AUNI COLLINGS

Variation in Connexin37, Methylenetetrahydrofolate Reductase and Upstream Transcription Factor 1 Genes in Relation to Early Atherosclerotic Vascular Changes in Young Adults

The Cardiovascular Risk
in Young Finns Study

■

ACADEMIC DISSERTATION
To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the Auditorium of Finn-Medi 1,
Biokatu 6, Tampere, on September 26th, 2008, at 12 o'clock.

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ACADEMIC DISSERTATION
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To my family

TIIVISTELMÄ

Tausta: Valtimonkovettumatauti on monitekijäinen sairaus, jolla on suuri kansanterveydellinen vaikutus sydän- ja verisuonitautisairastavuuteen ja kuolleisuuteen. Useat riskitekijät vaikuttavat valtimonkovettumataudin kulkuun, ja tiedetään, että altistuminen niille nuorena vaikuttaa myöhemmin sepelvaltimotaudin ja aivoverenkiertohäiriöiden kliinisten tapahtumien ilmenemiseen ja riskiin. Riskitekijöiden vaikutusta ja tasoja säätelevät myös useat perintötekijät. Näistä tekijöistä yksi on aukkoliitosten rakenneproteiinia Connexin 37 ilmentävä geeni *Cx37*. Connexin 37 -proteiini osallistuu aukkoliitoksissa solujen väliseen kommunikaatioon. Sen on myös havaittu vaikuttavan valkosolujen tarttumiseen verisuonen seinämään, mikä voi vaikuttaa varhaisten valtimonkovettumatautimuutoksien syntyyn verisuonissa. Metyleenitetrahydrofolaattireduktaasi (MTHFR) on avainentsyymi homokysteiinin aineenvaihduntareaktioketjussa, ja sen MTHFR-geenin perinnöllinen vaihtelu säätelee homokysteiinin pitoisuutta verenkierrossa. Seerumin suurentunutta homokysteiinipitoisuutta pidetään yhtenä riskitekijänä valtimonkovettumataudin kehittymisessä. *USF1* on yli 40:n sokeri- ja rasva-aineenvaihduntaan vaikuttavan geenin luentaan (transkriptioon) vaikuttava perintötekijä.

Tavoitteet: Väitöskirjatyön tavoitteena on ollut tutkia näiden kolmen edellä mainitun ehdokasgeenin variaatioiden (*Cx37* rs1764391, *MTHFR* rs1801133, *USF1* rs 3737787, rs2516838 ja rs2073658) yhteyksiä ultraäänimenetelmällä mitattaviin varhaisiin verisuonimuutoksiin sekä biokemiallisiin riskitekijöihin. Mitatut verisuonisuureet olivat kaulavaltimon seinämäpaksuus ja joustavuus sekä olkavaltimon sisäkalvon eli endoteelin toiminta.

Menetelmät: Tutkimus on osa laajaa Lasten sepelvaltimotaudin riskitekijät monikeskustutkimusta, joka on aloitettu vuonna 1980. Vuonna 2001 21-vuotisseurantatutkimukseen osallistui 2 283 henkilöä (63,5 % alkuperäisestä kohortista), jotka olivat iältään 24–39-vuotiaita. Tutkittavilta kerättiin riskitekijä- ja terveystiedot, heille tehtiin laaja joukko fysikaalisia mittauksia ja ultraäänitutkimukset, ja heiltä otettiin verinäytteet sekä biokemiallisia tutkimuksia että geenianalyysejä varten.

Tulokset: *Cx37* C1019T (rs1764391) -polymorfismi ei assosioitunut mitattuihin varhaisiin verisuonimuutoksiin (osatyö I). Homokysteiinin ja tupakoinnin vaikutus kaulavaltimon joustavuuteen ja olkavaltimon sisäkalvon toimintaan riippui *Cx37*-genotyypistä. Tämä *Cx37*:n ja riskitekijän välinen yhteisvaikutus (interaktio) osoittaa, että *Cx37* modifioi homokysteiinin ja tupakoinnin riskivaikutusta kyseisiin valtimomuutoksiin (osatyö II). Keskimääräiset seerumin homokysteiinipitoisuudet olivat korkeimmat henkilöillä joilla oli *MTHFR* C677T (rs1801133) TT-

genotyyppi, verrattuna muihin genotyyppeihin. T-alleeli oli myös yhteydessä kaulavaltimon joustavuuteen miehillä (osatyö III). *USF1*-geenin polymorfismeista kaksi (rs3737787 ja rs2516838) oli yhteydessä kaulavaltimon seinämäpaksuuteen: usf1s1:n (rs3737787) alleeli A mataliin arvoihin ja usf1s8:n (rs2516838) alleeli G korkeisiin arvoihin. Näiden lisäksi *USF1*-haplotyypypin (Hp2 G-C-G) kopioiden määrä (0, 1, 2) oli asteittain yhteydessä lisääntyneeseen kaulavaltimon seinämäpaksuuteen (osatyö IV).

Johtopäätökset: Tulosten perusteella tutkittu *MTHFR* (rs1801133) -polymorfismi säätelee seerumin homokysteiinipitoisuutta jo terveillä nuorilla aikuisilla, *MTHFR* ja *USF1*-geenivariaatioilla saattaa olla vaikutusta varhaisten oireettomien valtimomuutosten syntyyn. *Cx37*-polymorfismi mahdollisesti muuntaa homokysteiinin ja tupakoinnin riskivaikutusta suhteessa kaulavaltimon joustavuuteen ja olkavaltimon endoteelin toimintaan.

ABSTRACT

Background: Atherosclerosis is a complex multifactorial disease which has a profound public health influence on cardiovascular disease morbidity and mortality worldwide. There are several interacting environmental, biochemical and genetic risk factors that affect the progression of atherosclerosis and cardiovascular diseases (CVD). Exposure to these risk factors in early childhood may predispose to an increased risk of stroke and CVDs later in life. The genes involved in the aetiology of atherosclerosis can have an effect either directly in the artery wall or their common genetic variability may regulate the levels of biochemical CVD risk factors. One candidate gene for increased atherosclerosis risk is Connexin 37 (*Cx37*), which belongs to the connexin protein family and works as a functional gap junction protein. Cx37 mediates cellular communication and leukocyte adhesion to the vascular wall and may thus be involved in the initiation of atherosclerotic lesions. Methylenetetrahydrofolate reductase (MTHFR) is an enzyme which has a key role in the metabolic pathway involving homocysteine. A high serum homocysteine concentration is considered a risk factor for atherosclerosis. The upstream transcription factor 1 (*USF1*) has many links to atherosclerosis by regulating the transcription of more than 40 genes involved in lipid, carbohydrate and inflammatory pathways. *Cx37*, *MTHFR* and *USF1* are interesting candidate genes that may affect different atherosclerotic processes.

Aims: The study investigated the associations of single nucleotide polymorphisms in these candidate genes, *Cx37* (rs1764391), *MTHFR* (rs1801133) and *USF1* (rs3737787, rs2516838 and rs2073658), with vascular parameters of subclinical atherosclerosis - i.e. carotid artery intima-media thickness (IMT), carotid artery compliance (CAC) and brachial artery flow-mediated dilatation (FMD) - as well as with biochemical risk factors.

Subjects and methods: This thesis is part of the Cardiovascular Risk in Young Finns Study, a population-based follow-up study, where the first cross-sectional investigation was conducted in 1980. In the 21-year follow-up in 2001, the participating cohort comprised 2,283 subjects (63.5% of the original cohort) aged 24-39 years. The risk factor and health data was collected by means of a standardised questionnaire, physical examinations and carotid and brachial artery ultrasound measurements were performed and blood and DNA samples were collected for biochemical and genetic analyses.

Results: The *Cx37* C1019T (rs1764391) polymorphism did not associate with any of the studied vascular parameters (study I). The effects of homocysteine and smoking on CAC and FMD were found to be modified by the *Cx37* polymorphism. These interactions modified the effect of these

risk factors on the studied subclinical markers of atherosclerosis (study II). Serum homocysteine values were highest in subjects with the *MTHFR* C677T (rs 1801133) TT genotype, when compared to the other genotypes. Carriage of the T allele was also associated with higher CAC values in men (study III). Two polymorphisms of *USF1* (rs3737787 and rs2516838) associated with IMT; the minor allele A of usf1s1 (rs3737787) with low and the minor allele G of usf1s8 (rs2516838) with high IMT values. Moreover, an increasing copy number (0, 1, 2) of one of the *USF1* haplotypes studied (Hp2 G-C-G) was associated with gradually increasing values of carotid IMT (study IV).

Conclusions: These results suggest that the *MTHFR* (rs1801133) polymorphism regulates serum homocysteine levels already in young healthy adults and both *MTHFR* and *USF1* polymorphisms may have impact on the subclinical vascular changes of atherosclerosis. Moreover, the risk effect of homocysteine and smoking on CAC and FMD may be modified by the *Cx37* polymorphism.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications. In the text they are referred to by the Roman numerals I–IV.

I Collings A, Islam S, Juonala M, Rontu R, Kähönen M, Hutri-Kähönen N, Laitinen T, Marniemi J, Viikari JSA, Raitakari OT, Lehtimäki TJ. Associations between connexin37 gene polymorphism and markers of subclinical atherosclerosis: The Cardiovascular Risk in Young Finns Study. Atherosclerosis 2007;95:379-384.

II Collings A, Raitakari OT, Juonala M, Mansikkaniemi K, Kähönen M, Hutri-Kähönen N, Marniemi J, Viikari JSA, Lehtimäki TJ. The influence of smoking and homocysteine on subclinical atherosclerosis is modified by the connexin37 C1019T polymorphism – The Cardiovascular Risk in Young Finns Study. Clin Chem Lab Med 2008;46:1102-1108.

III Collings A, Raitakari OT, Juonala M, Rontu R, Kähönen, M, Hutri-Kähönen N, Rönnemaa T, Marniemi J, Viikari JSA, Lehtimäki TJ. Associations of methylenetetrahydrofolate reductase C677T polymorphism with markers of subclinical atherosclerosis: The Cardiovascular Risk in Young Finns Study. Scand J Clin Lab Invest 2008;68:22-30.

IV Collings A, Höyssä S, Fan M, Kähönen M, Hutri-Kähönen N, Marniemi J, Juonala M, Viikari JSA, Raitakari OT, Lehtimäki TJ. Allelic variants of upstream transcription factor 1 (*USF1*) gene associate with carotid artery intima-media thickness: The Cardiovascular Risk in Young Finns Study. Circ J 2008;72:1158-1164.

ABBREVIATIONS

ANCOVA	analysis of covariance
ANOVA	analysis of variance
apo	apolipoprotein
ATP	adenosinetriphosphate
BMI	body mass index
BP	blood pressure
CAC	carotid artery compliance
CAD	coronary artery disease
cDNA	complementary DNA
CHD	coronary heart disease
CRP	C-reactive protein
CVD	cardiovascular disease
Cx37	connexin37 protein
Cx37	connexin37 gene
D_d	diastolic diameter
DNA	deoxyribonucleic acid
DP	diastolic pressure
D_s	systolic diameter
FCHL	familial combined hyperlipidemia
FH	familial hypercholesterolemia
FMD	flow-mediated dilatation
GJIC	gap junctional intercellular communication
GWAS	genome-wide association study
HDL	high-density lipoprotein
HDL-C	high-density lipoprotein cholesterol
IDL	intermediate-density lipoprotein
IMT	intima-media thickness
LDL	low-density lipoprotein
LDL-C	low-density lipoprotein cholesterol
MI	myocardial infarction
MTHFR	methylenetetrahydrofolate reductase enzyme

MTHFR	methylenetetrahydrofolate reductase gene
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
PCR	polymerase chain reaction
R^2	correlation coefficient
SD	standard deviation
SE	standard error
SMC	smooth muscle cell
SNP	single nucleotide polymorphism
SP	systolic pressure
USF1	upstream transcription factor 1 protein
USF1	upstream transcription factor 1 gene
VLDL	very low density lipoprotein

1 INTRODUCTION

Atherosclerosis is a multifactorial disease of the artery wall. The clinical manifestations of atherosclerosis, including stroke, occlusive peripheral artery disease, coronary artery disease (CAD) and myocardial infarction (MI), are the leading causes of death and illness worldwide (Ross 1999). Atherosclerosis is characterised as a chronic, progressive inflammatory disease, where lipids and fibrous material accumulate in artery walls (Ross 1999, Lusis 2000). Autopsy studies have revealed that the pathogenesis of atherosclerosis begins in childhood and that preatherosclerotic lesions are found in children and young adults (Enos et al. 1953, Newman et al. 1986, Ylä-Herttuala et al. 1986, McGill and McMahan 1998).

Early life exposure to CAD risk factors may have an injuring influence on the vasculature, although clinical atherosclerotic diseases, e.g. stroke and CAD, emerge in middle age or later in life (Enos et al. 1953, Newman et al. 1986, McGill and McMahan 1998). The traditional risk factors of CAD are high age, male sex, high serum total and low-density lipoprotein (LDL) cholesterol concentration as well as diabetes, smoking and hypertension, and they are thought to contribute to roughly half of the disease cases (Lusis et al. 2004).

The asymptomatic phase of atherosclerosis is characterized by impaired endothelial function and a gradual thickening of the arterial wall in response to CAD risk factors (Ross 1999, Glass and Witztum 2001, Libby 2002, Hansson 2005). These common indices used to assess the subclinical state of atherosclerosis are carotid artery intima-media thickness (IMT), carotid artery compliance (CAC) and brachial artery flow-mediated dilatation (FMD) which can all be measured non-invasively by ultrasound. Elevated IMT values have been shown to predict future cardiovascular risk, such as stroke and MI (O'Leary et al. 1999, Chambless et al. 2000), and exposure to risk factors during childhood influences IMT in adulthood (Raitakari et al. 2003). Reduced arterial elasticity has been suggested to be an independent predictor of cardiovascular events (Blacher et al. 1998a, Boutouyrie et al. 2002). Exposure to risk factors during childhood has also been shown to predict decreased CAC in adulthood (Juonala et al. 2005). Early changes in the function of the endothelium can be studied by means of FMD (Celermajer et al. 1992) and dysfunction of the endothelium is considered an early result of atherosclerosis and is represented by impaired FMD (Neunteufl et al. 1997, Juonala et al. 2004a).

Along with several environmental and biochemical risk factors (Hopkins and Williams 1981), cardiovascular disease (CVD) susceptibility is modified by genetic predisposition (Lusis 2000). The important role of genetics is evidenced by familial aggregation of the disease, and

family and twin studies have shown that genetic factors explain ca. 20%–60 % of the observed variation in CVD mortality and morbidity, depending on the study population (Bak et al. 2002, Zdravkovic et al. 2002, Fox et al. 2003, Fischer et al. 2005, Moskau et al. 2005, Wienke et al. 2005, Cassidy-Bushrow et al. 2007).

Several genome-wide linkage and association studies have tried to find a chromosomal region or genes contributing to CVD (Pajukanta et al. 2000, Francke et al. 2001, Broeckel et al. 2002, Harrap et al. 2002, Chiodini and Lewis 2003, Hauser et al. 2004, Wang et al. 2004). Moreover, a large number of small and large-scale candidate gene studies have examined the effect of various genes in CAD-related pathways on CVD morbidity and mortality (Wellcome Trust Case Control Consortium 2007, Helgadottir et al. 2007, Larson et al. 2007, McPherson et al. 2007, Samani et al. 2007, Kathiresan et al. 2008, Willer et al. 2008).

In the present study, we selected three candidate genes due to their important role in the regulation of cell communication, homocysteine, carbohydrate and lipid metabolism and thus their possible role in the modulation of early atherosclerotic changes. From the selected genes, Connexin37 (*Cx37*) encodes for a gap junction protein, which has a polymorphism C1019T (rs1764391) that has been associated with carotid artery plaques (Boerma et al. 1999) and advanced atherosclerotic changes i.e., CAD (Yeh et al. 2001) and MI (Yamada et al. 2002). However, *Cx37* has not previously been studied in context with IMT, CAC or FMD.

A single nucleotide polymorphism (SNP), C677T (rs1801133), in the gene encoding for methylenetetrahydrofolate reductase (MTHFR), an enzyme in the biochemical pathway of methionine, leads to reduced enzyme activity and results in high circulating homocysteine values (Kang et al. 1991, Rosenblatt and Fenton 2001). Controversial results have been published on the *MTHFR* gene polymorphism in relation to IMT, some indicating a positive association (Kawamoto et al. 2001, Pallaud et al. 2001) and others not (McQuillan et al. 1999, Kelemen et al. 2004). There are no previous studies on the effect of this gene variant (rs1801133) on CAC, but in relation to FMD, two articles with negative results have been published after this thesis project was commenced (Pullin et al. 2002, Imamura et al. 2004).

Upstream transcription factor 1 (*USF1*), a new candidate for CAD, was originally shown to be associated with familial combined hyperlipidemia (FCHL) (Pajukanta et al. 2004). This ubiquitously expressed transcription factor has many functions in the regulation of various genes involved in inflammation, carbohydrate and lipid metabolism (Read et al. 1993, Iynedjian 1998, Ribeiro et al. 1999, Salero et al. 2003, Naukkarinen et al. 2005). The *USF1* gene variation has also been recently associated with autopsy-verified coronary atherosclerosis (Kristiansson et al. 2008) and CVD in women (Komulainen et al. 2006). However, to the best of our knowledge there are no

previous studies on the effects of the genetic variation in this gene on the subclinical atherosclerotic vascular indices, i.e., IMT, CAC and FMD. Therefore, the exact role of these three genetic markers in the development of early subclinical atherosclerosis is largely unknown.

The present study elucidated the relationship of the allelic variation of these three candidate genes, *Cx37*, *MTHFR* and *USF1*, with ultrasonographically measured surrogate markers of early atherosclerosis as well as with biochemical risk factors in the Cardiovascular Risk in Young Finns Study, a large ongoing population-based multicentre study on the risk factors of CAD and its determinants in children and adolescents which started in 1980.

2 REVIEW OF THE LITERATURE

2.1 The natural course of atherosclerosis

Atherosclerosis is described as a progressive, chronic inflammatory disease where progression leads to thickening of the inner vessel wall and can eventually lead to obstruction of the arterial lumen (Ross 1999). It causes different disease phenotypes; coronary artery disease, stroke and occlusive peripheral artery disease. Symptoms can be non-existent in the early phase, mild, chronic or acute as in plaque rupture and thrombosis. Atherosclerosis in its different forms is the leading cause of death in the western world accounting for approximately 50% of all deaths (Lusis 2000). As most of the symptomatic patients are middle-aged or elderly and as the disease typically takes years to develop until manifest symptoms arise, the pathogenesis of atherosclerosis starts already in childhood during the first decade of life. Post mortem autopsy studies have revealed that children and young adults have preatherosclerotic lesions in their arteries and that exposure to risk factors in early life are relevant to this initial pathophysiology (Enos et al. 1953, McNamara et al. 1971, Newman et al. 1986, McGill and McMahan 1998). It has been established that an adverse risk factor load during childhood is predictive of future cardiovascular risk in adulthood and it may increase the severity of the disease phenotype (Mahoney et al. 1996, Berenson et al. 1998, McGill et al. 2000, Berenson 2002, Raitakari et al. 2003).

The progression of atherosclerosis is characterized by an accumulation of lipids, T lymphocytes, macrophages and fibrous substances in artery walls. Many animal and human studies on the disease have suggested that atherosclerosis is an inflammatory disease and this reaction is due to responses to harmful agents – risk factors. The theory is known as the "response to injury" hypothesis, emphasising endothelial dysfunction as the initial step in the cascade of the different pathophysiological stages of this chronic inflammatory disease, which is present before any structural changes of the arterial wall occur. (Ross 1993, Ross 1999)

The development of atherosclerosis and its common risk factors are discussed briefly in the following sections.

2.1.1 Structure of the artery wall

Intima. The intima is the innermost layer of the artery wall. It includes the endothelial surface which is the luminal surface comprised of a monolayer of epithelial cells called the endothelium. The subendothelial part of the intima consists of two layers: the inner layer is called the

proteoglycan layer, which contains nonfibrous connective tissue. In this layer there are some macrophages and smooth muscle cells (SMC) of the synthesizing type. The outer layer is the musculoelastic layer and contains more SMCs, elastic fibres and collagen than the proteoglycan layer. The intima is separated from the media by the internal elastic lamina. (Stary et al. 1992)

Media. The second layer outward from the vessel lumen is the media. It is predominantly composed of synthesizing SMCs, which produce collagen, and contractile SMCs that are involved in the regulation of blood pressure by vasoconstriction and vasodilatation. (Ross and Glomset 1976) The external elastic lamina borders the media and the third layer, the adventitia.

Adventitia. The adventitia consists of loose connective tissue with fibroblasts, SMCs, mast cells, collagen fibres and proteoglycans. The outer part of this layer contains vasa vasorum, lymphatic vessels and nerves. (Ross and Glomset 1976)

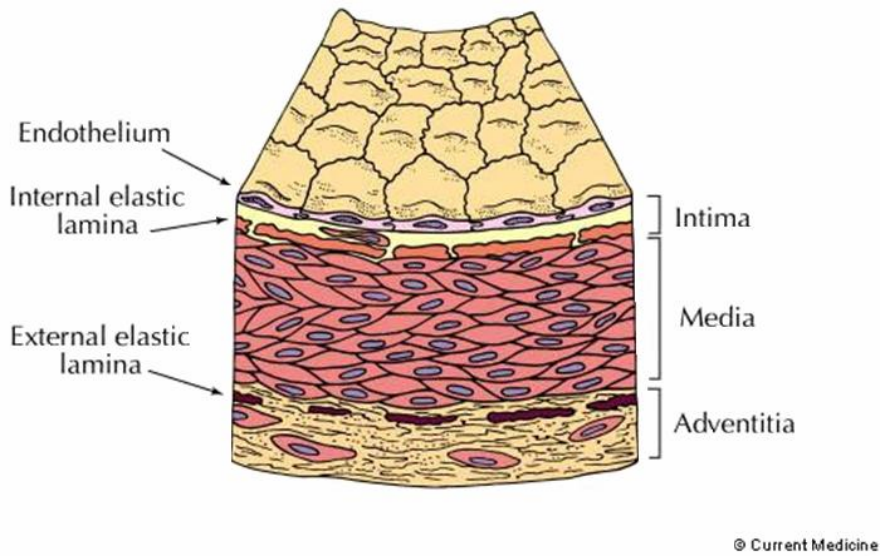

Figure 1. The struture of a normal muscular artery.

2.1.2 The endothelium, function and pathology

The endothelium consists of a monolayer of cells, the innermost part of the vessel wall. In normal healthy circumstances, the endothelium functions in many different ways. It acts as a selective and permeable barrier to the vessel wall and protects it from the circulating blood flow. The efflux of large molecules into the intima is limited, and the exchange of other substances between the subendothelial space and the plasma is regulated by the endothelium (Ross 1993). A healthy endothelium maintains vascular tone by releasing molecules such as nitric oxide (NO) and prostacyclin as well as endothelin, which modulate vasodilation or vasoconstriction, respectively; it maintains an antithrombotic setting by secreting factors with antiaggregatory (NO and prostacyclin), anticoagulatory (heparin, protein C and protein S) or fibrinolytic (tissue plasminogen

activator) properties. The endothelium also produces a number of factors such as different growth factors and cytokines that regulate cellular adhesion, smooth muscle cell migration and proliferation as well as vessel wall inflammation. (Ross 1993, Bonetti et al. 2003)

Endothelial dysfunction. Feasible causes for endothelial dysfunction include diabetes; via accumulation of advanced glycosylation end products which also occurs with aging; hypertension; infectious microbes; free radicals from, e.g., cigarette smoke; elevated and/or modified low density lipoprotein cholesterol (LDL-C); and genetic variability (Gimbrone 1999, Glass and Witztum 2001, Libby 2002, Hansson 2005). Under the effect of these destructive substances, at the cellular and molecular level, the function of the endothelium is disturbed and endothelial dysfunction appears. This can be seen as changes in endothelial function - e.g., decreased production of prostacyclin and NO, which act as vasodilators as well as increased secretion of leukocyte adhesion molecules and chemokines, leading to increased leukocyte adhesion to the endothelium and subsequent migration to the intima as well as increased expression of proinflammatory cytokines (e.g. tumour necrosis factor, inteleukin-1 and gamma interferon) and procoagulants (Gimbrone 1999). Biomechanical forces such as hydrostatic pressure and cyclic strains can also influence the structure and function of endothelial cells (Gimbrone 1999, Bonetti et al. 2003). This cascade of impaired vasodilation in addition to proinflammatory, proliferative and procoagulatory responses favours the development of atherosclerosis (Bonetti et al. 2003). The most vulnerable parts for plaque formation are regions where the vessel branches or curvs, these areas first begin to show increased permeability and attract monocytes and T lymphocytes (Gimbrone 1999). In the ongoing state of inflammation, the leukocytes themselves start to produce cytokines, chemokines and growth factors, inducing further damage through the accumulation of even more mononuclear cells, the proliferation of the smooth muscle cells and the formation of fibrous tissue. This cascade leads to the development of complicated lesions which can decrease blood supply in the vessel and can eventually rupture causing thrombosis and infarction. (Hansson 2005)

2.1.3 Atherosclerotic lesions

The Committee on Vascular Lesions of the Council of Atherosclerosis with the American Heart Association has classified the lesions of atherosclerosis:

Type I lesions. Type I lesions or initial lesions are the consequence of an accumulation of atherogenic lipoproteins and are particular to the regions of adaptive thickening of the intima. They have small isolated groups of a few macrophages, called foam cells, which contain lipid droplets. These lesions are found generally in infants and children; also in those adults with little

atherosclerosis or in lesion resistant areas. Most type I lesions are not visible to the naked eye. (Stary et al. 1994)

Type II lesions. Type II lesions consist mainly of foam cells present as adjacent layers (Figure 2). Smooth muscle cells in this type of lesion also contain lipid droplets. Most of the lipids are intracellular. Type II lesions are often synonymously called fatty streaks, because of their appearance. To be accurate, type II lesions include fatty streaks. Since all lesions of this type are not fatty streaks, the lesion should be determined by microscopic criteria. Type II lesions are divided further into progression-prone or type IIa and progression-resistant or type IIb groups. The smaller subgroup of type IIa lesions are found in atherosclerosis-prone sites and more often progress into advanced lesions; they include more smooth muscle cells and a greater accumulation of lipoproteins and macrophages, and the foam cells and extracellular lipid droplets are located deeper in the intimal layer. Type IIb lesions are more abundant and either do not develop further or do so slowly. Generally speaking, fatty streaks themselves are not clinically significant but may become so if they progress. Fatty streaks can be found during the first decade of life. (Stary et al. 1994)

