18 (IL-18) gene has a common single nucleotide polymorphism (SNP, rs187238) located at the promoter region in position −137 (G/C). We have previously shown that the C allele carriers of this polymorphism have a lower risk for SCD caused by CHD.[11] This polymorphism is in complete linkage disequilibrium with two other SNPs (+113T/G and +127C/T),[12] and it decreases the transcriptional activity of the gene and the production of IL-18.[12–14] IL-18 is a pro-inflammatory and pro-atherogenic cytokine, and it has been directly associated with the development of unstable atherosclerotic plaques.[15–18] The production of IL-18 leads to the secretion of interferon-γ (IFN-γ) and matrix metalloproteinases (MMPs), factors which are associated with instability in atherosclerotic plaques.[19,20] The development of unstable plaques increases the risk of acute cardiac events and may also lead to the development of coronary stenosis.[21–23] Similarly, IL-18 genetic variability and increased plasma concentrations of IL-18 have previously been associated with the occurrence of MI and acute fatal coronary events.[16,24–28]

The mechanism by which IL-18 genotype affects the occurrence of SCD remains unclear. It is possible that this genetic effect is due to an altered predisposition to traditional risk factors. We decided to examine whether traditional risk factors interact with IL-18 thus possibly impacting the occurrence of autopsy-verified CAD and SCD.

Methods

Subjects

Current study consists of two separate studies: The Helsinki Sudden Death Study (HSDS) and the Tampere Vascular Study (TVS). HSDS is an autopsy study comprising two independent autopsy series collected in 1981–1982 (A series, $n = 400$), and 1991–1992 (B series, $n = 300$). The autopsies were performed on Finnish Caucasian men (mean age of 53 years, range 33–70 years). All the men were subjected to a medicolegal autopsy because of their unexpected and often unwitnessed sudden death occurring outside a hospital. A medicolegal autopsy was not performed if the deceased had a previous clinical diagnosis of CAD complicated by, for example, severe chronic heart failure. All medicolegal autopsies were performed according to the same protocol at the Department of Forensic Medicine at the University of Helsinki, and the study was approved by the Ethics Committees of the Department and the University. The details of this study have been described in detail earlier.[29] The TVS material consists of arterial samples of atherosclerotic lesions (types V–VI)[30] from carotid arteries ($n = 9$), femoral arteries ($n = 4$), and the aortas ($n = 7$) of 20 patients subjected to vascular surgical procedures and healthy control samples of the left interior thoracic artery and the left interior mammary artery of patients ($n = 6$) undergoing coronary artery bypass surgery in Divisions of Vascular and Cardiothoracic Surgery, Tampere University Hospital.[31] Genome wide expression analysis (GWEA) was performed on all the samples. Of the patients, 15 were GG homozygotes, 9 GC heterozygotes, and 1 was a CC homozygote. The patients were divided into two groups: GG homozygotes and C allele carriers. The expression levels of IL-18 receptor α (IL-18Rα), IFN-γ, and IL-12 have been previously measured using real-time polymerase chain reaction (PCR) for a separate publication, and these measurements were incorporated into the data to obtain more accurate results.[31]

Genotyping of the IL-18 polymorphism

The DNA samples were genotyped using the 5′ nuclease assay for allelic discrimination with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Genotyping of the IL-18 SNP (rs187238) was successful for 663 (94.7%) of the 700 tissue samples in the HSDS and 25 (96.2%) of the 26 tissue samples in the TVS. The method of genotyping by PCR has been described in detail earlier.[11] Random duplicates were used as a quality control.

Post mortem verification and classification of varying causes underlying sudden cardiac death

Of all the SCDs in the present material, 220 (80%) were caused by CHD. Most frequently, the cause of death was acute or old MI ($n = 154$). Of these deaths, 101 were caused by an acute MI, 53 were associated with the scar of a prior MI and were most probably due to arrhythmias, and 64 men suffered a CHD-related SCD with no MI aetiology. Myocardial infarction was verified by means of a macroscopic and histological examination of the myocardium. The presence of a coronary thrombus was recorded during autopsy, when coronary arteries were opened longitudinally.