Type III lesions. Type III lesions are also known as intermediate lesions, transitional lesions or preathreomas (Figure 2). They are characterised as a morphological and chemical bridge between type II lesions and atheromas. They consist of extracellular lipid droplets and particles that form pools and disrupt the normal unity of the intimal smooth muscle. Type III lesions are present in the same locations as type IIa lesions earlier in life and are present from the third decade of life. (Stary et al. 1994)

Type IV lesions. Type IV lesions are called atheromas (Figure 2). They can be found in the fourth decade of life and are potentially symptomatic. In atheromas there is a dense accumulation of smooth muscle cells and extracellular lipids known as the lipid core which causes a severe disorganization of the intima. There may be calcification of cell organelles and calcium particles in the lipid core. At the site, the artery wall is thickened, but this does not often cause narrowing of the vascular lumen. These lesions are clinically significant as they may be susceptible to rupture and thrombosis. (Stary et al. 1995)

Type V lesions. Type V lesions are characterised by formation of new fibrous connective tissue (Figure 2). When a lesion with a lipid core gets a fibrous cap consisting of smooth muscle cells and extracellular matrix, it is called a fibroatheroma (type Va lesion). If there is calcification of the lipid core or other parts of the lesion, it may be referred to as type Vb (calcific lesions) or type VII. A type Vc (fibrotic) or a type VIII lesion has no lipid core and very little lipid accumulation. Fibroatheromas may become increasingly complex and develop fissures or haematomas, and they are clinically relevant. (Stary et al. 1995)

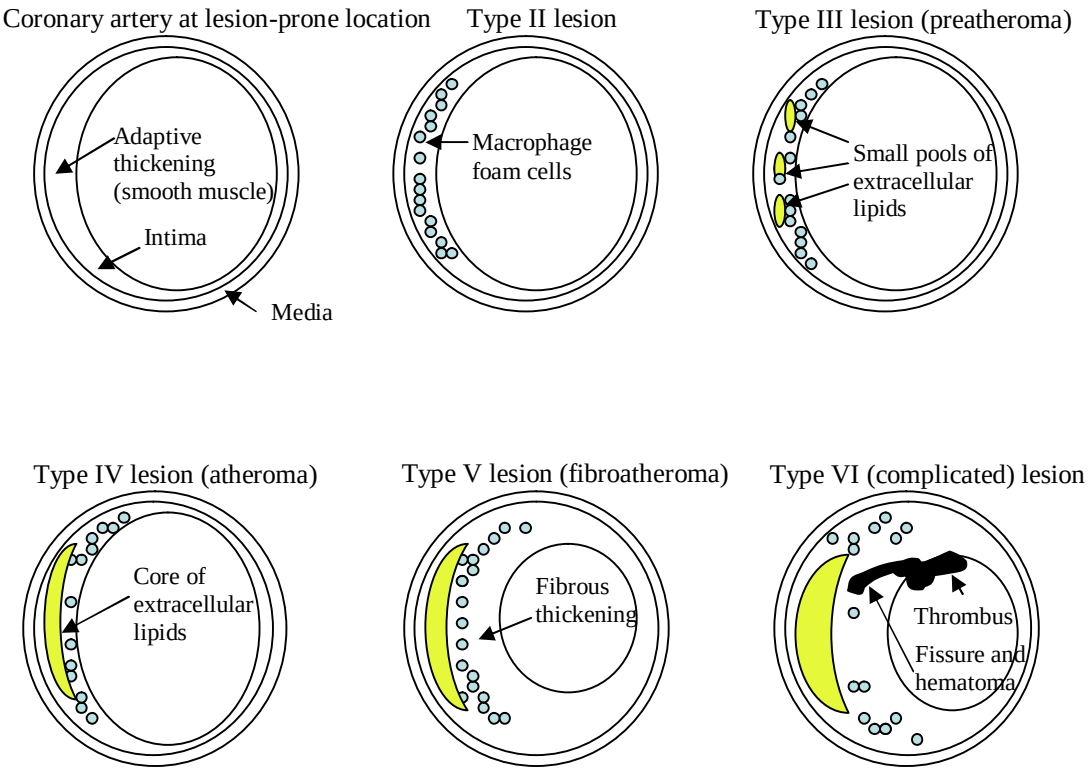

Figure 2. Cross section drawings of the different types of atherosclerotic lesions. Modified from Stary et al. 1994, and Stary et al. 1995.

Type VI lesions. Type VI lesions or complicated lesions are formed mainly from types IV and V and are complicated with disruption of the lesion surface (type VIa), haematoma or haemorrhage (type VIb) or thrombotic deposits (type VIc). These types of lesions are mostly the cause of morbidity and mortality in atherosclerosis. The most important clinical event is the acute occlusion of an artery caused by the added complications to the plaque and the subsequent thrombosis causing acute MI or stroke. (Stary et al. 1995)

2.2 Risk factors for atherosclerosis in children and young adults

Atherosclerotic diseases are multifactorial, affected by several interacting environmental, biochemical and genetic risk factors that effect the progression of atherosclerosis and CVDs. Early life exposure to these risk factors may predispose to increased risk of stroke and CVDs in later life. In 1981, Hopkins and Williams described in their review article a total of 246 factors that had been suggested to associate with CAD (Hopkins and Williams 1981). In the following sections, the most important risk factors affecting the pathophysiology of atherosclerosis are briefly introduced.

2.2.1 Lipoproteins and their lipids as risk factors for atherosclerosis

Low-density lipoprotein (LDL) and small dense LDL. Accumulation of LDL particles in the subendothelial matrix is considered to be a key initiating event in atherosclerosis. When circulating levels of LDL are raised, accumulation is greater as transport and retention are increased (Lusis 2000). The major cause of injury to the endothelium and underlying SMCs comes from the modified LDL particles. LDL can be modified by oxidation, glycation (in diabetes), lipolysis, aggregation, association with proteoglycans or by the incorporation of LDL to immune complexes (Ross 1999, Lusis 2000, Pentikäinen et al. 2000). The modification of LDL can lead either to the production of so-called minimally or fully modified LDL particles. The latter are taken up by macrophages through scavenger receptor mediated mechanisms (Kodama et al. 1990) and contribute to foam cell formation, while both modified LDL forms are also pro-inflammatory (Ross 1999, Lusis 2000, Pentikäinen et al. 2000). The most severely elevated circulating LDL levels are found in patients with LDL receptor defects which lead to familial hypercholesterolemia (FH), the mechanism of which was elucidated through the pioneering work of Brown and Goldstein describing the receptor mediating pathway of cholesterol homeostasis (Brown and Goldstein 1986). After this original discovery, FH has been the most studied model for atherosclerosis in humans, as the subjects develop atherosclerosis at a young age and have a highly elevated risk for CAD (Goldstein et al. 2001). Today, it has also been convincingly proven that lowering LDL by, for example, pharmacological treatment with statins decreases the risk of cardiovascular events and mortality (Scandinavian Simvastatin Survival Study 1994, Pedersen et al. 1998, Steinberg and Gotto 1999). In addition to modifications and an increased concentration of LDL cholesterol (LDL-C), the size and density of the LDL molecule have also been associated with the risk of atherosclerosis (Austin et al. 1988, Gardner et al. 1996). Small dense LDL particles are highly atherogenic, because they have an increased susceptibility to oxidative stress, a low binding affinity for the LDL receptor and a prolonged plasma half-life (Chapman et al. 1998).

Childhood levels of LDL-C have been shown to correlate positively with fatty streaks and raised atherosclerotic lesions on artery walls (McGill et al. 1997, Berenson et al. 1998). Considering the early phase of disease development, childhood LDL-C has also been associated with reduced endothelial function (Järvisalo et al. 2004a), predicting increased IMT values (Li et al. 2003, Raitakari et al. 2003) and decreased arterial elasticity in adulthood (Juonala et al. 2005). Small dense LDL has been reported to predict an impairment of flow-mediated dilatation in young men (Lupattelli et al. 2000) and to associate with IMT in asymptomatic patients with familial combined hyperlipidemia (Liu et al. 2002).

High-density lipoprotein (HDL). HDL cholesterol (HDL-C) is commonly referred to as the "good" cholesterol, because its high serum concentration is strongly protective against atherosclerosis. A large number of studies have shown that HDL-C levels are inversely associated with CAD (Gordon et al. 1977) and that a low plasma HDL-C is an independent risk factor for CAD mortality (Goldbourt et al. 1997). HDL has several antiatherogenic mechanisms - most importantly, HDL is involved in the reverse cholesterol transport where it participates in the removal of excess cholesterol from artery walls and other peripheral tissues back to the liver for elimination. The antiatherogenic properties of HDL also include the inhibition of lipoprotein oxidation in the artery wall and the inhibition of adhesion molecule expression in endothelial cells. In other words, it works in an anti-inflammatory manner. It may also affect endothelial function by stimulating the production of endothelial NO. (Barter et al. 2003)

HDL-C concentrations in children and young adults have been inversely associated with fatty streaks, raised lesions, calcification of the coronary artery and carotid IMT (Crouse et al. 1996, McGill et al. 1997, Knoflach et al. 2003). Low HDL-C associates with decreased arterial elasticity (Urbina et al. 2004), and adult arterial elasticity is predicted by low HDL-C levels in childhood (Juonala et al. 2005). Endothelial function has been shown to be impaired in young men with low HDL-C cholesterol values (Toikka et al. 1999).

Triglycerides. Many studies have failed to show an association between triglycerides and CAD after correction for other risk factors, making it difficult to demonstrate an independent role for them in CAD. The finding that high serum levels of triglycerides in combination with low HDL-C levels account for roughly twice as many cases of CAD as low HDL-C alone clearly indicates a role for triglycerides in this relationship (Castelli 1992). High serum levels of triglycerides may contribute to the development of atherosclerosis in several ways. Triglyceride-rich lipoproteins, very low density lipoproteins (VLDL) in particular, may yield a direct atherogenic effect, and on the other hand, hypertriglyceridemia may have an effect on other atherogenic lipoprotein profiles such as the presence of small dense LDL or low HDL levels (Grundy and Vega 1992). In two published meta-analyses, triglycerides were found to be an independent risk factor for CAD after adjusting for HDL-C, which has a strong inverse correlation with triglycerides (Hokanson and Austin 1996, Sarwar et al. 2007). Moreover, in a recent study, triglycerides were strongly associated with CAD risk in a population of healthy young men between the ages of 26 to 45 years. In the same study, a decrease in triglyceride levels associated with a decreased CAD risk. (Tirosh et al. 2007)

In young adults, triglycerides have been associated with the extent of fatty streaks and fibrous plaques in the aorta and coronary arteries (Berenson et al. 1998). Triglycerides have been

associated with IMT in a low-risk population (Ferrieres et al. 1999). An association between triglycerides and arterial elasticity has also been reported (Urbina et al. 2004), and endothelial dysfunction has been reported to be induced by postprandial hypertriglyceridemia (Bae et al. 2003).

Apolipoproteins. Apolipoproteins (apo) are structural components of lipoproteins which are molecules composed of lipids: cholesterol, cholesterol esters, triglycerides and phospholipids. The different apolipoproteins serve as enzyme activators in lipoprotein metabolism and mediate lipoprotein binding to cell surface receptors. ApoA-I and A-II are components of the antiatherogenic HDL particles. ApoB is the component of atherogenic lipoprotein particles; chylomicrons with $apoB_{48}$ and VLDL, intermediate density lipoprotein (IDL) and LDL with $apoB_{100}$. ApoE is present in chylomicrons, VLDL, IDL and HDL and is important in the clearance and metabolism of triglyceride-rich particles. Cholesterol transport may be assessed by measuring the apoB and apoA-I, and the ratio of these can be used to estimate the amount of atherogenic or antiatherogenic particles. This is why apolipoprotein analysis can improve the assessment of atherosclerosis. (Patsch and Gotto 1996, Srinivasan and Berenson 2001) ApoB and apoA-I levels may predict CAD risk possibly even better than LDL-C or HDL-C (Walldius et al. 2001). ApoB has been associated with increased IMT in middle-aged men (Wallenfeldt et al. 2004) and with impaired flow-mediated dilatation in combined hyperlipidemia (Sebestjen et al. 2005). Low apoA-I levels correlate with endothelial dysfunction in obese children (Tounian et al. 2001).

Lipoprotein(a). Lipoprotein(a) is an LDL-like particle in which $apoB_{100}$ has been linked to another glycoprotein, apo(a), with a varying number of kringles. Lipoprotein(a) resembles LDL with its lipid content. Apo(a) has a structural homology with plasminogen, and thus lipoprotein(a) therefore also has some prothrombotic features. Lipoprotein(a) can participate in the initiation and development of atherosclerotic plaque formation and possibly be involved in plaque rupture (Scanu et al. 1991, Dahlen and Stenlund 1997). Other deleterious effects include activation of monocytes, macrophages and SMCs, and in this manner lipoprotein(a) may induce inflammation (Scanu et al. 1991, Dahlen and Stenlund 1997). Lipoprotein(a) has been associated with CAD risk in the ARIC study (Chambless et al. 2003) and with increased risk of stroke and cardiovascular mortality in elderly men (Ariyo et al. 2003). In hypercholesterolaemic children, lipoprotein(a) levels have been associated with impaired FMD (Sorensen et al. 1994). In adult populations, lipoprotein(a) has failed to associate with carotid IMT or brachial FMD (Raitakari et al. 1999, Grebe et al. 2007). In elderly patients with type II diabetes mellitus, lipoprotein(a) has been shown to be an independent factor in aortic stiffness (Wakabayashi and Masuda 2006).

2.2.2 Lifestyle risk factors

Obesity. Obesity plays a significant role in the development of type 2 diabetes and metabolic syndrome, conditions that increase the risk for CVDs (Folsom et al. 1997, Meigs et al. 2003) and CVD mortality (Lehto et al. 1997, Malik et al. 2004). There may be many mechanisms by which excess body weight affects cardiovascular risk. In addition to the risk factors mentioned above, obesity associates with inflammation by inducing the production of proinflammatory cytokines and chemokines by adipocytes (Pradhan et al. 2001, Cai et al. 2005).

Obesity in childhood increases the risk of atherosclerotic lesions which may be a more important risk factor than obesity per se (Berenson et al. 1998). It has also been shown that childhood obesity associates with elevated carotid IMT during childhood and in adulthood (Berenson et al. 1998, Davis et al. 2001, Li et al. 2003, Raitakari et al. 2005). Obese children have also been reported to have impaired endothelial function (Tounian et al. 2001, Meyer et al. 2006), and their arterial elasticity in adulthood is decreased (Tounian et al. 2001, Juonala et al. 2005).

Weight and height are often used to calculate a ratio, the body mass index (BMI). This is a widely used index for assessing obesity, and it is preferred because it has the best correlation with body fat. Another means to evaluate the degree of body fat is the waist-to-hip ratio. A high waist-to-hip ratio is an indication of higher abdominal fat accumulation. (Bray and Gray 1988)

Smoking. Smoking is a major health hazard, and both active and passive smoking have a significant effect on cardiovascular morbidity and mortality. Nitric oxide is responsible for the endothelial vasodilatory function, and cigarette smoke causes a decreased NO availability. Exposure to smoke impairs the endothelial function and leads to increased levels of inflammatory markers. Platelet adherence and aggregation are also increased, as NO in normal circumstances acts as an inhibitor of platelet adherence. (Napoli and Ignarro 2001, Ambrose and Barua 2004) In addition, smoking alters the lipid profile. Smokers have higher total cholesterol, triglycerides and LDL-C and lower levels of HDL-C than non smokers (Craig et al. 1989). Furthermore, smoking increases LDL oxidation.

Smoking has been associated with an increased number of raised lesions of the abdominal aorta in young adults (McGill and McMahan 1998). Strong associations have been shown between smoking and IMT in men (Salonen and Salonen 1991, Ferrieres et al. 1999), and in young adults smoking has been related to IMT and endothelial dysfunction (Celermajer et al. 1993, Knoflach et al. 2003). Vascular elasticity can also be affected by smoking alone as reported in a population of young adults with no other cardiovascular risk factors (Li et al. 2005).

Physical activity. Low physical activity is considered a risk factor for atherosclerosis. Regular exercise has been shown to associate with higher FMD values in men, with athletes having

higher values than inactive subjects. Exercise also associated with preserved antioxidant defences of the endothelium, i.e. the endothelium function was better. (Franzoni et al. 2005) Physical activity has also been studied in relation to arterial compliance. In a study on men with different physical activity levels, the findings were the following: arterial compliance decreases with age but the amount of this decrease is attenuated in those who regularly performed vigorous endurance exercise, and a brief intervention of exercise can restore some arterial compliance and it is not dependant on changes in body weight, blood pressure or metabolic risk factors for atherosclerosis (Tanaka et al. 2000). The authors speculated that increased pulse pressure and distension of the vessel wall could stretch the collagen fibres and increase compliance. The other possibility discussed was the modulation of the sympathetic tone of the smooth muscle cells directly or by enhancing the effect of NO. With regard to physical activity and IMT, the results are somewhat discordant as discussed in a recent review: physical inactivity has been shown to associate with increased IMT, but intervention studies have conferred inconsistent results on the progression of intima-media thickening (Kadoglou et al. 2008).

2.2.3 Blood pressure

High blood pressure increases the development of atherosclerosis by inducing impaired endothelium-dependent vasodilatation and increasing leukocyte adherence to the endothelial surface (Chobanian and Alexander 1996). In addition, elevated blood pressure is associated with increased smooth muscle cell proliferation and macrophage accumulation in the intima and, furthermore, causes enhanced connective tissue synthesis in the vessel wall (Chobanian 1990). Hypertension can also stimulate growth factor and cytokine expression (Sarzani et al. 1989). Elevated blood pressure may also advance the inflammatory response by increasing oxidative stress and the production of free radicals by the arterial wall and hence recruit monocytes and cause endothelial dysfunction (Crawford and Blankenhorn 1991).

In children, high blood pressure has been shown to predict the occurrence of post mortem fatty streaks and fibrous lesions in the aorta and coronary arteries (Berenson et al. 1998, McGill and McMahan 1998). Hypertension in childhood has been shown to predict adulthood arterial stiffness (Li et al. 2004, Juonala et al. 2005) and increased carotid artery IMT (Raitakari et al. 2003). In adolescent boys, high blood pressure predicts endothelial dysfunction in adulthood (Juonala et al. 2006a).

2.2.4 Diabetes

The risk of cardiovascular morbidity and mortality is increased by both types of diabetes approximately two to four-fold, and the risk is correlated with the duration of the disease. The mechanism through which diabetic patients are at greater risk is proposed to be a decrease in endothelium-derived NO in response to hyperglycaemia. Hyperglycaemia increases the production of reactive oxygen species that inactivates NO. In physiological conditions, insulin increases NO-mediated vasodilation. High insulin levels in diabetic patients with insulin resistance impair the ability of the insulin to produce NO resulting in impaired vasodilation and endothelial dysfunction. Furthermore, the metabolic effects of diabetes, elevated free fatty acids, insulin resistance and hyperglycaemia, all have an impact on the atherosclerotic process. Diabetes also increases the migration of SMC and affects platelet function. (Marks and Raskin 2000, Creager et al. 2003)

Elevated IMT values and impaired elasticity of the carotid artery and endothelial dysfunction of the brachial artery have been measured in diabetic children (Parikh et al. 2000, Järvisalo et al. 2002a, Järvisalo et al. 2004b).

2.2.5 Other risk factors

In addition to the classic or common risk factors - high LDL-C, hypertension, smoking and diabetes - many other factors have also been linked to the development of atherosclerosis or to an increased risk of disease morbidity or mortality; these factors are also referred to as emerging risk factors.

Homocysteine. A substance widely studied in relation to cardiovascular risk is the serum homocysteine concentration. Homocysteine is an amino acid produced during the metabolism of methionine. There are reports on elevated homocysteine values associating with increased risk of CAD, stroke and peripheral vascular disease. There are some potential mechanisms through which homocysteine promotes atherosclerosis, which have been reviewed by Lawrence de Koning et al. (2003). Homocysteine enhances the production of several pro-inflammatory cytokines. Monocyte chemoattractant protein 1 is increased in endothelial cells, SMCs and monocytes, and it enhances monocyte endothelial binding and recruitment to the subendothelial space, an early step in fatty streak formation. Interleukin 8 expression has also been shown to be increased by homocysteine; this functions as a chemoattractant for T-lymphocytes and neutrophils. Homocysteine has been shown to impair normal endothelium-dependent vasodilation. This has been suggested to occur through the mechanism of oxidative stress, where homocysteine decreases the expression of antioxidant enzymes. It also impairs endothelial NO bioavailability by inhibiting glutathione peroxidase activity. Another suggested mechanism is endoplasmic reticulum stress. Homocysteine has been shown to elicit transient and chronic stress to the endoplasmic reticulum and thus adversly

affect cellular functions involved in, for example, lipid regulation, programmed cell death and inflammation, which are involved in the development and progression of atherosclerosis. (Lawrence de Koning et al. 2003) Hyperhomocysteinaemia has been associated with elevated carotid IMT in children and adolescents (Megnien et al. 1998).

C-reactive protein (CRP). Another risk marker that has received much attention is CRP, an acute phase protein. Small increases of circulating CRP have been positively associated with atherosclerosis, cardiovascular events, atherothrombosis and myocardial infarction. Atherogenic mechanisms may include complement activation, vascular cell activation, monocyte recruitment, lipid accumulation and thrombosis. (Paffen and DeMaat 2006) In children, increased CRP levels have been associated with increased carotid IMT and impaired brachial artery FMD (Järvisalo et al. 2002b).

In addition, there are several other studied factors: fibrinogen, platelet function, infections such as that of the bacterium *Chlamydia pneumoniae*, cytokines, myeloperoxidase, leptin, osteopontin, dietary factors and psychosocial factors. In all, more than two hundred and fifty factors have been associated with atherosclerosis (Hopkins and Williams 1981). Many of these associate with each other, and in many cases the conditional risk factors in addition to the conventional ones act in synergy to increase cardiovascular burden (Vinereanu 2006).

2.3 Ultrasound measurements of arteries

Ultrasound has become a common method to investigate early atherosclerotic changes in the vasculature. The technique is readily available, safe and non-invasive making it ideal for population studies. Furthermore, the technique is reliable, reproducible and relatively inexpensive compared to other imaging techniques. The most frequently used parameters obtained by ultrasound measurements that describe subclinical atherosclerosis include carotid artery intima-media thickness, carotid artery elasticity and brachial artery flow-mediated dilatation.

2.3.1 Intima-media thickness

Intima-media thickness is measured as the greatest distance between the lumen-intima interface and the media-adventitia interface. Measuring the intima-media thickness of the artery wall to assess atherosclerotic changes was first described with samples of the aorta and common carotid artery in a study where the samples of the vessels were studied in vitro by means of ultrasound and microscopy. No significant differences were found between the different measurements indicating that assessing intima media thickness by ultrasound imaging is a feasible approach to estimate atherosclerosis of arteries in vivo. (Pignoli et al. 1986) As the carotid artery is anatomically

conveniently situated for ultrasound measurements, it has become the artery of choice for examining IMT.

Increased carotid artery IMT values correlate with traditional risk factors for atherosclerosis: smoking (Poli et al. 1988, Heiss et al. 1991), total cholesterol, LDL-C, total triglycerides, blood pressure (Heiss et al. 1991), waist-to-hip ratio, abnormal glucose metabolism and physical inactivity (Folsom et al. 1994). In addition, increased IMT values have been shown to predict the likelihood of future cardiovascular events such as myocardial infarction and stroke in populations with no pre-existing history of cardiovascular events (O'Leary et al. 1999, Chambless et al. 2000).

Exposure to cardiovascular risk factors in childhood has been shown to relate to increased adulthood IMT values. In three prospective studies, the results are similar. Total cholesterol in both sexes and childhood BMI in females were significantly associated with adult IMT values in the Muscatine Study (Davis et al. 2001). Childhood LDL-C and BMI predicted carotid IMT values in young adults in the Bogalusa Heart Study (Li et al. 2003). In the Cardiovascular Risk in Young Finns Study, IMT in young adults associated significantly with childhood LDL-C, systolic blood pressure, BMI and smoking (Raitakari et al. 2003).

Earlier data suggest that a substantial factor causing variability in IMT is the pre-existing genetic background of the individual. In the Cardiovascular Risk in Young Finns study, it has been observed that young healthy adults who have a positive family history for CAD have increased carotid IMT (Juonala et al. 2006b). When studying IMT values in children and adolescents of parents with a history of premature myocardial infarction, increased IMT values were found independent of traditional cardiovascular risk factors (Cuomo et al. 2002). Moreover, in the Framingham Heart Study, it was estimated that a third of IMT variability is due to heritable factors (Fox et al. 2003).

2.3.2 Arterial elasticity

During the course of atherosclerosis, the stiffness of arteries increases, which is thought to be a marker of the disease process. Age is a major factor in the progressive stiffening of arteries, as the elasticity of the vessel wall decreases due to degeneration of the elastic fibres of the media. The loss of functional elasticity may also be a predisposing factor for atherosclerosis. (Avolio et al. 1998) There are different ways to assess arterial stiffness. One method is to calculate carotid artery distensibility or compliance, which indicates the ability of the arterial wall to expand in response to pulse pressure caused by the cardiac systole and diastole and is calculated from blood pressure and vessel diameter values (Salomaa et al. 1995). Other ultrasonically derived non-invasive indices include Young's elastic modulus that estimates arterial stiffness independently of IMT as it is

included in the formula (Riley et al. 1992, Salomaa et al. 1995) and the stiffness index which is a more complex formula that is considered independent of blood pressure (Hirai et al. 1989). All of the indices have a high correlation with each other. Pulse wave velocity, measuring the speed of pulse wave transmission between two arteries, and arterial pressure waveforms have also been used as measures of arterial elasticity. (Oliver and Webb 2003)

The elasticity of arteries has been associated with several cardiovascular risk factors. Total cholesterol, systolic blood pressure and positive parental history of MI have been related to arterial stiffness in adolescents (Riley et al. 1986). Obesity has been associated with arterial stiffness in children (Tounian et al. 2001). Childhood obesity, high LDL-C, high blood pressure and smoking predict decreased arterial elasticity in adults (Juonala et al. 2005). Arterial elasticity has also been suggested to be an independent predictor for cardiovascular events in hypertensive patients (Boutouyrie et al. 2002) in end-stage renal disease (Blacher et al. 1998a) or diabetes (Cruickshank et al. 2002). There is evidence that a positive parental history of MI relates to increased arterial stiffness (Riley et al. 1986). There also seems to be a relationship between elasticity and cardiovascular events even after adjustment for conventional risk factors (Laurent et al. 2001, Boutouyrie et al. 2002). This was also the case in The Strong Heart Study (North et al. 2002), where 950 adults were assessed for the heritability of carotid stiffness after accounting for covariate effects. The proportion of residual phenotypic variance due to additive genetic effects was reported to be 23%.

2.3.3 Endothelial function

Dysfunction of the endothelium, i.e. impaired arterial distensibility, is considered a key event in the early pathophysiology of atherosclerosis occurring before detectable structural changes in the vascular wall take place (Ross 1993). Most of the functional methods for endothelial testing examine the ability of endothelium to cause vasodilatation in response to pharmacologic and physiologic stimuli which might enhance the endothelial release of NO (Corretti et al. 2002). The measurement of coronary endothelial function in humans has been performed with quantitive angiography, before and after intracoronary infusion of acethylcoholine; in the different stages of atherosclerosis, a progressive impairment of the endothelial function to the end point of total loss of vasodilatation was found (Zeiher et al. 1991). A non-invasive method to assess endothelial function was introduced by Celermajer and colleagues in 1992. Here, the vessel dilatation is measured by ultrasound after reactive hyperaemia, and the parameter is known as flow-mediated dilatation (Celermajer et al. 1992). Brachial artery FMD has been found to correlate well with the endothelial

function of coronary arteries and can thus be used as a surrogate method for assessing coronary endothelial function (Anderson et al. 1995).

FMD is associated with risk factors such as hypercholesterolemia, smoking, hyperhomocystaeinemia, obesity and hypertension (Celermajer et al. 1993, Woo et al. 1997, Benjamin et al. 2004, Juonala et al. 2004a). Furthermore, brachial endothelial dysfunction relates to coronary atherosclerosis (Neunteufl et al. 1997) and can predict cardiovascular events in CAD patients (Chan et al. 2003). Family history seems to have an influence on FMD - young adults with a parental history of CAD had impaired FMD compared to controls (Clarkson et al. 1997). In the Cardiovascular Risk in Young Finns Study, however, no differences were found in FMD values between those who had a positive family history of CAD and those who did not (Juonala et al. 2006b). In a recent genome-wide association study where brachial artery FMD was one of the traits studied, the estimate for heritability was 19% (Vasan et al. 2007).

In previous results from the Cardiovascular Risk in Young Finns Study the findings support the hypothesis of the response-to-injury model where endothelial damage is required before anatomical changes of the arterial wall, i.e. increased IMT occur. FMD was found to be inversely correlated with IMT (Juonala et al. 2004a). A similar result has been reported in another healthy adult population, where all patients with elevated IMT (above 1 mm) had impaired FMD, but patients with reduced FMD did not necessarily have increased IMT (Campuzano et al. 2006). Changes in the ultrasound parameters used in this thesis study most probably occur in the order FMD, CAC and IMT during the course of atherosclerosis, but to the best of our knowledge, however, this has not yet been proven.

2.4 The role of genetics and the genes studied in atherosclerosis aetiology

The majority of atherosclerotic diseases result from a complex interplay between several genes and environmental risk factors although rare monogenic diseases leading to different CVDs also exist (Hopkins and Williams 1981). On the basis of familial aggregation of the CVDs, family and twin studies report heritability estimates for stoke and CAD mortality and morbidity that vary between 17% and 60%, depending on the study population (Goldbourt and Neufeld 1986, Jousilahti et al. 1996, Bak et al. 2002, Zdravkovic et al. 2002, Fox et al. 2003, Fischer et al. 2005, Moskau et al. 2005, Wienke et al. 2005, Cassidy-Bushrow et al. 2007). Results from the Framingham Heart Study suggest that a substantial proportion of variability in carotid IMT is explained by genetic factors, suggesting heritabilities between 35% and 67% depending on the carotid artery area and adjustment used in statistical analyses (Fox et al. 2003). In the offspring of parents with premature MI, there are structural and functional changes in the arteries in the form of impaired endothelial function and

increased carotid IMT (Gaeta et al. 2000). Parental history of stroke or MI has also been associated with IMT after controlling for other cardiovascular risk factors, suggesting a significant familial component (Jerrard-Dunne et al. 2003).

Several linkage studies have tried to establish chromosomal regions associated with CVDs (Pajukanta et al. 2000, Francke et al. 2001, Broeckel et al. 2002, Harrap et al. 2002, Chiodini and Lewis 2003, Hauser et al. 2004, Wang et al. 2004). (Table 1.)

In the last few years, genome-wide association scans using SNP arrays involving 0.5–1 million SNPs have also been performed in order to find genes and gene variations contributing to CVD (Table 1). The techniques allowing the investigation of large-scale structural genomic variation, i.e. gene copy number variation (Sebat et al. 2004, Sebat et al. 2007), are being established as new tools for identifying genes related to lipoprotein and metabolic phenotypes (Pollex and Hegele 2007a) as well as CVDs (Pollex and Hegele 2007b). There are also linkage studies on the phenotypes of subclinical atherosclerosis, i.e. IMT, FMD and different markers for arterial elasticity (Fox et al. 2004, Bielinski et al. 2005, Mitchell et al. 2005, O'Donnell et al. 2007, Sherva et al. 2007, Vasan et al. 2007, Franceschini et al. 2008). (Table 2.)

Moreover, a large number of small and large scale candidate gene studies have examined the effect of various genes in CAD-related pathways on CVD morbidity and mortality. These studies involve genes that may have a link to atherosclerosis, either through their function in lipid (HDL and LDL levels), amino acid or carbohydrate metabolism or in inflammation or endothelial function, or through mediating thrombotic activity.

The functionality of genes and SNPs have been widely studied by using genetically engineered knock-out and transgenic animal models as well as cell culture experiments combined with different molecular genetic techniques (Lusis 2000). The completion of the Human Genome Project and the HapMap Project as well as the development of techniques in molecular biology and bioinformatics have made it easier to investigate the genetic basis of complex traits, exponentially increasing the the genetic data during the last few years (Novelli et al. 2003, Puddu et al. 2005, Fortunato and Di Taranto 2007).

Table 1. Genes and chromosomal loci associated with different atherosclerosis phenotypes in genome-wide linkage or association studies.

Chromosomal locus / nearby gene / candidate gene*	Phenotype	Reference
Chromosomal region 2q21.1-22	CAD	Pajukanta et al. 2000
Chromosomal region Xq23-26	CAD	Pajukanta et al. 2000
Chromosomal region 16p13.3	CAD, MI	Francke et al. 2001
Chromosomal region 2q36-37.3	MI	Harrap et al. 2002
Chromosomal region 14	MI	Broeckel et al. 2002
Chromosomal region 3q26-27	CAD	Chiodini, et al. 2003
Chromosomal region 2q34-37	CAD	Chiodini, et al. 2003
Chromosomal region 3q13	Early onset CAD	Hauser et al. 2004
Chromosomal region 1p34-36	MI	Wang et al. 2004
Chromosomal region 9p21.3, near *CDKN2A* and *CDKN2B* (cyclin-dependent kinase inhibitor 2A and 2B)	MI, CAD	Helgadottir et al. 2007, McPherson et al 2007, Samani et al. 2007, WTCCC 2007
Chromosomal region 6q25.1, near *MTHFD1L* (methylenetetrahydrofolate dehydrogenase 1-like protein)	CAD	Samani et al. 2007
Chromosomal region 2q36.3	CAD	Samani et al. 2007
PSRC1/SORT1 (Proline/serine-rich coiled-coil1 / Sortilin1)	CAD	Willer et al. 2007
B3GALT4 (Beta-3-galactosyltransferase 4)	CAD	Willer et al. 2007
**ALOX5AP* (arachidonate 5-lipoxygenase-activating protein)	MI, CAD death	Larson et al. 2007
**GJA4* (gap junction protein alpha i.e. *Cx37*)	MI, CAD death	Larson et al. 2007
**MEF2A* (myocyte enhancer factor 2A)	MI, CAD death	Larson et al. 2007
*PCSK9*proprotein convertase subtilisin/kexin type 9	CAD, CVD	Willer et al. 2007, Larson et al. 2007, Kathiresan et al. 2008
LDLR (LDL receptor)	CAD, CVD	Willer et al. 2007, Kathiresan et al. 2008
APOE-APOC1-APOC4	CAD, CVD	Willer et al. 2007, Kathiresan et al. 2008
APOB	CAD, CVD	Willer et al. 2007, Kathiresan et al. 2008
CETP (cholesteryl ester transfer protein)	CVD	Kathiresan et al. 2008
LIPC (hepatic lipase)	CVD	Kathiresan et al. 2008
LPL (lipoprotein lipase)	CVD	Kathiresan et al. 2008
HMGCR (hydroxy-methylglutaryl-Coenzyme A reductase)	CVD	Kathiresan et al. 2008
ABCA1 (ATP-binding cassette, sub-family A, member 1)	CVD	Kathiresan et al. 2008

Abbreviations: CAD, coronary artery disease; MI, myocardial infarction; CVD, cardiovascular disease; WCCC, the Wellcome Trust Case Control Consortium. **Additional reference:** A catalogue of genome-wide association studies, National Human Genome Research Institute, National Institutes of Health http://www.genome.gov/page.cfm?pageID=26525384 (21.5.2008)

Table 2. Chromosomal regions and genes associated with different subclinical atherosclerosis phenotypes in genome-wide linkage or association studies.

Chromosomal locus / nearby gene / candidate gene*	Phenotype	Reference
SCARB1 (scavenger receptor, class b, type I)	internal carotid IMT	Fox et al. 2004
Chromosome 12	internal carotid IMT	Fox et al. 2004, O'Donnell et al. 2007
Chromosome 18	pulse pressure	Bielinski et al. 2005
Chromosome 20	pulse pressure	Bielinski et al. 2005
Chromosome 17	pulse pressure	Bielinski et al. 2005
Chromosome 21	pulse pressure	Bielinski et al.2005
Chromosome 2	pulse wave velocity	Mitchell et al. 2005
Chromosome 15	pulse wave velocity	Mitchell et al. 2005
Chromosome 13	pulse wave velocity	Mitchell et al. 2005
Chromosome 7	pulse pressure, pulse wave velocity	Bielinski et al. 2005, Mitchell et al. 2005, Franceschini et al. 2008
Chromosome 19	pulse pressure	Bielinski et al. 2005, Franceschini et al. 2008
ABI2 (abl interactor 2)	internal carotid intima media thickness	O'Donnell et al. 2007
PCSK2 (proprotein convertase subtilisin/kexin type 2)	common carotid IMT	O'Donnell et al. 2007
C20orf196 (chromosome 20 open reading frame 196)	common carotid IMT	O'Donnell et al. 2007
CFTR (cystic fibrosis transmembrane conductance regulator)	FMD	Vasan et al. 2007
*GPR-25** (G-protein coupled receptor 25)	pulse pressure-stroke volume ratio	Sherva et al. 2007
*SMOC-1** (secreted modular calcium binding protein)	pulse pressure-stroke volume ratio	Sherva et al. 2007
Chromosome 1	pulse pressure-stroke volume ratio	Sherva et el. 2008
Chromosome 14	pulse pressure-stroke volume ratio	Sherva et al. 2008

Abbreviations: IMT, intima media thickness; FMD, flow mediated dilatation

Linkage and association studies have been widely used to identify chromosomal regions and genes associated with CVDs. In linkage analysis, the starting point is to collect extended families with multiple members affected by the disease of interest. Genomes of the study subjects are analysed with a set of genomic markers such as, for example, microsatellites of SNPs. Those markers that segregate with disease more often than expected can help localize linked regions. This method has its limitations in studying polygenic features partly because it has only limited power to detect the effects of common alleles with modest effects on the disease (Risch and Merikangas 1996). Association studies look for particular disease-related markers on a population level; this approach has a greater power to detect effects of common variants (Risch and Merikangas 1996). If targeting the whole genome, association studies require more markers than linkage analysis and sample sizes of thousands of individuals, which results in higher costs. For this reason, association analyses were previously limited to candidate genes or regions of the genome. Candidate genes can be chosen by combining existing information on different biological pathways, previous genetic studies, linkage data, expression analysis and animal models (Hirschhorn 2005).

Resequencing of the candidate gene is used when an association between an SNP and the studied trait has been established. Resequencing the whole gene including its promoter region provides information of the linkage equilibrium between the variants. It can also be used to study the subpopulation associating with the studied trait in more detail. Technological advances in methods will enable large-scale resequencing projects.

The HapMap Project is an international collaboration to identify and catalogue genetic variabilities in the human genome. The most common type of genetic variation is the single nucleotide variation or SNP. HapMap also provides information on common haplotypes and tag SNPs that identify them. The international HapMap Project made human genome sequence variation data publicly available (www.hapmap.org 10.7.2008). Recent advances in genotyping technology have made the genotyping of hundreds of thousands of SNPs more cost-efficient, enabling large-scale regional and genome-wide association studies (GWAS).

The studies described in this thesis focus on the relationship between genetic variation in three candidate genes and on early markers of atherosclerosis as determined by changes in arterial wall parameters obtained by ultrasound measurements. The three candidate genes were selected due to their important roles in the regulation of cell communication as well as homocysteine, carbohydrate and lipid metabolism, therefore indicating their possible role in the modulation of early atherosclerotic changes. Another approach would have been to use HapMap data on tag SNPs and genotype for these, as they cover most of the genetic variation of the gene in question. The importance of these genes in atherosclerosis development is reviewed in the following sections.

2.4.1 Connexin37

Connexin37 belongs to a family of integral membrane proteins called connexins, which polymerize to form gap junctions between cells (Beyer et al. 1990, Reed et al. 1993). To date, there are over twenty known different connexin isoforms identified (Sohl and Willecke 2004). The human connexin37 gene was cloned by Reed and colleagues (1993). They found that it is a single-copy gene and that the cDNA for the gene encodes a 333-amino acid polypeptide with the predicted molecular mass of 37,238 D, which gives rise to the name human Cx37 (Reed et al. 1993). The gene was later mapped to chromosome 1p35.1 (Van Camp et al. 1995).

2.4.1.1 Connexin37 function

Connexin37 functions as a structural protein to form components of the subunit of a gap junction. Six connexin molecules form each hexagonal connexon hemichannel and two connexons form a gap junction between adjacent cells (Goodenough 1975, Perkins et al. 1997). This is depicted in Figure 3. The gap junction channel allows the passage of small molecules such as ions or second messengers between neighbouring cells (Gilula et al. 1972, Saez et al. 1989). Expression of *Cx37* has been found in the different tissues. In the avascular system in ovaries, Cx37 forms gap junctions between the oocyte and granulosa cells, and disruption of the *Cx37* gene results in infertility in female mice (Simon et al. 1997). In the vascular tissue, *Cx37* has been found to be expressed in the endothelial cells (Reed et al. 1993, Yeh et al. 1997) and smooth muscle cells (Li and Simard 1999, Nakamura et al. 1999) of the vessel wall. Cx37 has also been reported to be found in neutrophils (Zahler et al. 2003) and in monocytes, where the activity of Cx37 hemichannels was indicated to inhibit monocyte adhesion to the endothelium (Wong et al. 2006).

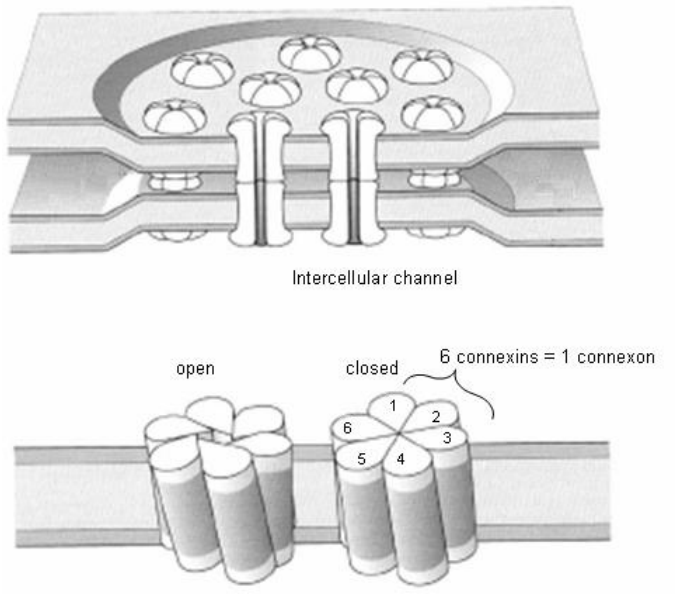

Figure 3. A schematic drawing of gap junction channels. Each connexon hemichannel is formed by six connexin subunits. Two opposing hemichannels form a gap junction. Modified from Sohl et al. 2004.

2.4.1.2 Connexin 37 polymorphism C1019T and atherosclerosis

The *Cx37* gene was a candidate gene for erythrokeratodermia in the genetic linkage studies performed by Richard et al. (1997). The study revealed a novel polymorphism at position 1019 of *Cx37,* (rs1764391) causing a cytosine-to-thymine nucleotide change and subsequently a proline-to-serine amino acid substitution at codon 319, which locates to the regulatory cytoplasmic tail of the protein (Richard et al. 1997). The same mutation was revealed and confirmed by Boerma et al. when studying whether the polymorphism recognises individuals with atherosclerotic plaques (Boerma et al. 1999). Subsequently, the *Cx37* 1019 polymorphism was suggested to be a risk factor for myocardial infarction in large-scale association (Yamada et al. 2002) and linkage studies (Wang et al. 2004). It has been shown that proinflammatory cytokines tumour necrosis factor-α and interleukin-1β selectively down-regulate gap junctional intercellular communication (GJIC). It was proposed that the selectivity was due to the presence of Cx37 in the myoendothelial gap junctions and that the inhibition of GJIC would serve as a link between inflammation and proliferation of smooth muscle cells in the vascular wall. (Hu and Cotgreave 1997) Atherogenic processes may also relate to changes in *Cx37* expression, such as in regeneration after injury (Yeh et al. 2000a), aging (Yeh et al. 2000b) and hyperlipidemia (Yeh et al. 2003). It has also been suggested that GJIC may be a factor in atherogenesis because cells in atherosclerotic plaques have been observed to express vascular wall connexins differentially (Kwak et al. 2002). More recently, it has been found that Cx37 hemichannels may be a factor in the initiation of the development of atherogenic plaques by regulating ATP-dependent monocyte adhesion. Mononuclear cells expressing the serine at codon 319 (1019T) have also showed stronger adhesion to endothelium than those expressing proline (1019C), suggesting that 1019C could function as a protective variant by hindering monocyte recruitment. (Wong et al. 2006)

Several association studies with different populations have been published, indicating a role for the C1019T polymorphism in coronary artery disease and myocardial infarction. The results have been contradictory. The C allele has been associated with carotid artery atherosclerotic plaques in Swedish men (Boerma et al. 1999), and the T allele has been shown to associate with coronary artery disease in Taiwanese men (Yeh et al. 2001) and in high risk Japanese men (Hirashiki et al. 2003). The T allele has also demonstrated an association with the risk of MI in Japanese (Yamada et al. 2002) and in Sicilian men (Listi et al. 2005). In a family-based Irish population, no association with CAD or MI was found for either genotype (Horan et al. 2006) and on the other hand, in a Swiss population, the C allele was associated with both CAD and MI and the T allele was identified as associating independently with MI (Wong et al. 2007). The studies are summarised in Table 3.

Table 3. Association studies of the *Cx37* C1019T polymorphism on different atherosclerosis phenotypes

Population	Male / female	Phenotype	Result	Reference
Swedish men	275 / 0	Carotid artery plaque	C allele positive	Boerma et al.
Taiwanese	120 / 57 CAD cases 70 / 32 controls	CAD	T allele positive in men	Yeh et al. 2001
Japanese	364 / 237	CAD	T allele positive in high CAD risk men	Hirashiki et al. 2003
Japanese	2858 / 1294	MI	T allele positive in men	Yamada et al. 2002
Italian	97 / 0 cases 196 / 0 controls	MI	T allele positive in men	Listi et al. 2005
Irish	N=1012	CAD, MI	negative	Horan et al. 2006
Swiss	N=781	CAD, MI	C allele positive for CAD, MI T allele positive for MI	Wong et al. 2007

Abbreviations: CAD, coronary artery disease; MI, myocardial infarction.

The studies of the C1019T polymorphism as a candidate gene for atherosclerosis have been performed on subjects with an established disease phenotype. There are no previous studies on the possible associations of the polymorphism with the early subclinical phase of the disease where the end point phenotypes include intima-media thickness or arterial elasticity.

2.4.2 Methylenetetrahydrofolate reductase (MTHFR)

5,10-Methylenetetrahydrofolate reductase is an enzyme which catalyses an NADPH-linked reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate which in turn is a cofactor for methylation of homocysteine to methionine. When this pathway is blocked due to a deficiency of MTHFR, it results in hyperhomocysteinaemia and homocystinuria with normal to low methionine levels. This distinguishes it from hyperhomocysteinemia caused by cystathionine synthase deficiency, a more common cause of homocystinuria. Patients with severe deficiency of MTHFR present with developmental delay, motor and gait abnormalities, seizures, psychiatric manifestations and vascular complications due to thromboembolisms at a young age. (Rosenblatt and Fenton 2001) Figure 4 shows the metabolism of homocysteine.

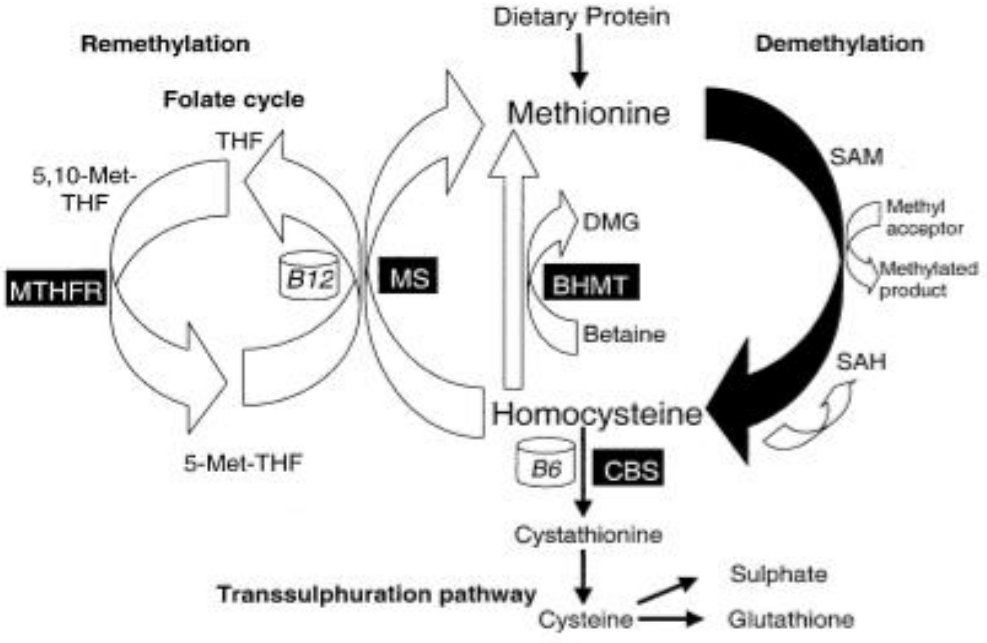

Figure 4. Metabolism of homocysteine. Dietary methionine is converted to S-adenosylmethionine (SAM) and is demethylated to S-adenosylhomocysteine (SAH) which is then cleaved into adenosine and homocysteine. Homocysteine is converted to cystathione and cysteine by cystathione β-synthase (CBS) and cofactor vitamin B6. Homocysteine can also be remethylated through the folate cycle and depends on methionine synthase (MS) and vitamin B12 as well as the enzyme methylenetetrahydrofolate reductase (MTHFR) and folate entering the cycle as tetrahydrofolate (THF). Remethylation in the liver and kidneys also happens via the enzyme betaine homocysteine methyltransferase (BHMT) transferring a methyl group via the demethylation of betaine to dimethylglycine (DMG). Modified from Lawrence de Koning et al. 2003.

2.4.2.1 MTHFR, homocysteine and a single nucleotide polymorphism

In subjects with severe hyperhomocysteinaemia caused by inborn errors in methionine metabolism (MTHFR deficiency), one of the phenotypes is premature development of atherosclerosis and intravascular thromboembolisms. This is why homocysteine has been proposed to be an atherogenic factor (McCully 1969). A thermolabile form of the MTHFR enzyme (ca 40% of residual MTHFR activity) was discovered in CAD patients. This caused a milder form of hyperhomocysteinaemia and it was therefore suggested that this deficient enzyme associates with CAD (Kang et al. 1988). In a further study by Kang and colleagues (1991), the thermolabile form of the enzyme was associated with higher homocysteine values and the development of atherosclerotic disease (Kang et al. 1991). Thereafter, the gene encoding MTHFR was studied further. First the cDNA of the gene was isolated (Goyette et al. 1994) and shortly after that, a common mutation in the gene was described (allele frequency of 0.38% in 114 French Canadians) which correlated with reduced enzyme activity and increased thermolability of MTHFR leading to high plasma homocysteine levels (Frosst et al.

1995). This newly found single nucleotide polymorphism was the C-to-T substitution of nucleotide 677 (rs1801133), which changes an alanine to a valine in the amino acid sequence of the enzyme. Individuals heterozygous for the polymorphism (genotype CT) had mean MTHFR activities of 65% and homozygotes (genotype TT) of approximately 30% compared to controls. (Frosst et al. 1995)

Since these findings, the polymorphism has been considered a putative risk factor for cardiovascular diseases, and numerous studies have been undertaken to investigate this prospect.

2.4.2.2 The *MTHFR* C677T polymorphism as a cardiovascular risk factor

In a review of the first studies on homocysteine in relation to the *MTHFR* genotypes and cardiovascular risk by Brattstrom and colleagues (1998), the C677T/MTHFR polymorphism was found to cause mildly elevated homocysteine values, those with the TT genotype having 25% higher levels than the subjects with the CC genotype. However, there was no evidence of causality between the mutation and the risk for CAD (Brattstrom et al. 1998). Elevated homocysteine values have been reported in patients with cerebrovascular or peripheral atherosclerosis (Brattstrom et al. 1984, Boers et al. 1985), and carotid artery intima-media wall thickening has been associated with higher levels of homocysteine (Malinow et al. 1993).