Autopsy measurement of coronary stenosis and atherosclerosis

At autopsy, a silicon rubber cast was made from the three main epicardial coronary arteries (the left anterior descending, left circumflex, and right coronary artery). The degree of coronary stenosis was determined from these rubber models. The cut-off value for the classification of CAD was over 50% stenosis in any part of one or several main coronary arteries. In order to analyse the areas of different types of atherosclerotic lesions and overall atherosclerosis, the coronary arteries were fixed in 10% buffered formalin and stained for fat by the Sudan IV staining method. The methods of these measurements have been described previously.[32]

Collection of risk factor data in the HSDS

A close friend, spouse, or relative of the deceased was interviewed by means of a questionnaire to obtain risk factor data. Complete interview data on risk factors were available in 402 (60.6%) of the 663 cases whose IL-18 genotype was successfully determined.[32] Risk factor data included the following variables: hypertensive (yes/no), diabetic (yes/no), smoker (yes/no) (smokers and ex-smokers were combined into the category of smokers for the statistical analysis), daily alcohol consumption, and body mass index (BMI). Body mass index

was calculated from the autopsy data. The victim was considered hypertensive if his hypertension had been diagnosed clinically by a physician and/or he was medically treated for hypertension, or if it was known that hypertensive blood pressure (BP) values had been measured from the subject prior to his death.

RNA isolation and genome wide expression analysis

The fresh tissue samples ($n = 26$) were immediately soaked to RNA-Later solution (Ambion Inc., Austin, TX, USA) and total-RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and RNAEasy Kit (Qiagen, Valencia, CA, USA). The GWEA Microarray experiments were performed by using Sentrix® Human-8 Expression BeadChips analysing over 23 000 known genes and gene candidates (Illumina, San Diego, CA, USA) according to the instructions given by the manufacturer. BeadChips were scanned with Illumina BeadArray Reader. The method has been more accurately described in our earlier work.[31] The accuracy of Illumina Sentrix® Human-8 Expression BeadChips microarray methodology to measure the gene expression was verified by real-time quantitative TaqMan PCR through quantifying the expression of 20 genes with both methods.[33]

Statistical analyses

Because of the small number of CC homozygotes ($n = 43$, 6.5%) in the HSDS, they were combined with the GC heterozygotes to form a group of C allele carriers for statistical analyses. However, the analyses were also repeated without pooling the genotypes.

In order to compare the characteristics between genotype groups, we used Pearson's χ^2-test for categorical variables and the Mann–Whitney *U* test for continuous variables. Binary logistic regression analysis was used to calculate the IL-18 genotype-by-risk-factor interactions. Analyses for each genotype group by risk factor interaction were carried out with and without adjustment for autopsy data (BMI and age) and interview data (alcohol consumption, smoking, diabetes, and hypertension). Covariates were included in the model in a stepwise manner. Only statistically significant ($P < 0.05$) covariates were accepted in the final adjusted model. If the interaction between IL-18 genotype group and a risk factor was found significant, the effect of the risk factor on the occurrence of SCD was studied separately using unadjusted and adjusted binary logistic regression analysis (stepwise procedure for accepting significant covariates) stratifying the population by IL-18 genotypes.

The control group consisted of men who had died of other diseases and men who had died of unnatural causes. This was included because the IL-18 genotype group did not associate with the rate of deaths caused by non-cardiac-related diseases or unnatural causes.

In the TVS material, the expression levels of IL-18 mRNA and IFN-γ mRNA were compared over IL-18 genotype groups, and patients with or without hypertension using adjusted analysis of variance (ANOVA). All ANOVAs were adjusted with the mRNA expression of surface structures expressed by antigen presenting cells (APC) [CD80 and CD86 transcript variant 1 (v1) and CD86 transcript variant 2 (v2)] and by T-cells (CD4, CD28, and CTLA-4).[34] Macrophages which act also as APCs are major producers of IL-18 and T cells are major producers of IFN-γ.[19] Only significant covariates were selected into the model by a backward elimination procedure. This allowed us to compare the expression of IL-18 and IFN-γ between samples with different inflammatory background, and it was necessary because the current material consist of atherosclerotic samples from different vascular beds with variation in inflammatory background. The expression of the mRNA of these factors is subjected to inflammatory stimuli.[34] Thus, they do not represent directly the quantity of the T-cells or APCs in the samples. However, at the same time, they provide some adjustment on the inflammatory activity within the samples. All analyses were repeated with log-transformation of the continuous variables, but this did not improve the predictive value of the analyses (measured by pseudo R^2-value), and thus the results of the analyses performed with crude values are reported. The study population was divided into two groups by median systolic arterial pressure (140 mmHg) in order to study the effect of hypertension on the dependent variables.

In both studies, the significance *P*-value < 0.05 was considered statistically significant. Values of significance on all non-parametric tests are presented as asymptomatic and two-tailed. The computations were carried out with SPSS for Windows software (Version 14.0, SPSS Inc., TX, USA).

Results

Characteristics of the study subjects in the HSDS

The mean age of the study population at the time of death was 53.2 ± 9.5 years (range 33–70 years). The characteristics of the study population are presented in *Table 1*. The genotype frequencies in the order of GG-GC-CC were: 359 (54.1%), 261 (39.4%), and 43 (6.5%). The allelic frequencies were 0.262 for the C allele and 0.738 for the G allele. The genotype distribution was in accordance with the Hardy–Weinberg equilibrium ($P = 0.89$ by χ^2-test). The genotype groups did not have any significant associations with any common CAD risk factors or with the occurrence of CAD, nor were there any significant differences (observed) between subjects with or without interview data available (data not shown).

The interactions between risk factors and IL-18 gene polymorphism on the risk of sudden cardiac death and coronary artery disease

According to unadjusted binary logistic regression analysis, IL-18 genotype group interacted significantly with hypertension (crude $P = 0.009$ and adjusted $P = 0.011$) as regards to the effect on the risk of SCD. After applying the Bonferroni correction, the interaction remained significant (crude $P = 0.045$ and adjusted $P = 0.055$). When the IL-18 genotype information was entered into the analysis without pooling CG heterozygotes and CC homozygotes, the result remained significant (crude $P = 0.022$ and adjusted $P = 0.029$). Interactions with daily alcohol consumption ($P = 0.600$), BMI ($P = 0.075$), smoking ($P = 0.998$), or diabetes ($P = 0.943$) were not statistically significant.

Similarly, in unadjusted binary regression analysis of the occurrence of CAD, there was a statistically significant interaction between IL-18 genotype group and hypertension ($P = 0.020$), which remained significant after an adjustment for other risk factors ($P = 0.026$). After Bonferroni correction, the interaction was no longer significant (crude $P = 0.100$ and adjusted $P = 0.130$). Interactions with daily alcohol consumption ($P = 0.701$), BMI ($P = 0.833$), smoking ($P = 0.805$), and diabetes ($P = 0.316$) were not statistically significant.

Table 1 General characteristics of the study subjects

	Valid n	All	IL-18 genotype groups		
			GG ($n = 359$)	GC/CC ($n = 304$)	*P*-Value
Age (years)[a]	663	53.2 ± 9.5	53.4 ± 9.5	52.9 ± 9.5	0.56
Body mass index (kg/m^2)[a]	663	24.7 ± 4.8	25.0 ± 4.7	24.3 ± 4.9	0.13
Alcohol use (portions/day)	432	7.6 ± 8.8	7.6 ± 8.6	7.7 ± 9.0	0.91
Hypertensive, *n* (%)	663	101 (15.2%)	62 (17.3%)	39 (12.8%)	0.13
Diabetic, *n* (%)	459	107 (23.3%)	54 (21.3%)	53 (25.7%)	0.32
Smokers, *n* (%)	473	390 (82.5%)	216 (83.1%)	174 (81.7%)	0.72
Coronary artery disease, *n* (%)	639	271 (42.4%)	150 (43.1%)	121 (41.6%)	0.75
Sudden cardiac death victims, *n* (%)[b]	608	220 (36.2%)	129 (40.1%)	91 (31.8%)	0.04