***MTHFR* and IMT.** Studies on the possible associations of the polymorphism with IMT have yielded controversial results. Results indicating a positive association between the *MTHFR* polymorphism and IMT have been reported in elderly Japanese with conventional vascular risk factors, i.e. hypertension, diabetes and smoking (Kawamoto et al. 2001), in Chinese end-stage renal disease patients (Lim et al. 2001), in elderly Italian women (Passaro et al. 2001) and in French men (Pallaud et al. 2001). Furthermore, it has been identified as a risk for carotid stenosis in Japanese women (Inamoto et al. 2003). On the other hand, there are reports with opposite results. In a random Australian population, the polymorphism did not independently predict increased IMT (McQuillan et al. 1999). The same was reported for Italian non-insulin-dependent diabetics (Mazza et al. 1999) and with pooled population data on South Asian, Chinese and European Canadian subjects (Kelemen et al. 2004). (Table 4.)

***MTHFR* and arterial elasticity.** There are no previous studies about the effects of *MTHFR* polymorphism on carotid artery elasticity. Circulating homocysteine concentrations have been shown to associate negatively with lower limb pulse wave velocity, a measure of arterial stiffness, in end stage renal disease patients (Blacher et al. 1998b) and to correlate with arterial stiffness, measured by pulse wave velocity, in hypertensive patients (Bortolotto et al. 1999). There are two studies where a methionine load was used to raise circulating homocysteine levels. In the first one, no association with levels of arterial stiffness was found when measured by pulse wave velocity

(Wilkinson et al. 2001), and in the second, there was a homocysteine concentration-related increase in arterial stiffness measured by systemic arterial compliance, calculated from arterial pressure waves and aortic flow (Nestel et al. 2003). Plasma homocysteine has also been found to associate with the arterial stiffness index of the brachial artery, (measured by a computerised oscillometry device) in end-stage renal disease patients on haemodialysis (Tsai et al. 2005). In one report, arterial stiffness (systemic arterial compliance) did not associate with the *MTHFR* genotype in a population of men during folic acid supplementation (Williams et al. 2005). (Table 4.)

***MTHFR* and FMD.** The association of *MTHFR* polymorphism with FMD has been studied in fairly small populations of healthy adults. In one study, the polymorphism did not associate with FMD (Pullin et al. 2002), and in another study of young men, subjects with the TT or CT genotype had similar FMD values as those with the CC genotype (Imamura et al. 2004). (Table 4.)

Table 4. Association studies of the *MTHFR* C677T polymorphism on different atherosclerosis phenotypes

Population	Male / female	Phenotype	Result	Reference
Japanese medical inpatients	147 / 179	IMT	positive	Kawamoto et al. 2001
Chinese haemodialysis patients	64 / 87	IMT	positive	Lim et al. 2001
French healthy cohort	77 / 84	IMT	positive for men	Pallaud et al. 2001
Italian healthy women	0 / 120	IMT	positive	Passaro et al. 2001
Japanese random population	1554 / 1693	IMT	positive for women	Inamoto et al. 2003
Australian random population	558 / 553	IMT	negative	McQuillan et al. 1999
Italian NIDDM patients	33 / 62	IMT	negative	Mazza et al. 1999
Canadian multiethnic population	432 / 386	IMT	negative	Kelemen et al. 2004
Australian healthy men	41 / 0	arterial stiffness	negative	Williams et al. 2005
Japanese healthy men	53 / 0	FMD	negative	Imamura et al. 2004

Abbreviations: IMT, intima media thickness; NIDDM, non-insulin dependent diabetes mellitus

2.4.3 Upstream transcription factor 1 (USF1)

Upstream transcription factors were first identified in HeLa cell nuclei as a factor able to bind and activate the adenovirus major late promoter in a study on transcription initiation (Sawadogo and Roeder 1985). Complete purification of the protein element discovered two polypeptides with molecular weights of 43 (USF1) and 44 (USF2) kDa, respectively (Sawadogo 1988, Sawadogo et al. 1988). USFs belong to the helix-loop-helix leucine zipper family of transcription factors (Murre et al. 1989, Gregor et al. 1990), which have highly conserved C-terminal domains responsible for dimerization and DNA binding. The gene encoding USF1 has been mapped to chromosome region 1q22-q23 (Shieh et al. 1993).

2.4.3.1 USF1 functions

USF1 is ubiquitously expressed and USF1 forms homo- and heterodimers with USF2. These complexes recognise and bind to a promoter sequence CACGTG, also called E-box (Gregor et al. 1990), which results in the activation of transcription and an enhanced expression of the target gene. USF1 regulates the expression of numerous genes, mostly those involved in carbohydrate and lipid metabolism such as: glucokinase (Iynedjian 1998), insulin (Read et al. 1993), glucagon receptor (Portois et al. 2002), plasminogen activator inhibitor-I (Kutz et al. 2006), hepatic lipase (Botma et al. 2001) apolipoproteins A-II (Ribeiro et al. 1999), A-V (Nowak et al. 2005), C-III (Pastier et al. 2002) and E (Salero et al. 2003). In addition, there are reports linking USF1 to the regulation of the expression of other types of molecules. USF1 has been shown to regulate osteopontin expression in smooth muscle cells (Malyankar et al. 1999). Osteopontin is a phosphoprotein, which is found in smooth muscle cells in atherosclerotic plaques (Ikeda et al. 1993). Furthermore, USF1 has been linked to the trascriptional regulation of hepcidin, an acute phase protein, induced by infection and proinflammatory cytokines (Bayele et al. 2006). Expression of CRP is also partially regulated by USF1 (Szalai et al. 2005). All the previous examples make *USF1* an interesting candidate gene for studying cardiovascular diseases in relation to lipid accumulation, inflammation and thrombosis.

2.4.3.2 *USF1* polymorphisms and atherosclerosis

A majority of patients suffering from premature CAD have an inherited lipoprotein disorder (Genest et al. 1992). FCHL is a common genetic dyslipidemia which is characterized by high levels of total cholesterol and/or triglycerides and an early onset atherosclerosis (Goldstein et al. 1973, Nikkilä and Aro 1973). In a linkage analysis study investigating the gene predisposing to FCHL, a new locus was identified on 1q21-q23 (Pajukanta et al. 1998). This is the same region as that found to contain the *USF1* gene (Shieh et al. 1993). A few years later in a Finnish population, FHCL was found to associate with *USF1* (Pajukanta et al. 2004). In this study, the *USF1* gene was sequenced and a total of 23 SNPs were identified, none of which resulted in amino acid changes. Two of them, named usf1s1 (in exon11, rs3737787) and usf1s2 (in intron7, rs2073658), showed linkage to FHCL and triglycerides (Pajukanta et al. 2004). In functional studies, expression profiles in fat biopsies were also found to differ according to the carrier status of the *USF1* haplotype (Pajukanta et al. 2004). Accordingly, it was suggested that usf1s2 is functionally important. It locates in a likely regulatory element and there is significant differential expression of apoE, ATP-binding cassette A1 and angiotensinogen genes based on the genotypes of usf1s2 (Naukkarinen et al. 2005).

Since these findings, linking *USF1* to FCHL, the gene polymorphisms have been studied in different populations. A study on *USF1* SNPs in lipid and glucose metabolism, (European

Atherosclerosis Research Study II) confirmed some of the previous results (Putt et al. 2004). Sequencing the gene region revealed no evidence of coding SNPs in the population of healthy young men, and the association with triglycerides was not statistically significant, although a trend was seen. There was however, a haplotype association with peak glucose values in the oral glucose tolerance test. (Putt et al. 2004)

Further confirmation came from other populations. In an American population studying the two SNPs, usf1s1 and usf1s2, the results found that FCHL, triglycerides and LDL-C associated with usf1s1 in the whole population, and in males associations with triglycerides and LDL-C were found with usf1s1, usf1s2 and the haplotype of the two (Coon et al. 2005). Relevant results have also been published with different ethnic populations: FCHL and triglycerides were shown to associate with usf1s1 and usf1s2 in Mexicans (Huertas-Vazquez et al. 2005). Among the Chinese, significant associations were found between different *USF1* SNPs and haplotypes with type 2 diabetes and metabolic syndrome (Ng et al. 2005). However, in a French case-control study, no evidence of associations between the *USF1* SNPs studied and type 2 diabetes was found, and the SNPs did not associated with biochemical parameters - glucose, triglycerides, total cholesterol, or apolipoproteins A-I or B - in non-diabetics (Gibson et al. 2005). Testing for *USF1* gene variant associations with CVD risk factors revealed significant associations between dense LDL particles in males and indices of adiposity (BMI, waist circumference, visceral and subcutaneous fat) in both female and male French Canadians (Choquette et al. 2007). In Dutch FCHL families, no associations were found with *USF1* polymorphisms when the FCHL was defined by nomogram, but when the traditional diagnostic criteria were used, suggestive associations with FCHL were found in addition to associations with total cholesterol and apoB (van der Vleuten et al. 2007). The usf1s1 polymorphism was found to influence lipid levels in a different population of Dutch FCHL patients and with CAD in America Caucasians in a sex-dependent manner (Lee et al. 2007). The common allele of usf1s1 in the males of both groups conferred risk, whereas in the American Caucasian group, the minor allele was associated with risk in females with CAD (Lee et al. 2007). At the population level, there is a report of *USF1* gene polymorphism, the minor allele of usf1s2, associating with increased risk of CVD and all cause mortality in females (Komulainen et al. 2006). (Table 5.)

Table 5. Association studies of *USF1* polymorphisms (usf1s1, rs3737787 and usf1f2, rs2073568) with different variables.

Population, size	SNP	Variables	Result	Reference
Americans with early CAD, stroke or hypertension, n=2195	usf1s1, usf1s2	FCHL, triglycerides, LDL-C	positive in the whole population, positive for triglycerides and LDL-C in men	Coon et al. 2005
Mexicans FHCL family members n=314	usf1s2, usf1s1	FCHL, triglycerides	positive	Huertas-Vazquez et al. 2005
Chinese healthy population n=175	usf1s1	type 2 DM, metabolic syndrome	positive	Ng et al. 2005
French type 2 DM patients n=744, controls, n=731	usf1s1, usf1s2	type 2 DM, glucose, triglycerides, total cholesterol, apoA-1, apo-B	negative	Gibson et al. 2005
French Canadians, n=760	usf1s1, usf1s2	different CVD risk factors	positive for BMI, waist circumference, visceral and subcutaneous fat in the whole population and dense LDL-particles in males	Choquette et al. 2007
Dutch FHCL family members, n=611	usf1s1, usf1s2	FHCL and different biochemical parameters	positive for total cholesterol and apo-B	van der Vleuten et al. 2006
Dutch FCHL family members, n=532	usf1s1	lipids	major allele positive for FCHL, triglycerides, total cholesterol and apo-B in men	Lee et al. 2007
American Caucasian, coronary angiography patients, n=1533	usf1s1	lipids	major allele positive for triglycerides in men, minor allele positive for triglycerides in women	Lee et al. 2007
Finnish random population, n=2225	usf1s2	CVD risk	positive in women	Komulainen et al. 2006

Abbreviations: SNP; single nucleotide polymorphism; FCHL familial combined hyperlipidemia; LDL-C low density lipoprotein cholesterol; DM, diabetes mellitus; apo, apolipoprotein; CVD, cardiovascular disease, BMI body mass index.

All studies so far have been conducted on populations with an established diagnosis of type 2 diabetes, FCHL or coronary artery disease, all of which are of a different phenotype from that of early atherosclerosis with no subjective symptoms. Komulainen and colleagues raised the question of whether the gene variants of *USF1* could have other ways than via lipid parameters to affect the

pathophysiology of atherosclerosis (Komulainen et al. 2006). Going back to the list of genes that are influenced by USF1, there are many that have been connected to earlier phenotypes in the course of atherosclerosis. ApoE (Salero et al. 2003), CRP (Szalai et al. 2005), plasminogen activator inhibitor-1 (Providence et al. 2002) and osteopontin (Malyankar et al. 1999), for example, are found in atherosclerotic plaques of the artery wall (Ylä-Herttuala et al. 1990, Stoop et al. 2000, Golledge et al. 2004, Norja et al. 2007). In addition, these genes have also been connected to the development of early atherosclerosis (Ilveskoski et al. 2000, Sakata et al. 2004, Kurata et al. 2006, Thakore et al. 2007).

There are no previous studies on the possible associations of the *USF1* polymorphisms or haplotypes with the vascular parameters IMT, CAC or FMD that may detect the early, subclinical phase of atherosclerosis.

2.4.4 Gene–environment interactions

Most of the multifactorial or complex diseases such as atherosclerosis, CAD and diabetes are known to be modified by both environmental and genetic factors. This is said to suggest that there is an interaction between an environmental factor or its consequence and the gene product or products. Therefore, the risk of the underlying genetic disposition may be modified by the environment. An interaction between a gene and the environment implies that, at the molecular level, the effect of the environmental factor modifies the function or the effect of the gene product. Typically, a gene-environment interaction is associated with greater than the expected additive effects of the genetic and environmental factors. Some show that the effect of a polymorphism on risk is only evident in association with certain environmental factors. Each person has a fixed predetermined genetic disposition to atherosclerosis which cannot be modified in addition to a certain environmental risk depending on lifestyle factors such as smoking, diet and exercise, all of which can be altered. In the event that a person with a high genetic risk occupies a high risk environment, the interaction effect may be great enough to cause the development of premature disease (Visvikis-Siest and Marteau 2006, Talmud 2007). The combination of genetic predisposition and interaction with an environmental factor can divide a phenotype into endophenotypes. An endophenotype is a hereditary characteristic which is associated with the disease but not a direct symptom of it. A model of gene-environment interaction is depicted in Figure 5.

There are many examples of gene-environment interactions in atherosclerosis. ApoE genotypes interact with smoking to affect CAD risk (Talmud et al. 2005). The genotype and smoking can also interact to influence carotid artery atherosclerosis (Djousse et al. 2002). For example, a certain allele of the angiotensin converting enzyme has an effect on carotid IMT in

smokers, but not in non-smokers (Sayed-Tabatabaei et al. 2004). As these examples show, the understanding of and interest in studying the complex diseases also from this point of view have increased recently, and this is a direction to be continued with. In future studies, it is important to also study the effects of environmental factors and to examine subpopulations of different profiles. This approach is likely to improve the understanding of the diseases and help to find specific and targeted therapies for high-risk subgroups. (Visvikis-Siest and Marteau 2006, Talmud 2007)

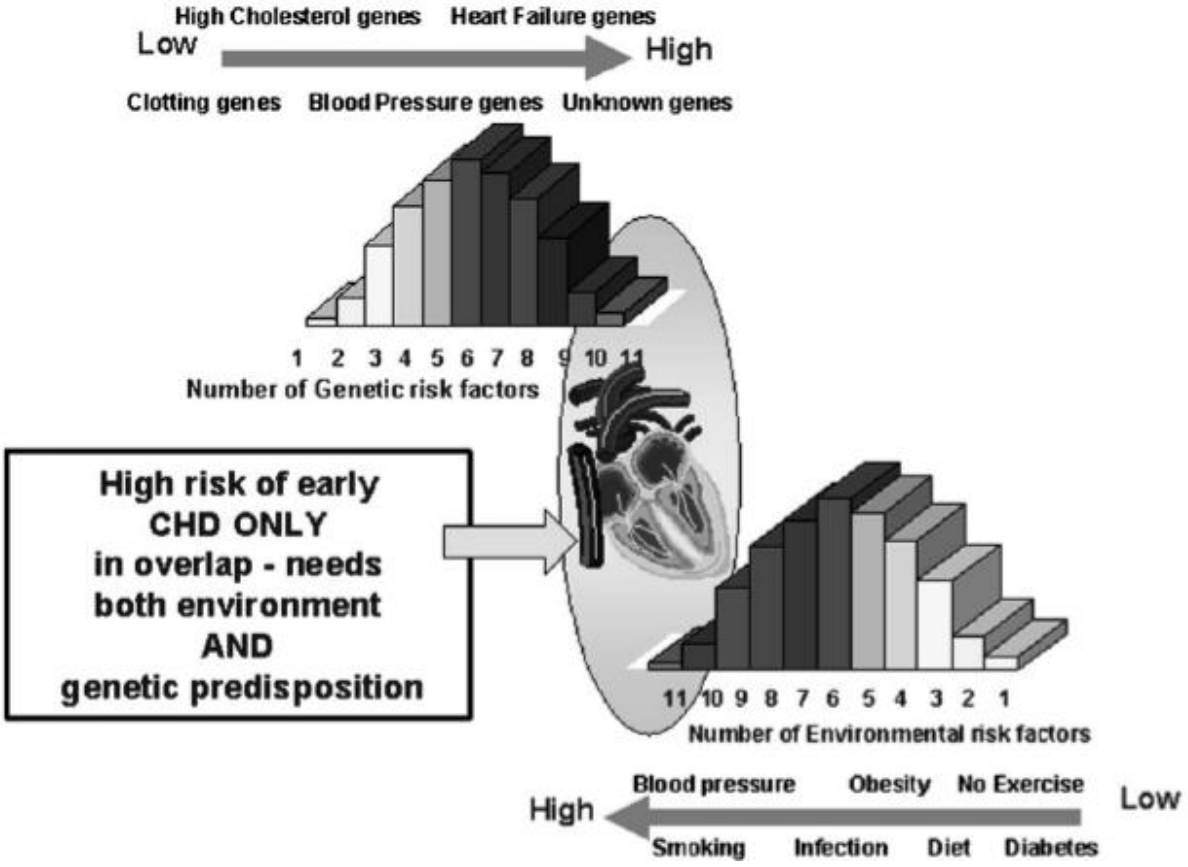

Figure 5. Model for gene-environment interaction. The model proposes that an individual has a position on the genetic risk spectrum depending on the number of inherited risk-increasing gene variants. They are also exposed to a variety of environmental risk factors. It is proposed that the risk of coronary heart disease (CHD) only occurs when the person at high genetic risk enters a high-risk environment and that genetic or environmental risks alone will not trigger a CHD event. Modified from Talmud 2007.

3 AIMS OF THE STUDY

The atherosclerotic process starting in childhood is a multifactorial disease and has many risk factors - metabolic, lifestyle-related and genetic. For early prevention, treatment or dietary intervention, it would be important to be able to detect subjects that are at high risk. The measured parameters, IMT, CAC and FMD, were used in the current studies as surrogate markers for the detection of subclinical atherosclerosis. Candidate genes, *Cx37*, *MTHFR* and *USF1*, were selected because the molecules they code may influence the pathogenesis of atherosclerosis. Connexin37 mediates cellular communication and is a factor in leukocyte adhesion to the vascular wall, the activity of MTHFR affects homocysteine metabolism and its blood concentration and USF1 has many links to atherosclerosis by regulating several genes in the lipid and carbohydrate pathways. However, the exact role of these genetic markers in the development of early subclinical atherosclerosis is largely unknown.

The present study elucidated the relationship of the allelic variation of the three candidate genes, *Cx37*, *MTHFR* and *USF1*, with ultrasonographically measured surrogate markers of early atherosclerosis as well as biochemical risk factors as a part of the Cardiovascular Risk in Young Finns Study. The specific aims of the present thesis are as follows:

1. To investigate whether the *Cx37* C1019T (rs1764391) polymorphism is associated with carotid artery IMT, CAC and brachial artery FMD and to examine the possible interaction effects of the polymorphism and common risk factors of atherosclerosis: age, smoking, physical activity, blood pressure, obesity, high homocysteine, insulin, total cholesterol, LDL-C, triglycerides, C-reactive protein or HDL-C on early markers of atherosclerosis i.e., IMT, CAC or FMD.

2. To analyse the *MTHFR* C667T (rs1801133) polymorphism in a Finnish population and to analyse whether there is an association between the polymorphism or serum homocysteine concentration and measured early markers of atherosclerosis IMT, CAC and FMD.

3. To study the associations of *USF1* gene polymorphisms usf1s1 (rs3737787), usf1s2 (rs2073658), and usf1s8 (rs2516838) as well as their haplotypes with carotid artery IMT, CAC and brachial artery FMD.

4 SUBJECTS AND METHODS

4.1 Subjects

The population studied in this thesis is from the ongoing prospective cohort study, The Cardiovascular Risk in Young Finns. It was started as a multicentre follow-up study of atherosclerosis risk factors in children and adolescents. The first cross-sectional examination of the study subjects was undertaken in 1980. The study is being carried out in five university cities with medical schools: Helsinki, Tampere, Turku, Kuopio and Oulu.

Figure 6. A map showing the locations of the five univeristy hospital cities where the Cardiovascular Risk in Young Finns Study has been carried out.

At the beginning of the study, 4,320 children and adolescents aged 3, 6, 9, 12, 15 and 18 years were randomly selected from the national population register (Åkerblom et al. 1985). Of those invited, 3,596 responded and took part in the study in 1980. Since then, four follow-up studies have been carried out at 3 year intervals until 1992. Physical examinations and blood sampling for the whole cohort were performed in 1983, 1986 and 1989. In 1992, the Helsinki, Kuopio and Turku region cohorts were included for blood sampling and the physical examination was carried out with the Turku cohort. The fifth follow-up examination was carried out between September 2001 and January 2002. At the time, the study subjects were between the ages of 24 and 39 years, and a total of 2,283 subjects participated, representing 63.5% of the original cohort (Juonala et al. 2004b). Non-invasive ultrasound measurements for vascular parameters were also introduced in 2001 (Raitakari et al. 2003, Juonala et al. 2004a, Juonala et al. 2005), providing detailed phenotypes for

study purposes. Blood samples for genetic analyses were also added to the study protocol. For this thesis project, first a subpopulation of 1,440 subjects of the 2001 cohort was studied for economical reasons concerning the genotyping (studies I, II and III). This group was randomly selected out of all subjects with complete follow-up data. Subsequently, the whole population of 2,283 subjects was used as the study population (study IV). The study was conducted under the guidelines of the declaration of Helsinki and was approved by local ethics committees. All participants gave written informed consent.

4.2 Methods

4.2.1 Physical examination and questionnaires

Participants filled in questionnaires on their general lifestyle including the following information: socioeconomic status, alcohol use, smoking, physical activity, their own and family medical history and eating behaviour. The duration, intensity and frequency of physical activity were assessed to calculate the physical activity index which was modified from a previously described method (Telama et al. 1985). Weight was measured with digital scales with an accuracy of 0.1 kg, and height using a wall-mounted statiometer with the accuracy of 0.5 cm. Body mass index was calculated from the measured height and weight data using the formula, BMI = weight (kg)/[height (m)]^2. Blood pressure (BP) was measured with a random zero sphygmomanometer. The average of three measurements with readings to the nearest even number was used in the analysis. In the analyses, mean BP was calculated using measured systolic (SP) and diastolic (DP) blood pressure values in the formula, mean BP = DP + 1/3 (SP - DP).

4.2.2 Blood collection and analyses

Venous blood samples were drawn from the antecubital vein after a 12-hour fast. All assays were done in duplicate in the laboratory of the Research and Development Unit of the Social Insurance Institution, Turku, Finland. Serum total cholesterol, HDL-C and triglycerides were determined by means of standard enzymatic methods (Olympus System Reagent; Germany). LDL-C concentration was calculated using the Friedewald formula (Friedewald et al. 1972). Serum apolipoproteins A-I and B were analyzed with an immunoturbidometric assay (Orion Diagnostica, Espoo, Finland). Serum glucose was measured enzymatically (glucose dehydrogenase, Olympus Diagnostica GmbH, Hamburg, Germany). Serum high sensitive CRP was measured using a latex turbidimetric immunoassay (Wako Chemicals GmbH, Neuss, Germany). The lower detection limit for the assay was 0.06 mg/l. A microparticle enzyme immunoassay kit was employed to measure serum homocysteine (Imx assay, Abbott Laboratories, Tokyo, Japan).

4.2.3 Ultrasound measurements

Ultrasound measurements were performed using Sequoia 512 ultrasound mainframes (Acuson, CA, USA) with 13.0 MHz linear array transducers.

Intima media thickness. The posterior wall of the left common carotid artery was scanned following a standardized protocol (Raitakari et al. 2003). A magnified image from the angle showing the greatest distance between the lumen-intima interface and the media-adventitia interface was recorded. A five-second moving scan, which included the beginning of the carotid bifurcation and the common carotid artery was recorded and stored in digital format for subsequent off-line analysis. Scans were analysed by a single reader blinded to subjects' details. Ultrasonic callipers were used to perform the analysis. The best-quality end-diastolic frame was selected from the clip image. From this image, at least four measurements of the common carotid far wall were taken approximately 10 mm proximal to the bifurcation to derive maximal carotid IMT. To assess the reproducibility of IMT measurements, 57 subjects were re-examined three months after the initial visit (2.5% random sample). The between visit coefficient of variation for IMT measurements was 6.4%. Below an ultrasound image of an IMT measurement (Figure 7) and a histological image of intimal thickening (Figure 8).

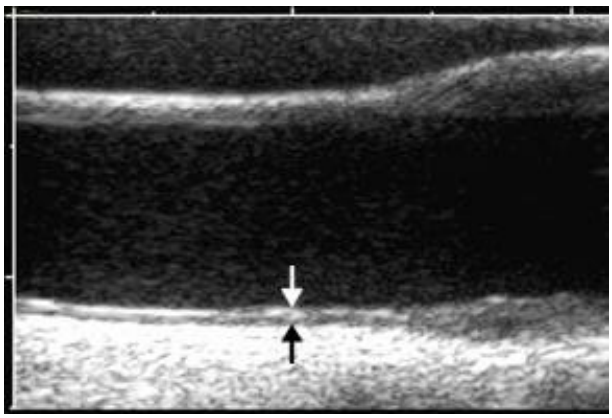

Figure 7. Carotid artery IMT measured at 10 mm proximal to the carotid bifurcation. Lumen-intima interface (white arrow) and media-adventitia interface (black arrow) are indicated.

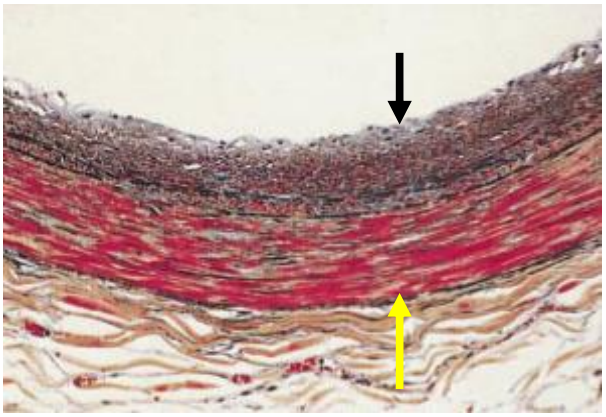

Figure 8. Intimal thickening. Lumen-intima interface (black arrow) and media-adventita interface (yellow arrow) are indicated. Modified from (Virmani et al. 2000)

Carotid artery compliance. Assessment of CAC was made by selecting the best-quality cardiac cycle from the 5- second clip images. The diameter of the common carotid artery 10 mm from the carotid bifurcation was measured at least twice in end-diastole and end-systole. The mean of the measurements was used as the end-diastolic and end-systolic diameters. Ultrasound measurements

and concomitant brachial blood pressure values were used to calculate carotid artery compliance = ([D_s - D_d] / D_d) / (SP - DP), where D_s is the systolic diameter, D_d the diastolic diameter, SP the systolic blood pressure and DP the diastolic blood pressure. The between-visit coefficient of variation was 2.7% for diastolic carotid diameter and 16.3% for CAC.