P-values have been derived with analysis of variance, the Mann–Whitney *U* test, and the chi-square test.
[a]Mean ± standard deviation.
[b]Coronary heart disease-related sudden cardiac death victims.

Table 2 Characteristics of hypertensive and normotensive men among GG homozygotes and C allele carriers of the IL-18 -137G/C polymorphism

	GG homozygotes		*P*-Value	C allele carriers		*P*-Value
	Hypertensive ($n = 62$)	Normotensive ($n = 297$)		Hypertensive ($n = 39$)	Normotensive ($n = 265$)	
Age (years)[a]	56.4 (9.5)	53.8 (9.7)	0.007	56.0 (9.4)	52.4 (9.5)	0.029
Body mass index (kg/m^2)[a]	26.8 (4.2)	24.6 (4.7)	0.001	26.5 (4.4)	23.9 (4.9)	0.002
Alcohol use (portions/day)[b]	6.9 (9.4)	7.8 (8.4)	0.533	5.4 (6.1)	8.2 (9.4)	0.081
Diabetic, *n* (%)	16 (25.8)	38 (19.9)	0.373	11 (28.2)	42 (25.1)	0.839
Smokers, *n* (%)	50 (84.7)	166 (82.6)	0.844	30 (78.9)	144 (82.3)	0.646

P-values have been derived with analysis of variance, the Mann–Whitney *U* test, and the chi-square test for categorical variables.
[a]Mean ± standard deviation.
[b]Mean ± standard error of mean.

The effect of hypertension on the risk of sudden cardiac death and coronary artery disease among IL-18 genotypes

Because of the statistically significant genotype-group-by-hypertension interaction, we divided the study population according to IL-18 genotype. Among GG homozygotes and C allele carriers, hypertensive men were statistically significantly older and had higher BMI compared with normotensive men (*Table 2*). The same associations were also observed separately among the 226 GC heterozygotes ($P = 0.023$ for age and $P = 0.001$ for BMI), but no longer among the 43 CC homozygous men ($n = 0.927$ for age and $P = 0.118$ for BMI). No other statistically significant differences were observed between hypertensive and normotensive men in either genotype group. Among SCD victims, 23.9% ($n = 55$) had suffered from hypertension. In the control group, the corresponding number was 10.4% ($n = 43$).

Hypertension was a major risk factor of SCD and CAD among GG homozygotes, but not among combined group of C allele carriers or among GC heterozygotes or CC homozygotes, when the genotypes were analysed separately (*Table 3*). Among GG homozygotes, hypertension associated significantly with SCD due to CHD (adjusted OR 3.75, $P = 0.0005$; *Figure 1*), SCD due to old or acute MIs (SCD due to arrhythmias caused by an old MI scar and/or AMI) (adjusted OR 4.56, $P = 0.0002$), fatal acute MI (adjusted OR 4.69, $P = 0.0004$), and with CAD (adjusted OR 3.08, $P = 0.0027$; *Figure 1*). Among the CC homozygotes, the effect of hypertension on the risk of SCD and CAD was evaluated by Fisher's exact χ^2-test because only five men had suffered from hypertension, one of them had suffered SCD, and only two had CAD. According to the χ^2-test, hypertension was not associated with SCD due to CHD ($P = 0.632$), SCD due to old or acute MI ($P = 1.00$), fatal acute MI ($P = 1.00$), or with CAD ($P = 1.00$).

The effect of hypertension on the different atherosclerotic plaque areas in the HSDS

In order to study how hypertension affects the atherosclerotic plaque formation and composition among IL-18 genotype

Table 3 The effect of hypertension on the risk of sudden cardiac death and coronary artery disease among GG homozygotes, C allele carriers and GC heterozygotes of the IL-18 −137 (G/C) polymorphism

	Unadjusted OR (95% CI)	*P*-Value	Adjusted OR (95% CI)	*P*-Value
GG homozygotes				
SCD by CHD	4.21 (2.28–7.78)	<0.00001	3.75 (1.78–7.91)	0.0005
SCD with MI aetiology	5.02 (2.63–9.57)	<0.000001	4.56 (2.05–10.11)	0.0002
SCD by fatal acute MI	4.75 (2.33–9.68)	0.00002	4.69 (2.01–10.95)	0.0004
CAD	4.42 (2.40–8.13)	<0.000002	3.08 (1.48–6.41)	0.0027
CG heterozygotes				
SCD by CHD	0.35 (0.04–3.45)	0.369	0.75 (0.28–2.00)	0.565
SCD with MI aetiology	1.69 (0.71–3.99)	0.233	1.06 (0.36–3.10)	0.922
SCD by fatal acute MI	1.88 (0.68–5.14)	0.222	1.30 (0.40–4.22)	0.661
CAD	1.54 (0.73–3.23)	0.259	0.83 (0.32–2.11)	0.687
C allele carriers				
SCD by CHD	1.19 (0.57–2.46)	0.643	0.78 (0.32–1.91)	0.583
SCD with MI aetiology	1.43 (0.64–3.18)	0.390	0.88 (0.32–2.40)	0.805
SCD by fatal acute MI	1.66 (0.66–4.20)	0.282	1.14 (0.39–3.37)	0.814
CAD	1.48 (0.73–2.97)	0.276	0.78 (0.32–1.89)	0.581

P-values have been derived with regression analysis. Autopsy data (BMI and age) and interview data (alcohol consumption, smoking, diabetes, and hypertension) were used to adjust the analyses. Covariates were included in the model in a stepwise manner. Only statistically significant ($P < 0.05$) covariates were accepted in the final adjusted model. SCD, sudden cardiac death; CHD, coronary heart disease; MI, myocardial infarction; CAD, coronary artery disease.