Brachial artery flow-mediated dilatation. The left brachial artery diameter was measured at rest and during reactive hyperaemia, which was induced by placing a tourniquet on the forearm and holding a pressure of 250 mmHg for 4.5 minutes and then released (Figure 9). The arterial diameter was measured three times at end-diastole at a fixed distance from an anatomic marker at rest and at 40, 60 and 80 seconds after cuff release. The average of the three measurements at each time point was used to derive the maximum FMD which is the greatest value between 40 to 80 seconds. The diameter of the vessel in scans after reactive hyperaemia was expressed as the percentage relative to the resting scan (100 percent). The between-visit coefficient of variation was 3.2% for brachial diameter and 26% for FMD.

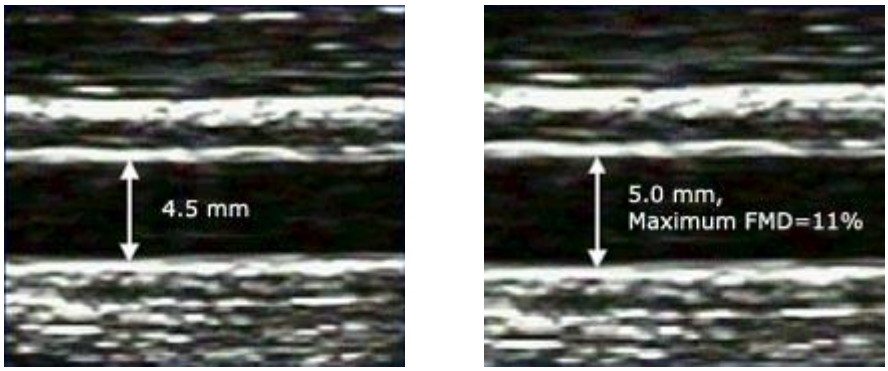

Figure 9. Brachial artery diameter measured at rest (left panel) and after reactive hyperaemia (right panel). FMD values were expressed as percentage relative to the resting scan (100%).

4.2.4 DNA extraction and genotyping

Genomic DNA was purified from samples of whole blood, which had been stored frozen at -20° C. Purification was performed using the Quiagen QIAmp DNA Blood Mini Kit (Quiagen Inc., Hilden Germany) or by the BioRobot M48 Workstation according to the manufacturer's instructions (Qiagen Inc., Hilden, Germany). Nucleotide sequences for the primers and allele-specific wild-type and variant probes labelled with the reporter dyes FAM™ or VIC® were deduced from sequences deposited in the GenBank database and synthesized in accordance with Applied Biosystems using the Assays by Design service. Samples were genotyped by employing the 5' nuclease assay in combination with specific fluorogenic TaqMan® MGB™ probes using the ABI Prism 7000 or the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Two

types of polymerase chain reactions (PCR) were employed. The first genotyping reactions were performed in a 25µl reaction volume containing genomic DNA, 1 x Universal Master Mix, 900 nM of each primer and 200 nM of each probe. The reactions were performed in 96-well plates following a standard protocol for TaqMan MGB probes. Later, new equipment was installed and a 5 µl reaction volume was used. These reactions were performed in 384-well plates using the standard method, and PCR pipetting steps were carried out with the Tecan© robot with Freedom EVOware, ver.1.0 programme (Tecan treding AG, Männedorf, Switzerland). Allele-specific fluorescence generated from each probe during the PCR amplification was measured with the allelic discrimination analysis module, resulting in the identification of three genotypes for each of the polymorphisms studied (Livak 1999). Random duplicates were used as a quality control.

4.3 Statistical methods and haplotype determination

For all the studies, continuous variables between different groups were compared by analysis of variance (ANOVA). For comparing categorical variables, the χ^2 test was used. Data which were not normally distributed were analyzed after logarithmical transformation, with results expressed as crude values. Linear regression analysis was used in the studies to determine the effects of the different genotypes or haplotypes on the vascular parameters IMT, CAC and FMD. Variables used in the multivariate models were chosen after stepwise analyses. In study II, interactions between the genotype and risk factors and their effects on the vascular parameters were analysed using analysis of covariance (ANCOVA). In study III, linkage disequilibrium analysis between polymorphisms was carried out with freely distributed software (Ding et al. 2003). Haplotypes were estimated from the studied single-nucleotide polymorphisms using the PHASE version 2.1.1 programme (Stephens et al. 2001, Stephens and Scheet 2005). This programme uses a Bayesian statistical method for reconstructing haplotypes from population genotype data and it lists the most likely pairs of haplotypes for each study subject.

Results are shown as mean ± standard deviation (SD), unstandardised regression coefficients (Beta) and standard errors (SE). P-values of less than 0.05 were considered statistically significant. All the statistical analyses were conducted using the statistical software SPSS (version 14.0 or 15.0, SPSS Inc., Chicago, IL, USA).

5 RESULTS

5.1 Characteristics of the population, candidate gene polymorphisms and haplotypes

The allele distributions for each of the genes were in Hardy-Weinberg equilibrium. There were no significant differences in age or sex among the different genotype groups. The genotype frequencies for the studied genes in the two populations were as stated in Table 6. There were 18 missing values for the Usf1s8 polymorphism due to unsuccessful genotyping.

Table 6. Frequencies of the studied genotypes for *Cx37* C1019T (studies I and II), *MTHFR* C677T (study III), and *USF1* usfs1, usfs2, usfs8 (study IV).

	Cx37 genotype	Number of subjects		*MTHFR* genotype	Number of subjects		*USF1* genotype	Number of subjects
C1019T	CC	486 (33.8 %)	C677T	CC	835 (58 %)	usf1s1	GG	929 (41.5 %)
	CT	717 (49.8 %)		CT	527 (36.6 %)		GA	1023 (45.7 %)
	TT	237 (16.4 %)		TT	78 (5.4 %)		AA	286 (12.5 %)
						usf1s2	CC	930 (41.6 %)
							CT	1023 (45.7 %)
							TT	284 (12.7 %)
						usf1s8	CC	1173 (52.8 %)
							CG	897 (40.4 %)
							GG	150 (6.8 %)

Six haplotypes (1-6) were presumed for the usf1s1 (rs3737787), usf1s2 (rs2073658) and usf1s8 (rs2516838) polymorphisms; they are 1(G-C-C), 2(G-C-G), 3(A-C-C), 4(A-T-C), 5(A-T-G) and 6(G-T-G). The haplotype analysis in our population resulted in only 5 haplotype pairs, each coded quantitatively as 1 = two copies of the haplotype, 2 = one copy of the haplotype and 3 = no copies. The haplotype frequencies were as follows: for haplotype 1, 1 = 321, 2 = 1077 and 3 = 884; for haplotype 2, 1 = 150, 2 = 908 and 3 = 1224; for haplotype 3, 1 = 0, 2 = 0 and 3 = 2281; for haplotype 4, 1 = 287, 2 = 1060 and 3 = 935; and for haplotype 5, 1 = 0, 2 = 1 and 3 = 2281. Haplotypes 3 and 5 were omitted from further analyses as almost the whole population was of only one type. Characteristics of the study groups are presented in Table 7.

Table 7. Characteristics of the two populations: 1,440 subjects in studies I, II and III; 2,238 subjects in study IV. Non-smokers have never smoked and smokers are current or ex-smokers. Values are means and standard deviations (SD).

Variable	Population of 1440 subjects	Population of 2238 subjects
	Mean ± SD	Mean ± SD
Subjects (men/women)	720 / 720	999 / 1239
Age (years)	31.9 ± 5.0	31.7 ± 5.0
Body mass index (kg/m²)	25.1 ± 4.4	25.1 ± 4.4
Smoking (no/yes)	647 / 754	1412 / 766
Systolic blood pressure (mmHg)	122.7 ± 14.6	122.1 ± 14.5
Diastolic blood pressure (mmHg)	73.4 ± 9.0	73.3 ± 9.0
Mean IMT (mm)	0.581 ± 0.093	0.581 ± 0.092
Maximal FMD (%)	8.05 ± 4.43	8.02 ± 4.43
CAC (mm/mmHg)	2.14 ± 0.74	2.17 ± 0.74
Total cholesterol (mmol/)	5.17 ± 0.98	5.17 ± 0.98
HDL cholesterol (mmol/l)	1.28 ± 0.31	1.29 ± 0.32
LDL cholesterol (mmol/l)	3.29 ± 0.86	3.28 ± 0.86
Triglycerides (mmol/l)	1.34 ± 0.84	1.34 ± 0.86
C-reactive protein (mg/l)	1.91 ± 4.03	1.95 ± 3.99
Glucose (mmol/l)	5.05 ± 0.83	5.05 ± 0.84
Homocysteine (µmol/l)	9.93 ± 3.84	9.80 ± 3.76
Apolipoprotein A-1 (g/l)	1.49 ± 0.25	1.50 ± 0.26
Apolipoprotein B (g/l)	1.07 ± 0.26	1.06 ± 0.27

Abbreviations: IMT, carotid artery intima media thickness; FMD, brachial artery flow mediated dilatation; CAC, carotid artery compliance; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

5.2 Effects of *Cx37* on risk factors and early markers of atherosclerosis (Study I)

Dividing the population of 1,440 by *Cx37* genotype and comparing the studied variables with ANOVA, the only significant difference between genotype groups was in BMI, with a p-value of 0.029. The results of the analysis are shown in Table 8. The same analyses were carried out for men and women separately. In women, the results indicated a difference between genotype groups in mean values of total cholesterol: CC 4.99 ± 0.91mmol/l, CT 5.18 ± 0.94 mmol/l and TT 5.03 ±1.01 mmol/l (p-value 0.042). In men, there were differences between genotype groups in mean values for BMI: CC 26.11 ± 4.46 kg/m^2, CT 25.3 ± 3.8 kg/m^2 and TT 26.2 ± 4.5 kg/m^2 (p-value 0.028); and for mean CRP values: CC 2.02 ± 4.94 mg/l, CT 1.37 ± 2.89 mg/l and TT 1.51 ± 2.54 mg/l (p-value 0.023). After correcting for multiple comparisons, the p-values for BMI in the whole population, total cholesterol in women, and BMI and CRP for men did not remain significant.

Table 8. Clinical characteristics and biochemical parameters according to the *Cx37* C1019T (rs1764391) polymorphism genotype. Values are means ± standard deviations.

Variable (unit)	*Cx37* genotype			p
	CC	CT	TT	
Subjects (men)	486 (241)	717 (350)	237 (129)	0.317
Subjects (smokers)	475 (249)	697 (377)	229 (128)	0.673
Age (years)	32.2 ± 5.0	31.7 ± 4.9	31.8 ± 5.2	0.232
Body mass index (kg/m^2)	25.2 ± 4.7	24.9 ± 4.2	25.8 ± 4.6	0.029
Mean blood pressure (mmHg)	89.7 ± 10.2	89.5±10.1	91.2 ± 10.7	0.089
Mean IMT (mm)	0.587 ± 0.098	0.576 ± 0.092	0.578 ± 0.083	0.159
Maximal FMD (%)	8.07 ± 4.44	8.13 ± 4.44	7.75 ± 4.38	0.547
CAC (mm/mmHg)	2.12 ± 0.73	2.17 ± 0.75	2.07 ± 0.72	0.151
Total cholesterol (mmol/l)	5.15 ± 0.95	5.18 ± 0.98	5.16 ± 1.06	0.829
LDL cholesterol (mmol/l)	3.28 ± 0.84	3.29 ± 0.85	3.26 ± 0.96	0.888
HDL cholesterol (mmol/l)	1.26 ± 0.31	1.30 ± 0.31	1.27 ± 0.32	0.083
Triglycerides (mmol/l)	1.34 ± 0.79	1.32 ± 0.88	1.40 ± 0.83	0.502
C-reactive protein (mg/l)	2.12 ± 5.08	1.73 ± 3.06	2.04 ± 4.17	0.222
Homocysteine (µmol/l)	10.10 ± 4.51	9.87 ± 3.49	9.80 ± 3.35	0.521
Apolipoprotein A-1 (g/l)	1.48 ± 0.25	1.51 ± 0.26	1.49 ± 0.25	0.200
Apolipoprotein B (g/l)	1.08 ± 0.26	1.07 ± 0.27	1.08 ± 0.26	0.698

Abbreviations: IMT, carotid artery intima media thickness; FMD, brachial artery flow mediated dilatation; CAC, carotid artery compliance; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

A model comparing the CC genotype to T allele carriers, the CT and TT genotypes combined, was also made. In the analysis, there were no significant differences between the groups in the whole population. In women, however, the mean values for total cholesterol were lower in the CC genotype compared to T allele carriers; mean values were 4.99 ± 0.91 mmol/l and 5.14 ± 0.96 mmol/l, respectively (p-value 0.043). In men, the T allele carriers had lower mean CRP values compared to the CC genotype; 1.40 ± 2.80 mg/l and 2.02 ± 4.94 mg/l, respectively (p-value 0.033), and also for mean IMT values, the T carriers had lower values than the CC genotype, 0.585 ± 0.097 mm and 0.604 ± 0.106 mm, respectively (p-value 0.017). After correction for multiple comparisons, only the p-value for IMT in T carrier men remained significant.

No significant associations were found between the *Cx37* C1017T genotypes and carotid artery IMT, CAC or brachial artery FMD either in the whole population or in the separate groups of men and women divided by genotype, or in the recessive models comparing CC to T allele carriers.

5.3 *Cx37*; interactions with risk factors and effects on vascular parameters (Study II)

In Study II the same population of 1,440 subjects and the same variables were used as in Study I. Interaction analyses between *Cx37* genotype and variables (age, BMI, current smoking, physical activity, mean blood pressure, total cholesterol, HDL-C, LDL-C, triglycerides CRP and homocysteine) with regard to the vascular parameters IMT, CAC and FMD were carried out for the whole population and for men and women separately by means of ANCOVA. The significant interaction effects found were *Cx37* and homocysteine on FMD (p-value 0.038) in the whole population, *Cx37* and physical activity on CAC in men (p-value 0.011) and *Cx37* and smoking on FMD in women (p-value 0.004).

Multivariate linear regression analyses were run with different models for adjustments. The interaction effect between homocysteine and *Cx37* on FMD was statistically significant in the whole population: higher levels of homocysteine tended to associate with higher FMD values in subjects with the TT genotype and somewhat lower FMD levels in subjects with CT genotype. This was the case in three different adjusted models (p for interaction 0.022 to 0.038), as seen in Table 9.

Table 9. The relationship between homocysteine and the Connexin37 (*Cx37)* rs1764391 genotype in predicting brachial artery flow-mediated dilatation (FMD) in the whole population.

	Cx37 CC (n=446)			*Cx37* CT (n=661)			*Cx37* TT (n=214)			P for
	β	SE	p	β	SE	p	β	SE	p	interaction
Model 1	-0.591	0.643	0.358	-1.713	0.570	0.003	1.263	1.035	0.224	0.038
Model 2	0.620	0.643	0.335	-0.940	0.575	0.103	2.504	1.020	0.015	0.022
Model 3	0.505	0.645	0.435	-0.896	0.589	0.129	2.414	1.056	0.023	0.038
Model 4	0.345	0.739	0.641	-0.793	0.637	0.215	2.273	1.090	0.039	0.093
Model 5	0.576	0.632	0.362	-0.916	0.566	0.106	2.467	1.012	0.016	0.025
Model 6	0.443	0.736	0.548	-0.872	0.649	0.180	2.192	1.138	0.056	0.088

Model 1 – no adjustment

Model 2 – adjusted for age, sex and brachial artery diameter

Model 3 – adjusted for age, sex, brachial artery diameter and current smoking

Model 4 – adjusted for age, brachial artery diameter and physical activity

Model 5 – adjusted for age, sex, brachial artery diameter, BMI, mean BP, HDL-C and LDL-C

Model 6 – adjusted for all of the above

Statistics: Multivariate models. Analysis of covariance was used to analyze *Cx37* genotype interactions with homocysteine and their effects on FMD. Adjusted with risk factors as shown in models 2-6.

Abbreviations: β, unstandardised regression coefficient, values are regression coefficients (expressed as %) for a 1-unit change in serum homocysteine; SE, standard error; BMI, body mass index; BP, blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

There was a significant interaction effect in women between smoking and *Cx37* on FMD. The results indicate that in female smokers with the CC genotype, FMD values are significantly increased as compared to non-smokers. For the TT genotype, on the other hand, the effect was the opposite, i.e. female smokers had lower FMD values compared to non-smokers, as seen in Table 10.

Table 10. The relationship between smoking (no/yes) and Connexin37 (*Cx37*) rs1764391 genotype in predicting brachial artery flow mediated dilatation (FMD) in women.

	Cx37 CC (n=228)			*Cx37* CT (n=350)			*Cx37* TT (n=102)			P for
	β	SE	p	β	SE	p	β	SE	p	interaction
Model 1	2.129	0.651	0.001	-0.139	0.531	0.794	-1.452	1.057	0.173	0.004
Model 2	2.200	0.623	0.001	-0.124	0.511	0.808	-1.933	1.009	0.058	0.001
Model 3	1.754	0.762	0.023	0.099	0.598	0.868	-1.349	1.154	0.247	0.059
Model 4	2.211	0.626	0.001	0.044	0.498	0.929	-2.349	1.017	0.023	0.001
Model 5	1.789	0.776	0.022	0.218	0.585	0.709	-2.433	1.209	0.049	0.041

Model 1 – no adjustment

Model 2 – adjusted for age and brachial artery diameter

Model 3 – adjusted for age, brachial artery diameter and physical activity

Model 4 – adjusted for age, brachial artery diameter, BMI, mean BP, HDL-C and LDL-C

Model 5 – adjusted for all the above

Statistics: Multivariate models. Analysis of covariance was used to analyze *Cx37* genotype interactions with smoking and their effects on FMD. Adjusted with risk factors as shown in models 2-5.

Abbreviations: β, unstandardised regression coefficients, values are regression coefficients (expressed in %) for the absence/presence of smoking. SE, standard error; BMI, body mass index; BP, blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

In men, there was a significant interaction effect between physical activity and *Cx37* on CAC both in the CT and the TT genotype group. The effect, however, did not remain significant in any of the adjusted models.

In Study II, we did not find any significant interaction effects between *Cx37* genotype and atherosclerosis risk factors on IMT.

5.4 Effects of *MTHFR* on homocysteine and early markers of atherosclerosis (Study III)

The population of 1,440 was divided by genotype and sex and the characteristics were analysed with ANOVA. In the comparison between *MTHFR* genotypes, there was an expected significant difference in the homocysteine values in both men and women, with highest values for the TT genotype. In men, mean homocysteine values for the genotypes were: 10.04 ± 2.27 µmol/l for CC,

10.93 ± 3.85 µmol/l for CT and 19.06 ± 8.62 µmol/l for TT (p-value < 0.001). In women the results were: 8.43 ± 2.3 µmol/l for CC, 9.29 ± 3.62 µmol/l for CT and 12.97 ± 6.35 µmol/l for TT (p-value < 0.001). Another difference between genotypes was found; men with the CC genotype had the lowest CAC values. Mean values for CAC were 1.95 ± 0.65 %/10mmHg for CC, 2.11 ± 0.67 %/10mmHg for CT and 2.07 ± 0.69 %/10mmHg for TT (p-value 0.008). None of the other vascular variables differed significantly between the genotype groups. The variables selected for each final linear model were derived from stepwise regression modelling.

Associations between atherosclerosis risk factors and homocysteine. Results of the final multivariate model for factors associating with homocysteine are presented in Table 11. In a parallel model in which apoA-I and apoB were replaced with HDL-C and LDL-C the adjusted R^2 values were 17.6% ($p < 0.001$) for the whole population and 15.8% ($p < 0.001$) for men and 8.6% ($p < 0.001$) for women.

Table 11. Multivariate linear regression model for the relationship between atherosclerosis risk factors and serum homocysteine (µmol/l) (dependent) in the whole population and divided by sex.

Explanatory variable	All subjects			Women			Men		
	Beta	SE	p	Beta	SE	p	Beta	SE	p
Sex (female/male)	1.615	0.214	<0.001	-	-	-	-	-	-
Age (years)	0.007	0.020	0.268	0.026	0.025	0.359	-0.012	0.031	0.792
Smoking (no/yes)	0.209	0.190	0.268	0.109	0.242	0.321	0.364	0.294	0.547
BMI (kg/m²)	0.013	0.027	0.548	0.007	0.032	0.526	0.019	0.045	0.939
MTHFR (CC>CT>TT)	2.098	0.157	<0.001	1.483	0.202	<0.001	2.707	0.239	<0.001
CRP (mg/l)	-0.022	0.024	0.444	-0.023	0.029	0.475	-0.012	0.040	0.971
Glucose (mmol/l)	-0.284	0.119	0.006	-0.326	0.166	0.050	-0.260	0.174	0.047
Insulin (mU/l)	0.005	0.023	0.548	0.029	0.026	0.386	-0.032	0.040	0.939
Triglycerides (mmol/l)	-0.008	0.151	0.270	-0.241	0.209	0.059	0.195	0.220	0.629
Apo A-1 (g/l)	-1.754	0.409	<0.001	-1.819	0.485	<0.001	-1.415	0.729	0.113
Apo B (g/l)	0.459	0.500	0.231	0.654	0.658	0.226	0.384	0.752	0.535

Abbreviations: Beta, unstandardised regression coefficient; SE, standard error; BMI, body mass index; MTHFR, methylenetetrahydrofolate reductase; CRP, C-reactive protein; Apo, apolipoprotein.

Statistics: Adjusted R^2=18% (p<0.001) for the total population model and R^2=9.3% (p<0.001) and R^2=15.8% (p<0.001) for women and men, respectively; model with crude values.

Associations between atherosclerosis risk factors and carotid artery CAC. For the model for variables associating with CAC, the results are given in Table 12. In models replacing apoA-I and apoB with HDL-C and LDL-C values, the R^2 for the total population was 27.1% ($p < 0.001$) and 26.1% ($p < 0.001$) and 25.4% ($p < 0.001$) for men and women, respectively.

Table 12. Multivariate linear regression model for the relationship between atherosclerosis risk factors and carotid artery compliance (%/10 mmHg) (dependent) in the whole population and divided by sex.

Explanatory variable	All subjects			Women			Men		
	Beta	SE	p	Beta	SE	p	Beta	SE	p
Sex (female/male)	-0.007	0.043	0.866	-	-	-	-	-	-
Age (years)	-0.034	0.004	<0.001	-0.037	0.005	<0.001	-0.036	0.005	<0.001
Smoking (no/yes)	0.046	0.034	0.180	0.039	0.052	0.451	0.048	0.044	0.277
BMI (kg/m²)	-0.002	0.005	0.627	0.001	0.007	0.937	-0.003	0.007	0.640
MTHFR (CC>CT>TT)	0.038	0.030	0.210	-0.010	0.045	0.832	0.091	0.039	0.020
Systolic BP (mmHg)	-0.020	0.001	<0.001	-0.025	0.002	<0.001	-0.017	0.002	<0.001
CRP (mg/l)	-0.004	0.004	0.390	-0.004	0.006	0.552	-0.002	0.006	0.748
Glucose (mmol/l)	0.031	0.025	0.202	0.037	0.036	0.380	0.022	0.033	0.498
Insulin (mU/l)	-0.011	0.004	0.010	-0.009	0.006	0.115	-0.014	0.006	0.024
Triglycerides (mmol/l)	0.035	0.027	0.202	0.045	0.045	0.314	0.025	0.033	0.446
Apo A-1 (g/l)	-0.035	0.075	0.634	-0.102	0.106	0.334	0.149	0.110	0.178
Apo B (g/l)	-0.273	0.090	0.003	-0.452	0.141	0.001	-0.099	0.114	0.386
Homocysteine (µmol/l)	0.012	0.005	0.014	0.020	0.008	0.013	0.004	0.006	0.444

Abbreviations: Beta, unstandardised regression coefficient; SE, standard error; BMI, body mass index; MTHFR, methylenetetrahydrofolate reductase; BP, blood pressure; CRP, C-reactive protein; Apo, apolipoprotein.
Statistics: Adjusted R^2=27.5% ($p<0.001$) for the total population model and R^2=26.1% ($p<0.001$) and R^2=25.8% ($p<0.001$) for women and men, respectively.

Associations between atherosclerosis risk factors and carotid artery IMT and brachial artery FMD. There were no associations between either *MTHFR* genotype or homocysteine and IMT or FMD in the whole population or in men or women separately in any of the different models.

5.5 Effects of *USF1* on risk factors and early markers of atherosclerosis (Study IV)

When comparing risk factor variables between genotype groups for the three *USF1* polymorphisms, no differences were found between the genotypes.

In the analyses for the vascular parameters, there was a significant difference between genotypes in IMT values for usf1s1 and usf1s8, as seen in Table 13. No differences were found between groups in CAC or FMD values. A comparison of minor allele carriers of the three polymorphisms to common allele homozygotes was also done, but no differences between these groups were found.

As the haplotypes 3 and 5 were mostly all of one type, these were omitted from further analyses. In testing for differences between the common risk factors, there were significant findings for current smoking and diastolic BP in haplotype 1; the p-values for ANOVA were 0.025 and 0.009, respectively. However, no differences were found between groups for haplotypes 2 and 4. Looking at the vascular parameters, there was a difference in IMT between groups in haplotypes 1 and 2, as demonstrated in Table 14. There were no differences between haplotype groups in CAC or FMD.

Table 13. Carotid artery intima-media thickness (IMT) values according to the genotypes of the three *USF1* polymorphisms.

USF1 genotype		Mean IMT (mm)
usf1s1 rs3737787	GG	0.578 ± 0.096
	GA	0.576 ± 0.090
	AA	0.582 ± 0.089
	p-value	0.046
usf1s2 rs2073658	CC	0.586 ± 0.096
	CT	0.576 ± 0.090
	TT	0.582 ± 0.089
	p-value	0.060
usf1s8 rs2516838	CC	0.577 ± 0.089
	CG	0.584 ± 0.093
	GG	0.597 ± 0.115
	p-value	0.021

Statistics: Analysis of variance.