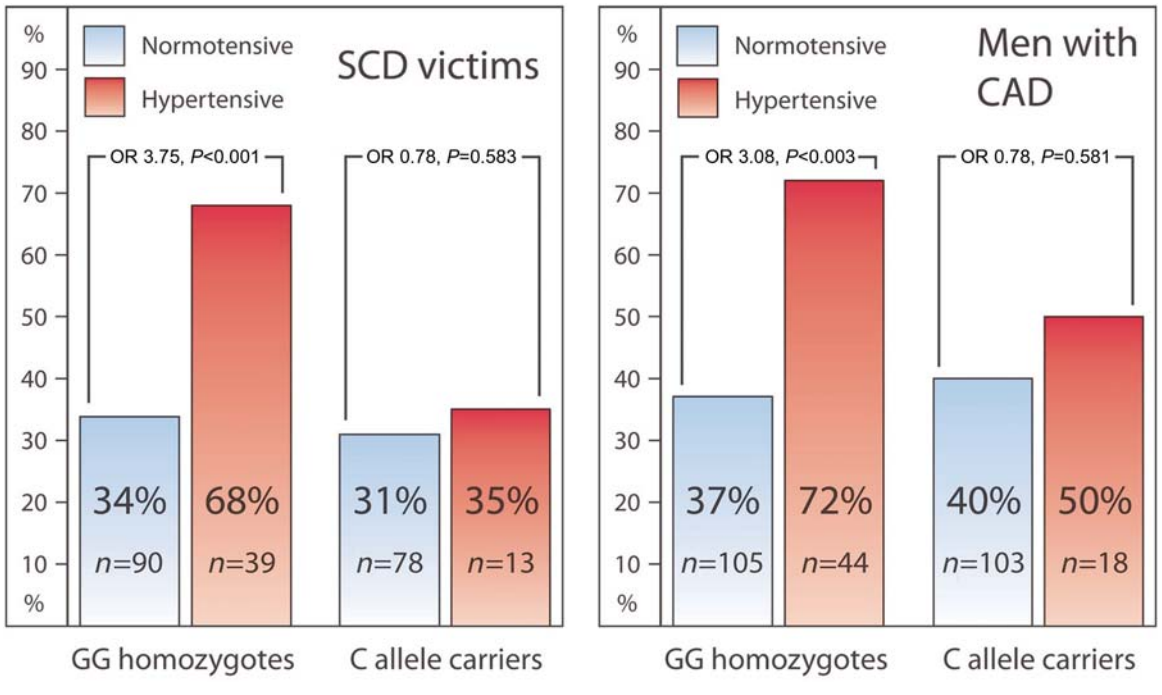

Figure 1 The effect of hypertension on the risk of sudden cardiac death (SCD) and coronary artery disease (CAD) among the GG homozygotes and C allele carriers of the IL-18 gene −137G/C polymorphism. Odds ratios (OR) are derived by logistic regression adjusted with traditional risk factors for CAD.

groups, we focused on the control group. This was done to avoid the evident selection bias presented by the significantly different mortality to CHD-related SCD among different IL-18 genotype groups. Among GG homozygotes, according to ANOVA adjusted with autopsy data (BMI and age), the coronary arteries of hypertensive men were more afflicted by overall atherosclerosis

(measured as the relative surface area covered by atherosclerotic lesions) [11.71% (SE 1.72) vs. 6.18% (SE 0.48), $P = 0.002$] when compared with normotensive men. Hypertensive men also had larger relative areas of fatty streaks [7.14% (SE 1.34) vs. 4.21% (SE 0.40), $P = 0.026$] and fibrotic plaques [4.57% (SE 0.66) vs. 1.97% (SE 0.21), $P < 0.001$]. According to adjusted regression analysis, hypertension was also a significant risk factor for the occurrence of complicated plaques (OR 8.38 with 95% CI 2.39–29.33, $P < 0.001$, covariates: BMI and age).

Among C allele carriers, hypertension was not associated with the overall atherosclerotic burden [9.34% (SE 1.08) vs. 8.38% (SE 0.72), $P = 0.701$], relative surface areas of fatty streaks [6.80% (SE 1.03) vs. 5.17% (SE 0.50), $P = 0.306$], surface areas of fibrotic plaques [2.53% (SE 0.62) vs. 3.21% (SE 0.43), $P = 0.268$], or with the occurrence of complicated plaques (OR 2.07 with 95% CI 0.73–5.89, $P = 0.174$). In addition, when the groups of GC heterozygotes and CC homozygotes were analysed separately, hypertension did not associate significantly with the plaque areas or with the occurrence of complicated plaques.

The effect of IL-18 genotype and hypertension on the expression of IL-18 and IFN-γ in atherosclerotic arterial samples (TVS)

According to adjusted ANOVA, the effect of hypertension on the expressions of IL-18 mRNA and IFN- γ mRNA were modulated by the IL-18 genotype [$P = 0.030$ (IL-18) and $P = 0.004$ (IFN- γ) for the interactions]. Among GG homozygotes, hypertension did not associate with the expression level of intracellular precursor IL-18 mRNA (0.89-fold increase, $P = 0.217$), whereas among C allele carriers hypertension augmented the expression of intracellular precursor IL-18 mRNA (1.31-fold increase, $P = 0.001$). Almost inversely, among GG homozygotes hypertension associated with a higher expression level of pro-atherosclerotic IFN-γ (1.58-fold increase, $P = 0.006$), whereas among C allele carriers hypertension seemed to associate with a lower expression level of IFN-γ (0.58-fold increase, $P = 0.047$).

In the atherosclerotic samples obtained from GG homozygotes, the expression levels of the intracellular precursor IL-18 mRNA were lower (0.68-fold increase, $P < 0.001$), but the expression levels of IFN-γ mRNA were higher (1.85-fold increase, $P < 0.001$) when compared with the atherosclerotic samples of the C allele carriers. Furthermore, according to unadjusted Mann–Whitney U test, the expression levels of the IL-18 mRNA seemed to be significantly higher in the healthy control samples when compared with the atherosclerotic samples (1.33-fold increase, $P = 0.052$).

Significant covariates associating with the expression of IL-18 mRNA were: CD80 ($P = 0.006$), CD86v1 ($P = 0.012$), CD4 ($P = 0.013$), CD86v2 ($P = 0.010$), Caspase-1 variant α ($P = 0.023$), and Caspase-1 variant ϵ ($P = 0.009$). The expression levels of Caspase-1 (Casp-1) variants were also introduced to this model because Casp-1 cleaves the IL-18 precursor protein into biologically active IL-18 resulting in the secretion of mature IL-18 protein. This intrinsic processing likely affects the amount of intracellular IL-18 precursor mRNA, which has a stable mRNA structure and is constitutively and intracellulary stored.[35]

Significant covariates associating with the expression of IFN-γ mRNA were: CD80 ($P < 0.001$), CD86v1 ($P < 0.001$), CD4 ($P = 0.014$), CTLA-4 ($P = 0.010$), IL-12 ($P = 0.004$), and IL-18Rα ($P = 0.003$). The expression levels of IL-12 and IL-18Rα were also included into this model because IL-12 in synergy with IL-18 augments the production of IFN-γ and IL-18Rα plays an important role in IL-18 signalling.[35] The expression of IL-18 binding protein, Casp-1vα, and Casp-1vϵ was also added to the analysis, but these covariates were not significant in the model and thus were not used for further adjustment.

Discussion

According to the present study, the IL-18 genotype significantly modifies the association of arterial hypertension with the occurrence of SCD due to CHD. We found that arterial hypertension was a major risk factor for SCD and severe CAD among GG homozygotes, but not among C allele carriers of the −137 (G/C) polymorphism (rs187238). When we studied the risk of SCD due to MI (SCD due to arrhythmias caused by an old MI scar and/or AMI) or the risk of fatal acute MI, the results were even more pronounced despite the lower sample sizes of the subgroups. The results of the autopsy data of the independent control group showed that hypertension associates significantly with development of overall coronary atherosclerosis as well as with the occurrence of complicated plaques among GG homozygotes, but not among C allele carriers. To confirm and investigate further this interaction, we performed detailed analysis of gene expression in atherosclerotic samples from live patients of the TV study. The results showed that the effect of hypertension on the expressions of IL-18 mRNA and IFN-γ mRNA is modulated by the IL-18 genotype.

Previously, it has been shown that circulating IL-18 levels are higher among patients with unstable angina pectoris and among MI victims.[24,26,36] Among CAD patients, higher IL-18 levels are associated with a higher risk of a future fatal coronary event.[25] Higher IL-18 levels have also been found to predict future coronary events among healthy middle-aged men.[37] The studied promoter G-to-C polymorphism of the IL-18 gene has been proven functional with the C allele, associating with lower transcriptional activity of the IL-18 gene and thus resulting in an attenuated production of IL-18.[12–14] Supporting these previous results, recent haplotype studies have shown that the variation in the IL-18 gene associates with circulating IL-18 levels.[16,38] The same studies also showed that a major haplotype which is the sole carrier of the −137C allele associates with lower IL-18 levels and with cardiovascular mortality during a follow-up of 5.9 years. One other major haplotype, the only one carrying the G allele of the A-to-G polymorphism at position +183, was similarly associated with the same endpoints. Other haplotypes or SNPs of this gene were not found to associate with circulating IL-18 levels or cardiovascular mortality.[16,38] Based on these previous results, it seems clear that IL-18 gene variation affects cardiovascular mortality. However, according to our new results, the

association between this polymorphism and CHD-related SCD seems more complex.