Table 14. Carotid artery intima-media thickness (IMT) values accorcing to the haplotypes derived from the three *USF1* polymorphisms.

USF1 haplotype		Mean IMT (mm)
Haplotype 1	2 copies	0.586 ± 0.095
	1 copy	0.575 ± 0.092
	no copies	0.586 ± 0.092
	p-value	0.011
Haplotype 2	2 copies	0.597 ± 0.115
	1 copy	0.584 ± 0.093
	no copies	0.577 ± 0.089
	p-value	0.028
Haplotype 3	2 copies	0.582 ± 0.088
	1 copy	0.576 ± 0.090
	no copies	0.586 ± 0.096
	p-value	0.057

Statistics: Analysis of variance.

Linear regression analyses were carried out without added variables to examine for linear associations between the vascular parameters and genotypes or haplotypes. Positive associations were found between usf1s8 (G allele) and IMT (p = 0.006) and haplotype 2 (copy number 0) and IMT (p = 0.009).

5.5.1 Associations between *USF1* polymorphisms and early markers of atherosclerosis

In the backward stepwise linear regression analyses for associations between the polymorphisms or haplotypes and IMT, the variables used were: age, sex, BMI, systolic BP, diastolic BP, smoking, CRP, glucose, HDL-C, LDL-C and triglycerides. The variables left in the final models in addition to the polymorphisms were: age, sex, BMI and systolic BP. For the haplotype models, the final variables were as above with smoking added for haplotypes 1 and 2. Results of these models are shown in Tables 15 and 16. Age, sex, BMI and systolic BP were significantly associated with IMT for all the polymorphisms. For usf1s1, the addition of the A allele associated inversely with IMT, in so that IMT values decreased in the order GG>GA>AA (p = 0.038). In other words, the major allele G associated with higher IMT values. For usf1s8 the values for IMT increased in the genotype order CC>CG>GG (p = 0.003) - here the minor allele associated with higher IMT values (Table 15).

Table 15. Multivariate linear regression models (backward stepwise, with the genotype forced into the model) for the relationships between risk factors and carotid artery intima-media thickness (mm) in three analyses depending on the different upstream transcription factor 1 (*USF1*) polymorphisms tested.

	Model for Usf1s1 rs3737787			Model for Usf1s2 rs2073658			Model for Usf1s8 rs2516838		
Explanatory variable	Beta	SE	p	Beta	SE	p	Beta	SE	p
Sex (female=1 male=2)	0.011	0.004	0.007	0.011	0.004	0.006	0.013	0.004	0.003
Age (years)	0.005	0.000	<0.001	0.005	0.000	<0.001	0.005	0.000	<0.001
BMI (kg/m²)	0.003	0.000	<0.001	0.003	0.000	<0.001	0.003	0.000	<0.001
Systolic BP (mmHg)	0.001	0.000	0.001	0.000	0.000	0.001	0.000	0.000	0.002
usf1s1 (GG=1 GA=2 AA=3)	-0.006	0.003	0.038	ND	ND	ND	ND	ND	ND
usf1s2 (CC=1 CT=2 TT=3)	ND	ND	ND	-0.005	0.003	0.069	ND	ND	ND
usf1s8 (CC=1 CG=2 GG=3)	ND	ND	ND	ND	ND	ND	0.009	0.003	0.003

Abbreviations: ND, not determined; usf1s1, *USF1* polymorphism rs3737787; usf1s2, *USF1* polymorphism rs2073658; usf1s8, *USF1* polymorphism rs2516838; Beta, unstandardised regression coefficient; SE, standard error; BMI, body mass index; BP, blood pressure.

Statistics: Variables used in the stepwise analyses were: age, sex, BMI, systolic BP, diastolic BP, smoking, C-reactive protein, glucose, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol and triglycerides. For the final models, adjusted R^2=13.5% (p<0.001) for usf1s1, R^2=13.1% (p<0.001) for usf1s2 and R^2=13.5% (p<0.001) for usf1s8.

In the final models for the haplotypes, sex, age, BMI and systolic BP were all also significantly associated with IMT, with the addition of smoking for haplotypes 1 and 2. There was a negative association between haplotype 2, and IMT values decreased in the order of 2 copies > 1 copy > no copies (p-value 0.006). This indicates that a higher copy number of haplotype 2 predicts higher IMT values (Table 16).

Table 16. Multivariate linear regression models (backward stepwise, with the haplotype forced into the model) for the relationships between risk factors and carotid artery intima-media thickness (mm) in three models depending on the different upstream transcription factor 1 (*USF1*) haplotypes tested.

	Model for Haplotype 1			Model for Haplotype 2			Model for Haplotype 4		
Explanatory variable	Beta	SE	p	Beta	SE	p	Beta	SE	p
Sex (female=1 male=2)	0.010	0.004	0.023	0.010	0.004	0.022	0.010	0.004	0.024
Age (years)	0.005	0.000	<0.001	0.005	0.000	<0.001	0.005	0.000	<0.001
BMI (kg/m²)	0.003	0.000	<0.001	0.003	0.000	<0.001	0.003	0.000	<0.001
Systolic BP (mmHg)	0.000	0.000	0.002	0.000	0.000	0.002	0.000	0.000	0.002
Smoking (no=0, yes=1)	0.008	0.004	0.044	0.008	0.004	0.044	NS	NS	NS
Haplo 1 (copies 2-1-0)	0.002	0.003	0.482	ND	ND	ND	ND	ND	ND
Haplo 2 (copies 2-1-0)	ND	ND	ND	-0.008	0.003	0.006	ND	ND	ND
Haplo 4 (copies 2-1-0)	ND	ND	ND	ND	ND	ND	0.005	0.003	0.054

Abbreviations: Haplo 1, haplotype 1 *USF* polymorphisms rs3737787 rs2073658 rs2516838 (nucleotide sequence G-C-C); Haplo 2, haplotype 2 nucleotide sequence (G-C-G) and Haplo 4, haplotype 4 nucleotide sequence (A-T-C) respectively; Beta, unstandardised regression coefficient; SE, standard error; BMI, body mass index; BP, blood pressure; ND, not determined; NS, not significant.

Statistics: Variables used in the backward stepwise analyses were: age, sex, BMI, systolic BP, diastolic BP, smoking, C-reactive protein, glucose, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol and triglycerides. For the final models, adjusted R^2=13.0% (p<0.001) for haplotype 1, R^2=13.3% (p<0.001) for haplotype 2 and R^2=13.1% (p<0.001) for haplotype 4.

6 DISCUSSION

6.1 Study population and dropout analysis

The study population of this thesis is that of the ongoing Cardiovascular Risk in Young Finns Study. This is an epidemiologic cohort study on risk factors and early signs of atherosclerosis. In the beginning of the study in 1980, a total of 4,320 randomly selected children and adolescents aged 3, 6, 9, 12, 15 and 18 years were invited to participate. The cohort was selected to represent equal amounts of boys and girls, from both rural and urban areas and different geographical locations in Finland. Of those invited to participate a total of 3,596 (83.2%) agreed; this was a high participation rate, and examining the non-participants in a separate questionnaire revealed no systematic reason not participating and the final sample was considered to represent the invited subjects well. (Åkerblom et al. 1985)

The study described in this thesis is based on the data obtained in the 21-year follow-up examination which was conducted in 2001. At the time of this fifth follow-up examination, the subjects were between the ages of 24 and 39 years and a total of 2,283, participated i.e. 63.5% of the original study cohort, participated. When the baseline characteristics of the participants in 2001 were compared to those who had dropped out, there were more women among the participants and the participants tended to be older than the dropouts. In age-adjusted analysis, however, no significant differences were found for men or women in total cholesterol, LDL, HDL, triglycerides, blood pressure, BMI or physical activity. Therefore, the 2001 cohort was considered representative of the original study population (Juonala et al. 2004b, Raitakari et al. 2008).

6.2 Methodological considerations

6.2.1 Candidate genes and association studies

There are different methods to be used when studying complex disorders such as atherosclerosis. Association studies have a greater power to detect effects of common variants than linkage analysis, but they require large sample sizes of thousands of individuals. In these types of studies, the genetic marker of interest is required to be in linkage disequilibrium with the disease allele, i.e. to be inherited together in the population. Genome-wide association studies are expensive to conduct. Therefore, association studies are most often limited to candidate genes, as has been the case with the present thesis. Candidate genes are chosen by assessing previous available studies, gene expression studies and knowledge of their function in biochemical pathways. The whole genome is

searched for genes whose products are also suggested to have a role in the pathology of the disease in question. The selection of the genes to be studied is made by using all the available data. (Novelli et al. 2003, Hirschhorn 2005)

Of the genes studied for this thesis, *Cx37* was chosen as it had previously been identified to be expressed in the vascular wall (Yeh et al. 1997, Li and Simard 1999, Nakamura et al. 1999) and the polymorphism in question had been associated with manifest atherosclerosis (Yeh et al. 2001, Hirashiki et al. 2003, Listi et al. 2005, Wong et al. 2007). The *MTHFR* polymorphism was chosen, because it causes an amino acid change in the enzyme and alters its function (Frosst et al. 1995). There was also putative evidence that this polymorphism has an association with IMT (Kawamoto et al. 2001, Passaro et al. 2001). For CAC and FMD, however, there were no previously found associations. *USF1* was chosen because of its involvement in many biochemical pathways that are connected to atherosclerosis risk factors.

Isolated populations are more commonly used in mapping for genes associated with complex diseases. The Finnish population may be particularly suitable for association studies as it has a genetically homogenous background (Peltonen et al. 1999). Many genetic studies need a large enough population to achieve a significant association, with sample sizes often in the thousands of subjects (Hirschhorn 2005). The strength in the studies included in this thesis is the fairly high sample size, 1,440 or 2,281, compared to many association studies where the number of study subjects is often in the hundreds. Another way to increase the power of an association analysis is to introduce haplotype analysis (Novelli et al. 2003), which was also used in Study IV. In Study II, the gene-environment interactions were investigated to reveal any associations which would only be seen in this context and which should be taken into account in further studies.

6.2.2 Ultrasound measurements

Measurements for the vascular parameters IMT, CAC and FMD were carried out using standardised protocols for ultrasound scanning of the carotid and brachial arteries. The digitally stored scans performed in the five centres were analysed by a single person who was blinded to the subjects details.

Carotid artery IMT has become a widely accepted method for assessing the degree of atherosclerosis. Increased IMT is significantly associated with common cardiovascular risk factors: hypercholesterolemia, smoking, LDL-C, triglycerides and blood pressure (Poli et al. 1988, Heiss et al. 1991) and with the level of atherosclerosis in other arteries of the vascular system (Allan et al. 1997). In addition, a high IMT value is predictive of future myocardial infarction and stroke in asymptomatic adults (O'Leary et al. 1999, Chambless et al. 2000). Measuring IMT in the common

carotid artery is easy and highly reproducible. Furthermore, the measurement gives a good estimate of the degree of atherosclerosis in the coronary arteries (Crouse et al. 1995). In the follow-up study in 2001, the reproducibility of the method was assessed by conducting a second examination for 57 subjects three months apart. The between-visit coefficient of variation was 6.4%, i.e. very similar to previous reports (Kanters et al. 1997).

Arterial elasticity was assessed by using the calculated parameter CAC, which measures the ability of the arterial wall to distend in response to the pulse pressure caused by contraction of the heart muscle. The values for the equation for CAC were derived from ultrasound measurements of the common carotid artery diameter during systole and diastole. The blood pressure measurements were derived from the brachial artery, and this can be argued as a limitation of the method. Preferably both variables, the diameter and pulse pressure, should be measured from the same artery. However, a close relationship between brachial pulse pressure and the relative diameter increase of the carotid artery during systole has been demonstrated (Reneman et al. 1986). This supports the assumption that brachial pulse pressure values can be used in calculating CAC. The between-visit coefficient of variation for carotid diameter in end-diastole was 2.7% and for CAC 16.4%. The values are in agreement with previous studies (Arnett et al. 1999).

Flow-mediated dilatation of the brachial artery was used as a marker for endothelial function. It has previously been correlated with coronary endothelial function (Anderson et al. 1995) and may therefore be used as an indirect way to assess coronary endothelial health. Low reproducibility is the greatest limitation for clinical use. In the present study, there was a relatively large long-term variation in FMD values. The between-visit coefficient of variation was 26%, comparable to a value described in another study (Järvisalo et al. 2006). Several factors, including temperature, diurnal pattern in vascular tone, fat-rich meals, sympathetic stimuli and menstrual cycle, among others, as well as reading variation, may affect the reproducibility of FMD (Corretti et al. 2002). However, the long-term variation in the brachial artery baseline diameter measurements was good. The coefficient of variation was 3%, which suggests that much of the variation in FMD is caused by physical variation and not by errors in measurements.

6.3 The effect of *Cx37* on early markers of atherosclerosis (Study I)

No relationship between the *Cx37* C1019T polymorphism and IMT was found (Study I). Previous studies have reported contradicting results. Both the C and the T allele have been shown to associate with IMT, CAD or MI (Boerma et al. 1999, Yeh et al. 2001, Yamada et al. 2002, Listi et al. 2005, Wong et al. 2007). In contrast to this, in one study neither allele was found to associate with either CAD or MI (Horan et al. 2006). Most of the studies on this polymorphism have been

performed with populations with detectable atherosclerosis phenotypes such as CAD or MI. In these cases, the phenotype is different from that studied in this thesis and the results are therefore not directly comparable. During the development of atherosclerosis there is a cumulative effect of risk factors and genetic interactions that must also be taken into account. The hypothesis that the C1019T polymorphism may associate with IMT in the early, asymptomatic phase of atherosclerosis is feasible, because it has been found that hemichannels may have a functional role in the initiation of plaque development, as they regulate monocyte adhesion (Wong et al. 2006).

There are no previous publications on the possible associations of the C1019T polymorphism with arterial elasticity or endothelial function in an apparently healthy adult population. In an animal study, it has been observed that *Cx37* expression is altered during adaptive arteriogenesis (Cai et al. 2001). Expression was induced in SMCs during collateral growth, and it was speculated that this may be an early signal indicating SMC response to haemodynamic changes. It has also been shown that Cx37 is one of the primary components of gap junctions between endothelial cells (Looft-Wilson et al. 2004). As vasodilatation is conducted along the endothelial layer, Cx37 may have a functional role. The polymorphism leads to an amino acid change, suggesting a reasonable hypothesis that the function of the protein may differ between the genotypes and possibly lead to detectable phenotypes as well. However, this study showed no evidence of association between the *Cx37* polymorphism and the other two variables of early atherosclerotic changes, CAC and FMD.

6.4 *Cx37* interacts with risk factors of atherosclerosis (Study II)

As a multifactorial disease, atherosclerosis is known to be affected by both genetic and environmental factors, and the underlying genetic risk may be modified by the environment. These possible interactions between *Cx37* and lifestyle factors and their effects on IMT, CAC and FMD were examined in Study II.

In the whole population, a significant interaction was found between *Cx37* and homocysteine that associated with FMD in the unadjusted model. Elevated levels of circulating homocysteine have previously been associated with endothelial dysfunction (Woo et al. 1997, Kanani et al. 1999). In the multivariate models a significant positive association between homocysteine and FMD was seen in the TT, genotype and the interaction between genotype and homocysteine remained significant in all the different models, except when physical activity was added as a variable. This indicates that the effect of physical activity on FMD outweighs that of the other variables. Our results in Study II indicate a possible effect of the genotype modulating the effect of homocysteine on FMD. In our study population, the effect of significantly higher than

normal levels of homocysteine could not be predicted as only a small percentage of the population had homocysteine values that can be considered pathological. The significance of this finding is unclear, as there is a network of other factors to take into consideration.

The observation was also made that the *Cx37* genotype modulates the effect smoking has on FMD in women. This interaction remained significant in different multivariate models. Study II showed that in smokers the CC genotype associated with higher FMD values than in non-smokers and in the TT genotype smoking associated with lower FMD, i.e. with impaired endothelial function. It is well known that smoking causes vascular dysfunction and that this effect is mediated by impaired nitric oxide production (Palmer et al. 1987, Tsuchiya et al. 2002). Function of the vascular wall requires cellular communication, and gap junctions are one route for cell-to-cell signalling. Previously, it has been shown that *Cx37* is expressed in endothelial cells as well as monocytes/macrophages (Yeh et al. 1997, Wong et al. 2006). The effect of the interaction of smoking and the *Cx37* polymorphism on endothelial function may lie in the fact that the polymorphism alters an amino acid. This may change the biophysical properties of the gap junctions and, consequently, have an effect on how environmental factors such as smoking influence the endothelium. There are data from a cell culture study indicating that NO inhibits the transfer of molecules through gap junctions by influencing *Cx37* (Bilsborough et al. 2003). Therefore, our suggestion is that the genetic variation in *Cx37* may modify the effect that smoking has on FMD by affecting the intercellular communication via gap junctions. Evidently, hormonal factors differ between the sexes and may interact causing a difference between men and women, and age can also partially explain why this result was only apparent in women. It has been shown that a sex-related difference in arterial calcification is age-related, as in a younger subgroup a more distinct difference between the sexes was found (Kardys et al. 2007). There may be many factors to be considered causing the different effects of *Cx37* in female non-smokers and smokers, but no further speculation can be made in the scope of the present study.

There are no previous reports on the effects or mechanisms of *Cx37* with regard to FMD. However, there are many studies on the role of gap junctions in endothelial function. A recent review (Schmidt et al. 2008) discussed the mechanism of vascular tone in microcirculation. Gap junctions provide a longitudinal coupling of cells that allows changes in membrane potential, and thus in vasomotor responses, to spread along the vessel wall. This type of response is called conducted dilatation which is different from flow-induced dilation, but both may contribute to dilations of up-stream vessels to provide efficient blood flow to meet tissue needs. Gap junctions may also provide communication between endothelial cells and SMCs, allowing the transfer of dilator molecules, such as NO, between the cells. One link between our results concerning the

interaction of homocysteine and smoking, with *Cx37* to associate with FMD is NO. Homocysteine (Lawrence de Koning et al. 2003) as well as smoking (Ambrose and Barua 2004) have both been shown to impair NO bioavailability, leading to impaired endothelial function. The effect that these interactions may have on the endothelial function can only be speculated. One possible explanation, in view of the studies included in the present thesis, is that the polymorphism may alter the function of the connexin protein in gap junctions, which then modifies the effect NO has on the endothelium.

Another finding in Study II was the interaction of physical activity and the polymorphism which associated with CAC. This was found in men in the unadjusted model. An increase in physical activity associated positively with CAC in the CT genotype in all the multivariate models, but the interaction, however, did not remain significant in these adjusted models. This association with CAC is in accordance with previous reports, as it has been shown that subjects who are more physically active have better arterial compliance at baseline, and an increase in exercise can increase CAC values for those who normally do not exercise (Tanaka et al. 2000). *Cx37* genotype and physical activity had a strongly significant positive effect on CAC in the CT genotype and a lesser effect in the TT genotype. One explanation for this may be positive heterosis, where heterozygotes of a certain allele show a greater effect on a studied feature than homozygous subjects. Molecular heterosis is discussed in a review by Comings and MacMurray, where molecular heterosis is defined to occur when subjects heterozygous for a certain polymorphism of a single gene show a significantly greater or lesser effect for a quantitative or dichotomous trait than homozygotes (Comings and MacMurray 2000). One of the examples given of the heterozygous advantage concerns sickle cell disease, in which heterozygotes are protected from malaria infection when compared to noncarriers of the trait and homozygotes are at risk of dying from sickle cell disease (Comings and MacMurray 2000).

No interactions between gene and risk factors were found to associate with IMT. This was surprising to some extent, as in Study I we found a trend for the CC genotype to have higher IMT values than the other genotypes, and we hypothesized that this maybe explained by an underlying interaction. Monocytes that express the T allele have been shown to be more adhesive to the endothelium (Wong et al. 2006) than those expressing the C allele and this may lead to earlier initiation of plaque development and higher IMT values.

The limitations of Study II are similar to those mentioned for the previous study in terms of the study population. A lower p-value for the significance of the interactions can also be argued. If the level of significance had been set at 0.01 the only significant finding would have been between smoking and *Cx37* genotype and FMD in women.

6.5 The effect of *MTHFR* on early markers of atherosclerosis (Study III)

In Study III the same concept was used as in Study I. Associations between the *MTHFR* C677T polymorphism and early markers of subclinical atherosclerosis were studied. The effect of the polymorphism on circulating homocysteine concentration was also analysed. Previous studies have indicated that the T allele of this polymorphism associates with higher values of circulating homocysteine (Frosst et al. 1995). In Study III, a similar association was found for *MTHFR* genotype and homocysteine: homocysteine values increased in the genotype order CC, CT and TT.

No associations were found between homocysteine concentration or the *MTHFR* C677T polymorphism and IMT. This finding is in line with previous data reviewed by Durga and colleagues, in which an analysis of eight independent studies lead to the conclusion that the association between homocysteine values and IMT in the general population is absent or weak at the most (Durga et al. 2004). There have been other reports publishing opposite results, and elevated homocysteine values have been associated positively with developing atherosclerosis based on increased IMT values. In a multiethnic population, homocysteine values of higher than 11.7 µmol/l have been associated with a significant increase in IMT, but in the same population the TT genotype of the polymorphism did not associate with IMT (Kelemen et al. 2004). Other studies support this finding, as reported in Italian, Australian and Dutch populations (McQuillan et al. 1999, Mazza et al. 2000, Durga et al. 2005) in which the polymorphism did not associate with IMT. In several reports, however, the TT genotype has been associated with elevated IMT levels (Kawamoto et al. 2001, Pallaud et al. 2001, Passaro et al. 2001), and it has been found to be an independent risk factor for carotid artery stenosis (Inamoto et al. 2003). In these studies, where positive associations have been found, study populations have been older than our study population. The age of the population plays a significant role, as age was the most significant factor associating with IMT in our study. In meta-analyses studying the *MTHFR* polymorphism in relation to atherosclerosis, mostly negative results were reported. No evidence of an association was found between the polymorphism and CAD (Lewis et al. 2005), but, on the other hand, there was a greater risk for stroke in the TT genotype versus the CC genotype (Casas et al. 2005). As there are many phenotypes during the course of the disease and a multitude of interactions and cumulative effects to be considered, it is not surprising that these results vary.

There are few studies on *MTHFR* polymorphism associations with arterial elasticity. In a Taiwanese population no association was found between the T allele and stiffness in haemodialysis patients (Tsai et al. 2005), and no associations were found in an Australian study population on folate supplementation between *MTHFR* genotype and changes in blood pressure or large artery

stiffness (Williams et al. 2005). Our study population was healthy, with no chronic illnesses, and only 5.6% of the subjects had homocysteine values above 15 µmol/l, which is generally considered the upper limit of the reference range (Ueland et al. 1993). The results of Study III propose, however, that the association between *MTHFR* and CAC is independent of homocysteine values. This, to the best of our knowledge, is the first report of an association between *MTHFR* genotype and CAC.

No associations were found between *MTHFR* C677T polymorphism and FMD in either the whole population or in men or women separately. This is in accordance with previous study reports (Pullin et al. 2002, Imamura et al. 2004).

Homocysteine was found to be directly associated with CAC in the whole population and remained a significant predictor of CAC in women. A novel result in Study III was a significant association between the *MTHFR* polymorphism and CAC in men. Men with the CC genotype had the lowest CAC values as well as the lowest homocysteine values. In a linear model, homocysteine values were not significantly associated with CAC.

Homocysteine has previously been associated with arterial stiffness. It has been shown to influence pulse wave velocity in the lower limbs and aortic stiffness (Blacher et al. 1998a, Bortolotto et al. 1999). In two studies where a methionine load was used to raise homocysteine levels, one of the studies found no association with arterial stiffness (Wilkinson et al. 2001) and the other found a homocysteine-related increase in stiffness in central arteries (Nestel et al. 2003). Based on these previous findings, the conclusion could be drawn that the TT genotype, which associates with higher homocysteine values, would also associate with increased arterial stiffness. Our findings were the opposite, with the TT genotype having the highest homocysteine values associated with greater CAC values - i.e. the arteries were more elastic – and therefore the association with stiffness would be negative. There is a previous study that found a similar result when examining associations between *MTHFR* genotypes and the number of gaps in the internal elastic lamina of arterial walls (Hämelahti et al. 2002). It was found that TT genotypes with higher homocysteine values had fewer defects in the elastic lamina as compared to CC genotypes. This was also an unexpected result. The possible mechanism underlying these results is unclear. Values of circulating homocysteine do not necessarily compare to those in the vascular wall. It has also been suggested that cells may react in different ways to intracellular and extracellular changes in homocysteine values (Hultberg et al. 1998). The effect of homocysteine on the vascular wall during the development atherosclerosis most probably is a result of a network of different factors and their interactions, not only a straightforward case of high circulatory values.

Possible limitations to the study include the young study population. Age is a significant predictive factor for the vascular parameters, and only very mild clinical findings for atherosclerosis would be expected. Nevertheless, our hypothesis was to see if any subtle changes in association with genotypes could be seen in the stage of subclinical atherosclerosis. The newly found result concerns only a small part of the study group as the number of TT genotype subjects was 78, 5.4% of the whole population in this study. The small size of the subgroup decreases the power of the result. As this was also an unexpected result, a chance finding cannot be ruled out. New studies are needed to replicate these results, and different study approaches are necessary to further understand the possible biological mechanism behind the phenomenon.

6.6 The effect of *USF1* on early markers of atherosclerosis (Study IV)

USF1 was chosen as a candidate gene because of its association with FCHL, metabolic disease and CVD (Pajukanta et al. 2004, Ng et al. 2005, Komulainen et al. 2006). The hypothesis was that functional polymorphisms of *USF1* may associate with early vascular changes of atherosclerosis, IMT, CAC and FMD. In Study IV, we found associations between two of the common *USF1* single nucleotide polymorphisms and IMT. There was also an association with one of the derived haplotypes and IMT in this population. Study IV showed that the major allele G of polymorphism usf1s1 was positively associated with IMT values and the minor allele A was negatively associated with IMT values. For the polymorphism usf1s8, however, the minor allele G was positively associated with IMT values. An increasing copy number of haplotype 2 which comprised nucleotides G-C-G (polymorphisms usf1s1, usf1s2 and usf1s8, respectively) associated positively with IMT. In the case of haplotype 2, however, subjects with two copies of haplotype 2 were in the smallest group. Therefore, it can be concluded that only a minority of the population is in the risk group for elevated IMT as the majority of the study population has no copies of haplotype 2.