High BP values have previously been associated with high circulating IL-18 concentrations.[39,40] Furthermore, circulating IL-18 values along with greater intima media thickness values could be treated effectively by lowering morning BP peaks with a 12 month treatment with the unselective β-blocker carvedilol.[40] Nevertheless, this does not explain why hypertension seems to associate with the risk for SCD only among IL-18 GG homozygotes.

In the present study, we found that IL-18 genotype significantly modulates the effect of hypertension on the expression of IL-18 mRNA and IFN-γ mRNA in atherosclerotic tissue samples obtained from live patients. It is especially noteworthy that among GG homozygotes, hypertension associated with higher expression level of the pro-atherosclerotic IFN-γ. In the group of C allele carriers, hypertension actually associated with lower expression level of IFN-γ despite the fact that among them hypertension did associate with a higher expression level of the intracellular IL-18 mRNA. Previously, it has been shown that β_2-adrenergic receptor stimulation activates both basal and inducible IL-18 promoter activity in endothelial cells leading to upregulation of IL-18 mRNA expression, and more stable IL-18 mRNA.[41] Also, it has been shown in an *in vitro* study that aldosterone in cardiomyocytes, through the production of angiotensin II and endothelin-1, increase IL-18 mRNA and protein expression.[42] Unfortunately, it is not possible to deduct more conclusions from our expression analysis results because the regulation of the different genetic pathways is highly complex, and thus more accurate and extensive data would be required.

Hypertension might also have a more general effect on the occurrence of SCD and formation of atherosclerosis. The overall pro-inflammatory effects of higher BP on the vessel wall[43] may lead to increased production of mature IL-18 by endothelial cells and macrophages. Also the C allele carriers might be better protected against this because of their impaired production of IL-18.[12–14] Furthermore, Sahar *et al.*[44] have shown that pre-treatment of vascular smooth muscle cells with angiotensin II increases the expression of the ligand-binding subunit of IL-18Rα of the IL-18 receptor, and thus leads to IL-18-mediated induction of the transcription factor nuclear factor-κB (pathway leading to the expression of several pro-inflammatory cytokines and chemokines and the production of pro-inflammatory cytokines such as IL-6 and IL-8).

A major limitation of our study is the fact that we do not have the genotype data concerning the +183A/G polymorphism (rs5744292) which has also been found to significantly affect circulating IL-18 levels. However, as the haplotype carrying the +183G allele is also associated with attenuated production of IL-18, it only emphasizes our results because these two SNPs do not co-exist in a same haplotype.[16] Therefore, among GG homozygotes of the −137G/C polymorphism, there are more G allele carriers of the +183A/G polymorphism than among the C allele carriers.[16,38] Furthermore, we had no data on the serum IL-18, cholesterol, and other lipid values of the deceased men. In addition, we were unable to obtain complete interview data on all subjects, and thus we were unable to analyse the possible effect of pre-mortem medication on the IL-18 genotype-by-hypertension interaction. The information on hypertension in the present study was based on interview data which cannot be considered a valid data based on recorded clinical measurements. However, previous studies have shown that the validity of interview data concerning hypertension is high,[45,46] and moreover, it is more likely for hypertensive men to have been misdiagnosed as normotensive, than normotensive men as hypertensive. This suggests that the reliability of our results would not be significantly weakened by this limitation. More likely, our results would be more pronounced if the accuracy of the diagnosis would be better. Other limitations concerning the study population have been discussed earlier.[11] The strength of the present study is the fact that it comprises almost all SCDs which occurred in the Helsinki region during the time of the collection of the autopsy series, making our study population a valid representative of a population at the risk of SCD.

In conclusion, our results suggest that the effect of hypertension on the development of CHD leading to an untimely SCD and the development of overall coronary atherosclerosis as well as the occurrence of complicated plaques is modulated by the IL-18 gene promoter region −137G/C polymorphism. Hypertension seems to be a major risk factor for these endpoints among GG homozygous men, but not among C allele carriers.

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