Effects of *USF1* polymorphisms on gene expression have been studied in fat tissue of FCHL patients and both up- and down-regulation of genes has been detected (Pajukanta et al. 2004). In the study it was suggested that the possible effects of *USF1* risk alleles on gene transcription regulation may be due to differences in tissues or cell types which may also be affected by local stimuli (Pajukanta et al. 2004). There is biological relevance to the hypothesis that *USF1* might associate with subclinical atherosclerosis. It regulates several genes that have a functional role in the accumulation of lipids, inflammation and thrombosis, such as apoE (Salero et al. 2003) and CRP (Szalai et al. 2005). These molecules have also been connected to the development of early atherosclerosis (Ilveskoski et al. 2000, Thakore et al. 2007).

In previous studies on usf1s1, there has been a sex-dependent association between the common allele and triglycerides and related metabolic traits in Dutch and American men (Lee et al. 2007). In addition, the minor allele has been associated with lower total and LDL-C as well as a decreased risk of coronary artery calcium, and as there was only a small decrease when adjusted for cholesterol, it was suggested that the influence of *USF1* on CVD risk may be due to other pathways than lipids alone (Reiner et al. 2007). Study IV showed a similar association, as the minor allele associated with lower IMT. It may be speculated that as *USF1* has many roles and influences different biochemical risk factor parameters, it would also associate with early markers of atherosclerosis. However, a direct association of two *USF1* polymorphisms with CVD and mortality has been found in women, and the authors suggest that there may be other ways for gene variants to affect CVD risk than via lipid parameters (Komulainen et al. 2006). The results of Study IV indicate that the gene variants do seem to influence IMT directly as the lipid values were not significant variables in the models. There is no speculation as to what the mechanism behind this phenomenon may be. It has been stated that there is an increased risk for CVD mortality in relatives of FCHL patients and that the predetermined genetic factors may effect the disease progression independently of other risk factors (Austin et al. 2000). The fact that *USF1* is involved in the regulation of glucose metabolism may also be one possible factor. Advanced glycosylation end products, formed in diabetes, promote inflammation in the artery wall during the initial phase of the formation of atherosclerotic lesions (Lusis 2000). Gene-gene and gene-environment interactions have been speculated as reasons for the heterogenic results in association studies of *USF1* polymorphisms as risk factors for atherosclerosis (Reiner et al. 2007). In addition, the changes in the expression or function of *USF1* may have an atherogenic influence on other metabolic routes besides lipids (Lee et al. 2007).

No evidence of associations of the studied polymorphisms and haplotypes with CAC or FMD was found in the present study. In our population a relatively large within-subject long-term variation has been reported in these parameters (Juonala et al. 2004a, Juonala et al. 2005), which may be one reason as to why the *USF1* gene variants were only associated with IMT.

Only three of the known *USF1* polymorphisms and the haplotypes derived from them were investigated in Study IV. Other significant associations may be found as more data accumulate on the effects that different polymorphisms may have on the gene. The possibly limiting factor is also our young and healthy study population, as age is a significant predicting factor for the vascular parameters used. The present study was the first to assess possible associations of *USF1* polymorphisms and vascular parameters used in the diagnosis of atherosclerosis. Therefore, the findings need to be confirmed by repeating the analyses in a different population.

6.7 Clinical implications and future perspectives

Atherosclerosis is a complex and multifactorial disease that has a number of traditional risk factors. Epidemiologic studies have identified the most common risk factors to be age, sex, hypercholesterolemia, diabetes, smoking and hypertension, which are said to account for a large fraction of the cases (Lusis et al. 2004). In further studies new risk factors have been recognised, such as small dense LDL particles, homocysteine and inflammatory cytokines (Libby 2001). Many of the risk factors are genetically determined. The Human Genome Project and the HapMap project have provided the means to search for candidate genes and the polymorphisms in them. There is a multitude of single nucleotide polymorphisms under investigation, and new data are constantly being published. The studies in this thesis are the first to investigate the polymorphisms of *Cx37*, *MTHFR* and *USF1* in relation to early signs of atherosclerosis measured by by means of ultrasound in a Finnish population. The results are an addition to this vast area of research where hundreds of genes and thousands of their polymorphisms are being investigated. The results of our studies found associations between *MTHFR* and CAC, *USF1* and IMT, and for *Cx37* interactions with homocysteine and smoking were found to affect FMD. The presumption that FMD, CAC and IMT measure different phases of atherosclerosis could be an explanation to why the three SNPs associated with different parameters. It is important to replicate these results in independent studies, because multiple testing was carried out and many different parameters and covariates were used. The next step would be to resequence the genes and look for additional variation, in addition, to determining linkage equilibrium between the variants. Further investigation of different phenotypes is also required to assess whether the SNPs studied in this thesis are indeed of future clinical importance in risk stratification or diagnosis for atherosclerosis.

Ideally, the goal is to identify those gene variants that have a significant and indisputable effect on atherosclerosis. In addition to searching for polymorphisms in candidate genes, haplotypes should be investigated as haplotype analysis increases the power of association analyses to detect effects of alleles inherited together (Novelli et al. 2003). As the information on candidate genes keeps expanding, chip technology can be used to screen for a number of polymorphisms simultaneously. New techniques are being employed, and another novel approach is the microarray analysis, where the expression pattern of a number of genes can be assayed in the same analysis (Fortunato and Di Taranto 2007). This, combined with candidate gene analysis, has proven to be effective in the study of complex diseases (Novelli et al. 2003).

In the health care system, in primary prevention or during therapy for a manifest disease, the aim is to stop disease progression and reduce the risk of the disease developing into the next stage.

Understanding the genomic profile of each patient might be a means to understand the disease mechanism and help identify those at high risk (Seo et al. 2006). In future, screening for a set of specific polymorphisms with established associations with atherosclerosis would enable the characterisation of the patient in question and the determination of a personal risk profile. Results on the gene variants that affect disease development at different stages could be identified and tailored preventive measures for the individual taken. This type of approach has recently been published by Kathiresan and colleagues. In their study, the hypothesis was that a combination of SNPs associated with LDL-C or HDL-C contributes to the risk of cardiovascular disease. The study showed that the genotype score did not improve risk prediction, but a significant improvement in risk classification was seen in models using the genotype score (Kathiresan et al. 2008). If this type of screening was undertaken in childhood, primary prevention via education and counselling could impede disease progression and lengthen disease-free time. A similar strategy could also be used for tailoring pharmacotherapy in later stages of the disease, to find out which combination of drugs at which stage of the disease is the most beneficial. It is also important to detect the significant interactions of specific polymorphisms with risk factors, as this combination may be of consequence. A futuristic view could be that genetic screening is carried out in childhood and these potential risks are then evaluated in combination with the environmental risk in normal age-related clinical check ups over the course of the following years, with the provision of targeted counselling according to the risks. When the disease does manifest itself, the correct and most effective treatment can be utilised for each patient individually. This is certainly a far-fetched scenario and would lead to many ethical questions, as there are so many factors to be taken into account in the disease process. A plausible idea for the near future would be to add some genetic markers to the risk stratification equations and gain a more precise prediction for the patient.

The results of the studies included in this thesis need to be followed with more studies to confirm their potential clinical importance. In the Cardiovascular Risk in Young Finns Study, the next follow-up has already been conducted. Six years have passed since the previous study, making the oldest cohort now 45 years old. This group may have developed more pronounced changes in the vascular measurements and function or even some cases of clinical CAD. A change in phenotype will enable further studies on the *Cx37*, *MTHFR* and *USF1* polymorphisms in a new context.

7 SUMMARY AND CONCLUSIONS

The Cardiovascular Risk in Young Finns study population data were used to investigate the effect of candidate gene polymorphisms and that of their interactions with lifestyle factors, on early markers of subclinical atherosclerosis IMT, CAC and FMD. The candidate genes studied in this thesis were *Cx37*, *MTHFR* and *USF1*. The studied candidate genes encode molecules that have very different types of physiological functions, and they also differ in the way they affect arterial wall functions. *Cx37* mediates cellular communication and is a factor in leukocyte adhesion to the vascular wall, *MTHFR* effects homocysteine metabolism and *USF1* has many links to atherosclerosis by regulating several genes in the lipid and carbohydrate pathways. The main findings and conclusions of these studies are as follows:

I In the case of the C1019T polymorphism (rs1764391) of the *Cx37* gene, no associations were found between the genotype and carotid artery compliance, brachial artery FMD or carotid artery IMT (Study I). Homocysteine, smoking and physical activity modified the effect of *Cx37* polymorphism on CAC and FMD. Significant interactions were found between *Cx37* and homocysteine, affecting FMD in the whole population; between *Cx37* and physical activity, affecting CAC in men; and between *Cx37* and smoking, affecting FMD in women (Study II). Variation of the allele may change the effect these risk factors have on CAC and FMD, resulting in a different risk depending on genotype. No interactions were found to predict IMT. More interaction studies are required to better understand the complexity of gene-environment interactions in atherosclerosis.

II Homocysteine concentration differed significantly between genotype groups for the *MTHFR* C677T polymorphism (rs1801133), the T allele increasing the levels as expected based on earlier studies (Study III). There was also a positive association between homocysteine concentration and CAC in the whole population and in women. An unexpected finding was the significant positive association between the T allele and CAC values in men. As the TT genotype had the highest homocysteine values in the whole population and among women, the finding suggests that the genotype-CAC association found in men is independent of homocysteine values. No associations were found between homocysteine or the polymorphism and IMT or FMD.

III Two polymorphisms and one of the studied haplotypes of transcription factor *USF1* associated with carotid artery IMT (Study IV). The major allele G of usf1s1 (rs3737787) associated with high IMT and the minor allele G of usf1s8 (rs2516838) with high IMT values. An increasing copy number of haplotype 2, including the major usf1s1 allele (G), the major usf1s2 allele (C) and the minor usf1s8 allele (G), associated with higher IMT values. The association was independent of lipid or glucose concentrations. No associations were found between the polymorphisms or haplotypes and CAC or FMD. As the results were the first to describe such associations they need to be confirmed in another population.

The results of these included studies indicate the complexity of investigating multifactorial diseases like atherosclerosis. Associations between polymorphisms in these candidate genes and markers of subclinical atherosclerosis were found.

It can be concluded that the risk for endothelial dysfunction is increased in smoking women with the TT genotype of *Cx37* polymorphism. On the other hand, when homocysteine is increased, this genotype may serve as a protective factor. When determining *MTHFR* genotypes, homocysteine does not have to be considered a risk factor in men with the TT genotype, since this genotype is protective against subclinical atherosclerosis measured by CAC. In determining *USF1* polymorphisms to detect the risk of increased IMT, it would be important to determine all three polymorphisms of this gene, since their combined effect can be dramatically different from the effect of single alleles.

This was the first time these candidate gene polymorphisms were studied in a large Finnish population. The results are complex and difficult to interpret and their future significance is yet to be determined. The findings need to be confirmed in other populations and in different phenotypes. Further functional research is also required to assess whether these associations are of future clinical importance in risk stratification or diagnosis for atherosclerosis.

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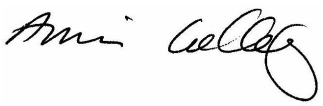

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9 REFERENCES

Åkerblom HK, Viikari J, Uhari M, Räsänen L, Byckling T, Louhivuori K, Pesonen E, Suoninen P, Pietikäinen M, Lähde PL, Dahl M, Aromaa A, Sarna S and Pyörälä K (1985): Atherosclerosis precursors in Finnish children and adolescents. I. General description of the cross-sectional study of 1980, and an account of the children's and families' state of health. Acta Paediatr Scand Suppl 318:49-63.

Allan PL, Mowbray PI, Lee AJ and Fowkes FG (1997): Relationship between carotid intima-media thickness and symptomatic and asymptomatic peripheral arterial disease. The Edinburgh Artery Study. Stroke 28:348-353.

Ambrose JA and Barua RS (2004): The pathophysiology of cigarette smoking and cardiovascular disease: an update. J Am Coll Cardiol 43:1731-1737.

Anderson TJ, Uehata A, Gerhard MD, Meredith IT, Knab S, Delagrange D, Lieberman EH, Ganz P, Creager MA, Yeung AC and et al. (1995): Close relation of endothelial function in the human coronary and peripheral circulations. J Am Coll Cardiol 26:1235-1241.

Ariyo AA, Thach C and Tracy R (2003): Lp(a) lipoprotein, vascular disease, and mortality in the elderly. N Engl J Med 349:2108-2115.

Arnett DK, Chambless LE, Kim H, Evans GW and Riley W (1999): Variability in ultrasonic measurements of arterial stiffness in the Atherosclerosis Risk in Communities study. Ultrasound Med Biol 25:175-180.

Austin MA, Breslow JL, Hennekens CH, Buring JE, Willett WC and Krauss RM (1988): Low-density lipoprotein subclass patterns and risk of myocardial infarction. Jama 260:1917-1921.

Austin MA, McKnight B, Edwards KL, Bradley CM, McNeely MJ, Psaty BM, Brunzell JD and Motulsky AG (2000): Cardiovascular disease mortality in familial forms of hypertriglyceridemia: A 20-year prospective study. Circulation 101:2777-2782.

Avolio A, Jones D and Tafazzoli-Shadpour M (1998): Quantification of alterations in structure and function of elastin in the arterial media. Hypertension 32:170-175.

Bae JH, Schwemmer M, Lee IK, Lee HJ, Park KR, Kim KY and Bassenge E (2003): Postprandial hypertriglyceridemia-induced endothelial dysfunction in healthy subjects is independent of lipid oxidation. Int J Cardiol 87:259-267.

Bak S, Gaist D, Sindrup SH, Skytthe A and Christensen K (2002): Genetic liability in stroke: a long-term follow-up study of Danish twins. Stroke 33:769-774.

Barter P, Kastelein J, Nunn A and Hobbs R (2003): High density lipoproteins (HDLs) and atherosclerosis; the unanswered questions. Atherosclerosis 168:195-211.

Bayele HK, McArdle H and Srai SK (2006): Cis and trans regulation of hepcidin expression by upstream stimulatory factor. Blood 108:4237-4245.

Benjamin EJ, Larson MG, Keyes MJ, Mitchell GF, Vasan RS, Keaney JF, Jr., Lehman BT, Fan S, Osypiuk E and Vita JA (2004): Clinical correlates and heritability of flow-mediated dilation in the community: the Framingham Heart Study. Circulation 109:613-619.

Berenson GS (2002): Childhood risk factors predict adult risk associated with subclinical cardiovascular disease. The Bogalusa Heart Study. Am J Cardiol 90:3L-7L.

Berenson GS, Srinivasan SR, Bao W, Newman WP, 3rd, Tracy RE and Wattigney WA (1998): Association between multiple cardiovascular risk factors and atherosclerosis in children and young adults. The Bogalusa Heart Study. N Engl J Med 338:1650-1656.

Beyer EC, Paul DL and Goodenough DA (1990): Connexin family of gap junction proteins. J Membr Biol 116:187-194.

Bielinski SJ, Lynch AI, Miller MB, Weder A, Cooper R, Oberman A, Chen YD, Turner ST, Fornage M, Province M and Arnett DK (2005): Genome-wide linkage analysis for loci affecting pulse pressure: the Family Blood Pressure Program. Hypertension 46:1286-1293.

Bilsborough W, Green DJ, Mamotte CD, van Bockxmeer FM, O'Driscoll GJ and Taylor RR (2003): Endothelial nitric oxide synthase gene polymorphism, homocysteine, cholesterol and vascular endothelial function. Atherosclerosis 169:131-138.

Blacher J, Pannier B, Guerin AP, Marchais SJ, Safar ME and London GM (1998a): Carotid arterial stiffness as a predictor of cardiovascular and all-cause mortality in end-stage renal disease. Hypertension 32:570-574.

Blacher J, Demuth K, Guerin AP, Safar ME, Moatti N and London GM (1998b): Influence of biochemical alterations on arterial stiffness in patients with end-stage renal disease. Arterioscler Thromb Vasc Biol 18:535-541.

Boerma M, Forsberg L, Van Zeijl L, Morgenstern R, De Faire U, Lemne C, Erlinge D, Thulin T, Hong Y and Cotgreave IA (1999): A genetic polymorphism in connexin 37 as a prognostic marker for atherosclerotic plaque development. J Intern Med 246:211-218.

Boers GH, Smals AG, Trijbels FJ, Fowler B, Bakkeren JA, Schoonderwaldt HC, Kleijer WJ and Kloppenborg PW (1985): Heterozygosity for homocystinuria in premature peripheral and cerebral occlusive arterial disease. N Engl J Med 313:709-715.

Bonetti PO, Lerman LO and Lerman A (2003): Endothelial dysfunction: a marker of atherosclerotic risk. Arterioscler Thromb Vasc Biol 23:168-175.

Bortolotto LA, Safar ME, Billaud E, Lacroix C, Asmar R, London GM and Blacher J (1999): Plasma homocysteine, aortic stiffness, and renal function in hypertensive patients. Hypertension 34:837-842.

Botma GJ, Verhoeven AJ and Jansen H (2001): Hepatic lipase promoter activity is reduced by the C-480T and G-216A substitutions present in the common LIPC gene variant, and is increased by Upstream Stimulatory Factor. Atherosclerosis 154:625-632.

Boutouyrie P, Tropeano AI, Asmar R, Gautier I, Benetos A, Lacolley P and Laurent S (2002): Aortic stiffness is an independent predictor of primary coronary events in hypertensive patients: a longitudinal study. Hypertension 39:10-15.

Brattstrom L, Wilcken DE, Ohrvik J and Brudin L (1998): Common methylenetetrahydrofolate reductase gene mutation leads to hyperhomocysteinemia but not to vascular disease: the result of a meta-analysis. Circulation 98:2520-2526.

Brattstrom LE, Hardebo JE and Hultberg BL (1984): Moderate homocysteinemia--a possible risk factor for arteriosclerotic cerebrovascular disease. Stroke 15:1012-1016.

Bray GA and Gray DS (1988): Obesity. Part I--Pathogenesis. West J Med 149:429-441.

Broeckel U, Hengstenberg C, Mayer B, Holmer S, Martin LJ, Comuzzie AG, Blangero J, Nurnberg P, Reis A, Riegger GA, Jacob HJ and Schunkert H (2002): A comprehensive linkage analysis for myocardial infarction and its related risk factors. Nat Genet 30:210-214.

Brown MS and Goldstein JL (1986): A receptor-mediated pathway for cholesterol homeostasis. Science 232:34-47.

Cai D, Yuan M, Frantz DF, Melendez PA, Hansen L, Lee J and Shoelson SE (2005): Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. Nat Med 11:183-190.

Cai WJ, Koltai S, Kocsis E, Scholz D, Schaper W and Schaper J (2001): Connexin37, not Cx40 and Cx43, is induced in vascular smooth muscle cells during coronary arteriogenesis. J Mol Cell Cardiol 33:957-967.

Campuzano R, Moya JL, Garcia-Lledo A, Tomas JP, Ruiz S, Megias A, Balaguer J and Asin E (2006): Endothelial dysfunction, intima-media thickness and coronary reserve in relation to risk factors and Framingham score in patients without clinical atherosclerosis. J Hypertens 24:1581-1588.

Casas JP, Bautista LE, Smeeth L, Sharma P and Hingorani AD (2005): Homocysteine and stroke: evidence on a causal link from mendelian randomisation. Lancet 365:224-232.

Cassidy-Bushrow AE, Bielak LF, Sheedy PF, 2nd, Turner ST, Kullo IJ, Lin X and Peyser PA (2007): Coronary artery calcification progression is heritable. Circulation 116:25-31.

Castelli WP (1992): Epidemiology of triglycerides: a view from Framingham. Am J Cardiol 70:3H-9H.

Celermajer DS, Sorensen KE, Gooch VM, Spiegelhalter DJ, Miller OI, Sullivan ID, Lloyd JK and Deanfield JE (1992): Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. Lancet 340:1111-1115.

Celermajer DS, Sorensen KE, Georgakopoulos D, Bull C, Thomas O, Robinson J and Deanfield JE (1993): Cigarette smoking is associated with dose-related and potentially reversible impairment of endothelium-dependent dilation in healthy young adults. Circulation 88:2149-2155.

Chambless LE, Folsom AR, Clegg LX, Sharrett AR, Shahar E, Nieto FJ, Rosamond WD and Evans G (2000): Carotid wall thickness is predictive of incident clinical stroke: the Atherosclerosis Risk in Communities (ARIC) study. Am J Epidemiol 151:478-487.

Chambless LE, Folsom AR, Sharrett AR, Sorlie P, Couper D, Szklo M and Nieto FJ (2003): Coronary heart disease risk prediction in the Atherosclerosis Risk in Communities (ARIC) study. J Clin Epidemiol 56:880-890.

Chan SY, Mancini GB, Kuramoto L, Schulzer M, Frohlich J and Ignaszewski A (2003): The prognostic importance of endothelial dysfunction and carotid atheroma burden in patients with coronary artery disease. J Am Coll Cardiol 42:1037-1043.

Chapman MJ, Guerin M and Bruckert E (1998): Atherogenic, dense low-density lipoproteins. Pathophysiology and new therapeutic approaches. Eur Heart J 19 Suppl A:A24-30.

Chiodini BD and Lewis CM (2003): Meta-analysis of 4 coronary heart disease genome-wide linkage studies confirms a susceptibility locus on chromosome 3q. Arterioscler Thromb Vasc Biol 23:1863-1868.

Chobanian AV (1990): 1989 Corcoran lecture: adaptive and maladaptive responses of the arterial wall to hypertension. Hypertension 15:666-674.

Chobanian AV and Alexander RW (1996): Exacerbation of atherosclerosis by hypertension. Potential mechanisms and clinical implications. Arch Intern Med 156:1952-1956.

Choquette AC, Bouchard L, Houde A, Bouchard C, Perusse L and Vohl MC (2007): Associations between USF1 gene variants and cardiovascular risk factors in the Quebec Family Study. Clin Genet 71:245-253.

Clarkson P, Celermajer DS, Powe AJ, Donald AE, Henry RM and Deanfield JE (1997): Endothelium-dependent dilatation is impaired in young healthy subjects with a family history of premature coronary disease. Circulation 96:3378-3383.

Comings DE and MacMurray JP (2000): Molecular heterosis: a review. Mol Genet Metab 71:19-31.

Coon H, Xin Y, Hopkins PN, Cawthon RM, Hasstedt SJ and Hunt SC (2005): Upstream stimulatory factor 1 associated with familial combined hyperlipidemia, LDL cholesterol, and triglycerides. Hum Genet 117:444-451.

Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J and Vogel R (2002): Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. J Am Coll Cardiol 39:257-265.

Craig WY, Palomäki GE and Haddow JE (1989): Cigarette smoking and serum lipid and lipoprotein concentrations: an analysis of published data. Bmj 298:784-788.

Crawford DW and Blankenhorn DH (1991): Arterial wall oxygenation, oxyradicals, and atherosclerosis. Atherosclerosis 89:97-108.

Creager MA, Luscher TF, Cosentino F and Beckman JA (2003): Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: Part I. Circulation 108:1527-1532.

Crouse JR, 3rd, Craven TE, Hagaman AP and Bond MG (1995): Association of coronary disease with segment-specific intimal-medial thickening of the extracranial carotid artery. Circulation 92:1141-1147.

Crouse JR, Goldbourt U, Evans G, Pinsky J, Sharrett AR, Sorlie P, Riley W and Heiss G (1996): Risk factors and segment-specific carotid arterial enlargement in the Atherosclerosis Risk in Communities (ARIC) cohort. Stroke 27:69-75.

Cruickshank K, Riste L, Anderson SG, Wright JS, Dunn G and Gosling RG (2002): Aortic pulse-wave velocity and its relationship to mortality in diabetes and glucose intolerance: an integrated index of vascular function? Circulation 106:2085-2090.

Cuomo S, Guarini P, Gaeta G, De Michele M, Boeri F, Dorn J, Bond M and Trevisan M (2002): Increased carotid intima-media thickness in children-adolescents, and young adults with a parental history of premature myocardial infarction. Eur Heart J 23:1345-1350.

Dahlen GH and Stenlund H (1997): Lp(a) lipoprotein is a major risk factor for cardiovascular disease: pathogenic mechanisms and clinical significance. Clin Genet 52:272-280.

Davis PH, Dawson JD, Riley WA and Lauer RM (2001): Carotid intimal-medial thickness is related to cardiovascular risk factors measured from childhood through middle age: The Muscatine Study. Circulation 104:2815-2819.

Ding K, Zhou K, He F and Shen Y (2003): LDA--a java-based linkage disequilibrium analyzer. Bioinformatics 19:2147-2148.

Djousse L, Myers RH, Province MA, Hunt SC, Eckfeldt JH, Evans G, Peacock JM and Ellison RC (2002): Influence of apolipoprotein E, smoking, and alcohol intake on carotid atherosclerosis: National Heart, Lung, and Blood Institute Family Heart Study. Stroke 33:1357-1361.

Durga J, Verhoef P, Bots ML and Schouten E (2004): Homocysteine and carotid intima-media thickness: a critical appraisal of the evidence. Atherosclerosis 176:1-19.

Durga J, Bots ML, Schouten EG, Kok FJ and Verhoef P (2005): Low concentrations of folate, not hyperhomocysteinemia, are associated with carotid intima-media thickness. Atherosclerosis 179:285-292.

Enos WF, Holmes RH and Beyer J (1953): Coronary disease among United States soldiers killed in action in Korea; preliminary report. J Am Med Assoc 152:1090-1093.

Ferrieres J, Elias A, Ruidavets JB, Cantet C, Bongard V, Fauvel J and Boccalon H (1999): Carotid intima-media thickness and coronary heart disease risk factors in a low-risk population. J Hypertens 17:743-748.

Fischer M, Broeckel U, Holmer S, Baessler A, Hengstenberg C, Mayer B, Erdmann J, Klein G, Riegger G, Jacob HJ and Schunkert H (2005): Distinct heritable patterns of angiographic coronary artery disease in families with myocardial infarction. Circulation 111:855-862.

Folsom AR, Eckfeldt JH, Weitzman S, Ma J, Chambless LE, Barnes RW, Cram KB and Hutchinson RG (1994): Relation of carotid artery wall thickness to diabetes mellitus, fasting glucose and insulin, body size, and physical activity. Atherosclerosis Risk in Communities (ARIC) Study Investigators. Stroke 25:66-73.

Folsom AR, Szklo M, Stevens J, Liao F, Smith R and Eckfeldt JH (1997): A prospective study of coronary heart disease in relation to fasting insulin, glucose, and diabetes. The Atherosclerosis Risk in Communities (ARIC) Study. Diabetes Care 20:935-942.

Fortunato G and Di Taranto MD (2007): Polymorphisms and the expression of genes encoding enzymes involved in cardiovascular diseases. Clin Chim Acta 381:21-25.

Fox CS, Polak JF, Chazaro I, Cupples A, Wolf PA, D'Agostino RA and O'Donnell CJ (2003): Genetic and environmental contributions to atherosclerosis phenotypes in men and women: heritability of carotid intima-media thickness in the Framingham Heart Study. Stroke 34:397-401.

Fox CS, Cupples LA, Chazaro I, Polak JF, Wolf PA, D'Agostino RB, Ordovas JM and O'Donnell CJ (2004): Genomewide linkage analysis for internal carotid artery intimal medial thickness: evidence for linkage to chromosome 12. Am J Hum Genet 74:253-261.

Franceschini N, MacCluer JW, Rose KM, Rutherford S, Cole SA, Laston S, Goring HH, Diego VP, Roman MJ, Lee ET, Best LG, Howard BV, Fabsitz RR and North KE (2008): Genome-wide linkage analysis of pulse pressure in American Indians: the Strong Heart Study. Am J Hypertens 21:194-199.

Francke S, Manraj M, Lacquemant C, Lecoeur C, Lepretre F, Passa P, Hebe A, Corset L, Yan SL, Lahmidi S, Jankee S, Gunness TK, Ramjuttun US, Balgobin V, Dina C and Froguel P (2001): A genome-wide scan for coronary heart disease suggests in Indo-Mauritians a susceptibility locus on chromosome 16p13 and replicates linkage with the metabolic syndrome on 3q27. Hum Mol Genet 10:2751-2765.

Franzoni F, Ghiadoni L, Galetta F, Plantinga Y, Lubrano V, Huang Y, Salvetti G, Regoli F, Taddei S, Santoro G and Salvetti A (2005): Physical activity, plasma antioxidant capacity, and endothelium-dependent vasodilation in young and older men. Am J Hypertens 18:510-516.

Friedewald WT, Levy RI and Fredrickson DS (1972): Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 18:499-502.

Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJ, den Heijer M, Kluijtmans LA and van den Heuvel LP (1995): A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 10:111-113.

Gaeta G, De Michele M, Cuomo S, Guarini P, Foglia MC, Bond MG and Trevisan M (2000): Arterial abnormalities in the offspring of patients with premature myocardial infarction. N Engl J Med 343:840-846.

Gardner CD, Fortmann SP and Krauss RM (1996): Association of small low-density lipoprotein particles with the incidence of coronary artery disease in men and women. Jama 276:875-881.

Genest JJ, Jr., Martin-Munley SS, McNamara JR, Ordovas JM, Jenner J, Myers RH, Silberman SR, Wilson PW, Salem DN and Schaefer EJ (1992): Familial lipoprotein disorders in patients with premature coronary artery disease. Circulation 85:2025-2033.

Gibson F, Hercberg S and Froguel P (2005): Common polymorphisms in the USF1 gene are not associated with type 2 diabetes in French Caucasians. Diabetes 54:3040-3042.

Gilula NB, Reeves OR and Steinbach A (1972): Metabolic coupling, ionic coupling and cell contacts. Nature 235:262-265.

Gimbrone MA, Jr. (1999): Endothelial dysfunction, hemodynamic forces, and atherosclerosis. Thromb Haemost 82:722-726.

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

Goldbourt U and Neufeld HN (1986): Genetic aspects of arteriosclerosis. Arteriosclerosis 6:357-377.

Goldbourt U, Yaari S and Medalie JH (1997): Isolated low HDL cholesterol as a risk factor for coronary heart disease mortality. A 21-year follow-up of 8000 men. Arterioscler Thromb Vasc Biol 17:107-113.

Goldstein JL, Schrott HG, Hazzard WR, Bierman EL and Motulsky AG (1973): Hyperlipidemia in coronary heart disease. II. Genetic analysis of lipid levels in 176 families and delineation of a new inherited disorder, combined hyperlipidemia. J Clin Invest. 52:1544-1568.

Goldstein JL, Hobbs HH and Brown MS (2001): Familial Hypercholesterolemia. In: The Metabolic & Molecular Bases of Inherited Disease, pp. 2863-2913. Eds. CR Scriver, AL Beaudet, WS Sly and Valle, D, McGraw-Hill.

Golledge J, McCann M, Mangan S, Lam A and Karan M (2004): Osteoprotegerin and osteopontin are expressed at high concentrations within symptomatic carotid atherosclerosis. Stroke 35:1636-1641.

Goodenough DA (1975): The structure of cell membranes involved in intercellular communication. Am J Clin Pathol 63:636-645.

Gordon T, Castelli WP, Hjortland MC, Kannel WB and Dawber TR (1977): High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. Am J Med 62:707-714.

Goyette P, Sumner JS, Milos R, Duncan AM, Rosenblatt DS, Matthews RG and Rozen R (1994): Human methylenetetrahydrofolate reductase: isolation of cDNA, mapping and mutation identification. Nat Genet 7:195-200.

Grebe MT, Schoene E, Schaefer CA, Boedeker RH, Kemkes-Matthes B, Voss R and Tillmanns HH (2007): Elevated Lipoprotein(a) does not promote early atherosclerotic changes of the carotid arteries in young, healthy adults. Atherosclerosis 190:194-198.

Gregor PD, Sawadogo M and Roeder RG (1990): The adenovirus major late transcription factor USF is a member of the helix-loop-helix group of regulatory proteins and binds to DNA as a dimer. Genes Dev 4:1730-1740.

Grundy SM and Vega GL (1992): Two different views of the relationship of hypertriglyceridemia to coronary heart disease. Implications for treatment. Arch Intern Med 152:28-34.

Hämelahti P, Järvinen O, Sisto T, Wirta V, Ilveskoski E, Koivula T and Lehtimäki T (2002): Methylenetetrahydrofolate reductase gene C677T mutation is related to the defects in the internal elastic lamina of the artery wall. Eur J Clin Invest 32:869-873.

Hansson GK (2005): Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 352:1685-1695.

Harrap SB, Zammit KS, Wong ZY, Williams FM, Bahlo M, Tonkin AM and Anderson ST (2002): Genome-wide linkage analysis of the acute coronary syndrome suggests a locus on chromosome 2. Arterioscler Thromb Vasc Biol 22:874-878.

Hauser ER, Crossman DC, Granger CB, Haines JL, Jones CJ, Mooser V, McAdam B, Winkelmann BR, Wiseman AH, Muhlestein JB, Bartel AG, Dennis CA, Dowdy E, Estabrooks S, Eggleston K, Francis S, Roche K, Clevenger PW, Huang L, Pedersen B, Shah S, Schmidt S, Haynes C, West S, Asper D, Booze M, Sharma S, Sundseth S, Middleton L, Roses AD, Hauser MA, Vance JM, Pericak-Vance MA and Kraus WE (2004): A genomewide scan for early-onset coronary artery disease in 438 families: the GENECARD Study. Am J Hum Genet 75:436-447.

Heiss G, Sharrett AR, Barnes R, Chambless LE, Szklo M and Alzola C (1991): Carotid atherosclerosis measured by B-mode ultrasound in populations: associations with cardiovascular risk factors in the ARIC study. Am J Epidemiol 134:250-256.

Helgadottir A, Thorleifsson G, Manolescu A, Gretarsdottir S, Blondal T, Jonasdottir A, Sigurdsson A, Baker A, Palsson A, Masson G, Gudbjartsson DF, Magnusson KP, Andersen K, Levey AI, Backman VM, Matthiasdottir S, Jonsdottir T, Palsson S, Einarsdottir H, Gunnarsdottir S, Gylfason A, Vaccarino V, Hooper WC, Reilly MP, Granger CB, Austin H, Rader DJ, Shah SH, Quyyumi AA, Gulcher JR, Thorgeirsson G, Thorsteinsdottir U, Kong A and Stefansson K (2007): A common variant on chromosome 9p21 affects the risk of myocardial infarction. Science 316:1491-1493.

Hirai T, Sasayama S, Kawasaki T and Yagi S (1989): Stiffness of systemic arteries in patients with myocardial infarction. A noninvasive method to predict severity of coronary atherosclerosis. Circulation 80:78-86.

Hirashiki A, Yamada Y, Murase Y, Suzuki Y, Kataoka H, Morimoto Y, Tajika T, Murohara T and Yokota M (2003): Association of gene polymorphisms with coronary artery disease in low- or high-risk subjects defined by conventional risk factors. J Am Coll Cardiol 42:1429-1437.

Hirschhorn JN (2005): Genetic approaches to studying common diseases and complex traits. Pediatr Res 57:74R-77R.

Hokanson JE and Austin MA (1996): Plasma triglyceride level is a risk factor for cardiovascular disease independent of high-density lipoprotein cholesterol level: a meta-analysis of population-based prospective studies. J Cardiovasc Risk 3:213-219.

Hopkins PN and Williams RR (1981): A survey of 246 suggested coronary risk factors. Atherosclerosis 40:1-52.

Horan PG, Allen AR, Patterson CC, Spence MS, McGlinchey PG and McKeown PP (2006): The connexin 37 gene polymorphism and coronary artery disease in Ireland. Heart 92:395-396.

Hu J and Cotgreave IA (1997): Differential regulation of gap junctions by proinflammatory mediators in vitro. J Clin Invest 99:2312-2316.

Huertas-Vazquez A, Aguilar-Salinas C, Lusis AJ, Cantor RM, Canizales-Quinteros S, Lee JC, Mariana-Nunez L, Riba-Ramirez RM, Jokiaho A, Tusie-Luna T and Pajukanta P (2005): Familial combined hyperlipidemia in Mexicans: association with upstream transcription factor 1 and linkage on chromosome 16q24.1. Arterioscler Thromb Vasc Biol 25:1985-1991.

Hultberg B, Andersson A and Isaksson A (1998): Higher export rate of homocysteine in a human endothelial cell line than in other human cell lines. Biochim Biophys Acta 1448:61-69.

Ikeda T, Shirasawa T, Esaki Y, Yoshiki S and Hirokawa K (1993): Osteopontin mRNA is expressed by smooth muscle-derived foam cells in human atherosclerotic lesions of the aorta. J Clin Invest 92:2814-2820.

Ilveskoski E, Loimaala A, Mercuri MF, Lehtimäki T, Pasanen M, Nenonen A, Oja P, Bond MG, Koivula T, Karhunen PJ and Vuori I (2000): Apolipoprotein E polymorphism and carotid artery intima-media thickness in a random sample of middle-aged men. Atherosclerosis 153:147-153.

Imamura A, Okumura K, Matsui H, Mizuno T, Ogawa Y, Imai H, Numaguchi Y, Sakai K and Murohara T (2004): Endothelial nitric oxide synthase and methylenetetrahydrofolate reductase gene polymorphisms are associated with endothelial dysfunction in young, healthy men. Can J Cardiol 20:1229-1234.

Inamoto N, Katsuya T, Kokubo Y, Mannami T, Asai T, Baba S, Ogata J, Tomoike H and Ogihara T (2003): Association of methylenetetrahydrofolate reductase gene polymorphism with carotid atherosclerosis depending on smoking status in a Japanese general population. Stroke 34:1628-1633.

Iynedjian PB (1998): Identification of upstream stimulatory factor as transcriptional activator of the liver promoter of the glucokinase gene. Biochem J 333 (Pt 3):705-712.

Järvisalo MJ, Putto-Laurila A, Jartti L, Lehtimäki T, Solakivi T, Rönnemaa T and Raitakari OT (2002a): Carotid artery intima-media thickness in children with type 1 diabetes. Diabetes 51:493-498.

Järvisalo MJ, Harmoinen A, Hakanen M, Paakkunainen U, Viikari J, Hartiala J, Lehtimäki T, Simell O and Raitakari OT (2002b): Elevated serum C-reactive protein levels and early arterial changes in healthy children. Arterioscler Thromb Vasc Biol 22:1323-1328.

Järvisalo MJ, Lehtimäki T and Raitakari OT (2004a): Determinants of arterial nitrate-mediated dilatation in children: role of oxidized low-density lipoprotein, endothelial function, and carotid intima-media thickness. Circulation 109:2885-2889.

Järvisalo MJ, Raitakari M, Toikka JO, Putto-Laurila A, Rontu R, Laine S, Lehtimäki T, Rönnemaa T, Viikari J and Raitakari OT (2004b): Endothelial dysfunction and increased arterial intima-media thickness in children with type 1 diabetes. Circulation 109:1750-1755.

Järvisalo MJ, Jartti L, Marniemi J, Rönnemaa T, Viikari JS, Lehtimäki T and Raitakari OT (2006): Determinants of short-term variation in arterial flow-mediated dilatation in healthy young men. Clin Sci (Lond) 110:475-482.

Jerrard-Dunne P, Markus HS, Steckel DA, Buehler A, von Kegler S and Sitzer M (2003): Early carotid atherosclerosis and family history of vascular disease: specific effects on arterial sites have implications for genetic studies. Arterioscler Thromb Vasc Biol 23:302-306.

Jousilahti P, Puska P, Vartiainen E, Pekkanen J and Tuomilehto J (1996): Parental history of premature coronary heart disease: an independent risk factor of myocardial infarction. J Clin Epidemiol 49:497-503.

Juonala M, Viikari JS, Laitinen T, Marniemi J, Helenius H, Rönnemaa T and Raitakari OT (2004a): Interrelations between brachial endothelial function and carotid intima-media thickness in young adults: the cardiovascular risk in young Finns study. Circulation 110:2918-2923.

Juonala M, Viikari JS, Hutri-Kähönen N, Pietikäinen M, Jokinen E, Taittonen L, Marniemi J, Rönnemaa T and Raitakari OT (2004b): The 21-year follow-up of the Cardiovascular Risk in Young Finns Study: risk factor levels, secular trends and east-west difference. J Intern Med 255:457-468.

Juonala M, Järvisalo MJ, Mäki-Torkko N, Kähönen M, Viikari JS and Raitakari OT (2005): Risk factors identified in childhood and decreased carotid artery elasticity in adulthood: the Cardiovascular Risk in Young Finns Study. Circulation 112:1486-1493.

Juonala M, Viikari JS, Rönnemaa T, Helenius H, Taittonen L and Raitakari OT (2006a): Elevated blood pressure in adolescent boys predicts endothelial dysfunction: the cardiovascular risk in young Finns study. Hypertension 48:424-430.

Juonala M, Viikari JS, Räsänen L, Helenius H, Pietikäinen M and Raitakari OT (2006b): Young adults with family history of coronary heart disease have increased arterial vulnerability to metabolic risk factors: the Cardiovascular Risk in Young Finns Study. Arterioscler Thromb Vasc Biol 26:1376-1382.

Kadoglou NP, Iliadis F and Liapis CD (2008): Exercise and carotid atherosclerosis. Eur J Vasc Endovasc Surg 35:264-272.

Kanani PM, Sinkey CA, Browning RL, Allaman M, Knapp HR and Haynes WG (1999): Role of oxidant stress in endothelial dysfunction produced by experimental hyperhomocyst(e)inemia in humans. Circulation 100:1161-1168.

Kang SS, Wong PW, Zhou JM, Sora J, Lessick M, Ruggie N and Grcevich G (1988): Thermolabile methylenetetrahydrofolate reductase in patients with coronary artery disease. Metabolism 37:611-613.

Kang SS, Wong PW, Susmano A, Sora J, Norusis M and Ruggie N (1991): Thermolabile methylenetetrahydrofolate reductase: an inherited risk factor for coronary artery disease. Am J Hum Genet 48:536-545.

Kanters SD, Algra A, van Leeuwen MS and Banga JD (1997): Reproducibility of in vivo carotid intima-media thickness measurements: a review. Stroke 28:665-671.

Kardys I, Vliegenthart R, Oudkerk M, Hofman A and Witteman JC (2007): The female advantage in cardiovascular disease: do vascular beds contribute equally? Am J Epidemiol 166:403-412.

Kathiresan S, Melander O, Anevski D, Guiducci C, Burtt NP, Roos C, Hirschhorn JN, Berglund G, Hedblad B, Groop L, Altshuler DM, Newton-Cheh C and Orho-Melander M (2008): Polymorphisms associated with cholesterol and risk of cardiovascular events. N Engl J Med 358:1240-1249.

Kawamoto R, Kohara K, Tabara Y, Miki T, Doi T, Tokunaga H and Konishi I (2001): An association of 5,10-methylenetetrahydrofolate reductase (MTHFR) gene polymorphism and common carotid atherosclerosis. J Hum Genet 46:506-510.

Kelemen LE, Anand SS, Hegele RA, Stampfer MJ, Rosner B, Willett WC, Montague PA, Lonn E, Vuksan V, Teo KK, Devanesen S and Yusuf S (2004): Associations of plasma homocysteine and the methylenetetrahydrofolate reductase C677T polymorphism with carotid intima media thickness among South Asian, Chinese and European Canadians. Atherosclerosis 176:361-370.

Knoflach M, Kiechl S, Kind M, Said M, Sief R, Gisinger M, van der Zee R, Gaston H, Jarosch E, Willeit J and Wick G (2003): Cardiovascular risk factors and atherosclerosis in young males: ARMY study (Atherosclerosis Risk-Factors in Male Youngsters). Circulation 108:1064-1069.

Kodama T, Freeman M, Rohrer L, Zabrecky J, Matsudaira P and Krieger M (1990): Type I macrophage scavenger receptor contains alpha-helical and collagen-like coiled coils. Nature 343:531-535.

Komulainen K, Alanne M, Auro K, Kilpikari R, Pajukanta P, Saarela J, Ellonen P, Salminen K, Kulathinal S, Kuulasmaa K, Silander K, Salomaa V, Perola M and Peltonen L (2006): Risk alleles of USF1 gene predict cardiovascular disease of women in two prospective studies. PLoS Genet 2:e69.

Kristiansson K, Ilveskoski E, Lehtimäki T, Peltonen L, Perola M and Karhunen PJ (2008): Association analysis of allelic variants of USF1 in coronary atherosclerosis. Arterioscler Thromb Vasc Biol 28:983-989.

Kurata M, Okura T, Watanabe S, Fukuoka T and Higaki J (2006): Osteopontin and carotid atherosclerosis in patients with essential hypertension. Clin Sci (Lond) 111:319-324.

Kutz SM, Higgins CE, Samarakoon R, Higgins SP, Allen RR, Qi L and Higgins PJ (2006): TGF-beta 1-induced PAI-1 expression is E box/USF-dependent and requires EGFR signaling. Exp Cell Res 312:1093-1105.

Kwak BR, Mulhaupt F, Veillard N, Gros DB and Mach F (2002): Altered pattern of vascular connexin expression in atherosclerotic plaques. Arterioscler Thromb Vasc Biol 22:225-230.

Larson MG, Atwood LD, Benjamin EJ, Cupples LA, D'Agostino RB, Sr., Fox CS, Govindaraju DR, Guo CY, Heard-Costa NL, Hwang SJ, Murabito JM, Newton-Cheh C, O'Donnell CJ, Seshadri S, Vasan RS, Wang TJ, Wolf PA and Levy D (2007): Framingham Heart Study 100K project: genome-wide associations for cardiovascular disease outcomes. BMC Med Genet 8 Suppl 1:S5.

Laurent S, Boutouyrie P, Asmar R, Gautier I, Laloux B, Guize L, Ducimetiere P and Benetos A (2001): Aortic stiffness is an independent predictor of all-cause and cardiovascular mortality in hypertensive patients. Hypertension 37:1236-1241.

Lawrence de Koning AB, Werstuck GH, Zhou J and Austin RC (2003): Hyperhomocysteinemia and its role in the development of atherosclerosis. Clin Biochem 36:431-441.

Lee JC, Weissglas-Volkov D, Kyttala M, Sinsheimer JS, Jokiaho A, de Bruin TW, Lusis AJ, Brennan ML, van Greevenbroek MM, van der Kallen CJ, Hazen SL and Pajukanta P (2007): USF1 Contributes to High Serum Lipid Levels in Dutch FCHL Families and U.S. Whites With Coronary Artery Disease. Arterioscler Thromb Vasc Biol 27:2222-2227.

Lehto S, Rönnemaa T, Haffner SM, Pyörälä K, Kallio V and Laakso M (1997): Dyslipidemia and hyperglycemia predict coronary heart disease events in middle-aged patients with NIDDM. Diabetes 46:1354-1359.

Lewis SJ, Ebrahim S and Davey Smith G (2005): Meta-analysis of MTHFR 677C->T polymorphism and coronary heart disease: does totality of evidence support causal role for homocysteine and preventive potential of folate? Bmj 331:1053.

Li H, Srinivasan SR, Chen W, Xu JH, Li S and Berenson GS (2005): Vascular abnormalities in asymptomatic, healthy young adult smokers without other major cardiovascular risk factors: the Bogalusa Heart Study. Am J Hypertens 18:319-324.

Li S, Chen W, Srinivasan SR, Bond MG, Tang R, Urbina EM and Berenson GS (2003): Childhood cardiovascular risk factors and carotid vascular changes in adulthood: the Bogalusa Heart Study. Jama 290:2271-2276.

Li S, Chen W, Srinivasan SR and Berenson GS (2004): Childhood blood pressure as a predictor of arterial stiffness in young adults: the bogalusa heart study. Hypertension 43:541-546.

Li X and Simard JM (1999): Multiple connexins form gap junction channels in rat basilar artery smooth muscle cells. Circ Res 84:1277-1284.

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

Libby P (2002): Inflammation in atherosclerosis. Nature 420:868-874.

Lim PS, Hung WR and Wei YH (2001): Polymorphism in methylenetetrahydrofolate reductase gene: its impact on plasma homocysteine levels and carotid atherosclerosis in ESRD patients receiving hemodialysis. Nephron 87:249-256.

Listi F, Candore G, Lio D, Russo M, Colonna-Romano G, Caruso M, Hoffmann E and Caruso C (2005): Association between C1019T polymorphism of connexin37 and acute myocardial infarction: a study in patients from Sicily. Int J Cardiol 102:269-271.

Liu ML, Ylitalo K, Nuotio I, Salonen R, Salonen JT and Taskinen MR (2002): Association between carotid intima-media thickness and low-density lipoprotein size and susceptibility of low-density lipoprotein to oxidation in asymptomatic members of familial combined hyperlipidemia families. Stroke 33:1255-1260.

Livak KJ (1999): Allelic discrimination using fluorogenic probes and the 5' nuclease assay. Genet Anal 14:143-149.

Looft-Wilson RC, Payne GW and Segal SS (2004): Connexin expression and conducted vasodilation along arteriolar endothelium in mouse skeletal muscle. J Appl Physiol 97:1152-1158.

Lupattelli G, Lombardini R, Schillaci G, Ciuffetti G, Marchesi S, Siepi D and Mannarino E (2000): Flow-mediated vasoactivity and circulating adhesion molecules in hypertriglyceridemia: association with small, dense LDL cholesterol particles. Am Heart J 140:521-526.

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

Lusis AJ, Fogelman AM and Fonarow GC (2004): Genetic basis of atherosclerosis: part II: clinical implications. Circulation 110:2066-2071.

Mahoney LT, Burns TL, Stanford W, Thompson BH, Witt JD, Rost CA and Lauer RM (1996): Coronary risk factors measured in childhood and young adult life are associated with coronary artery calcification in young adults: the Muscatine Study. J Am Coll Cardiol 27:277-284.

Malik S, Wong ND, Franklin SS, Kamath TV, L'Italien GJ, Pio JR and Williams GR (2004): Impact of the metabolic syndrome on mortality from coronary heart disease, cardiovascular disease, and all causes in United States adults. Circulation 110:1245-1250.

Malinow MR, Nieto FJ, Szklo M, Chambless LE and Bond G (1993): Carotid artery intimal-medial wall thickening and plasma homocyst(e)ine in asymptomatic adults. The Atherosclerosis Risk in Communities Study. Circulation 87:1107-1113.

Malyankar UM, Hanson R, Schwartz SM, Ridall AL and Giachelli CM (1999): Upstream stimulatory factor 1 regulates osteopontin expression in smooth muscle cells. Exp Cell Res 250:535-547.

Marks JB and Raskin P (2000): Cardiovascular risk in diabetes: a brief review. J Diabetes Complications 14:108-115.

Mazza A, Motti C, Nulli A, Pastore A, Andreotti F, Ammaturo V, Bianco P, Santoro E, Federici G and Cortese C (1999): Serum homocysteine, MTHFR gene polymorphism, and carotid intimal-medial thickness in NIDDM subjects. J Thromb Thrombolysis 8:207-212.

Mazza A, Motti C, Nulli A, Marra G, Gnasso A, Pastore A, Federici G and Cortese C (2000): Lack of association between carotid intima-media thickness and methylenetetrahydrofolate reductase gene polymorphism or serum homocysteine in non-insulin-dependent diabetes mellitus. Metabolism 49:718-723.

McCully KS (1969): Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. Am J Pathol 56:111-128.

McGill HC, Jr. and McMahan CA (1998): Determinants of atherosclerosis in the young. Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Research Group. Am J Cardiol 82:30T-36T.

McGill HC, Jr., McMahan CA, Malcom GT, Oalmann MC and Strong JP (1997): Effects of serum lipoproteins and smoking on atherosclerosis in young men and women. The PDAY Research Group. Pathobiological Determinants of Atherosclerosis in Youth. Arterioscler Thromb Vasc Biol 17:95-106.

McGill HC, Jr., McMahan CA, Herderick EE, Malcom GT, Tracy RE and Strong JP (2000): Origin of atherosclerosis in childhood and adolescence. Am J Clin Nutr 72:1307S-1315S.

McNamara JJ, Molot MA, Stremple JF and Cutting RT (1971): Coronary artery disease in combat casualties in Vietnam. Jama 216:1185-1187.

McPherson R, Pertsemlidis A, Kavaslar N, Stewart A, Roberts R, Cox DR, Hinds DA, Pennacchio LA, Tybjaerg-Hansen A, Folsom AR, Boerwinkle E, Hobbs HH and Cohen JC (2007): A common allele on chromosome 9 associated with coronary heart disease. Science 316:1488-1491.

McQuillan BM, Beilby JP, Nidorf M, Thompson PL and Hung J (1999): Hyperhomocysteinemia but not the C677T mutation of methylenetetrahydrofolate reductase is an independent risk determinant of carotid wall thickening. The Perth Carotid Ultrasound Disease Assessment Study (CUDAS). Circulation 99:2383-2388.

Megnien JL, Gariepy J, Saudubray JM, Nuoffer JM, Denarie N, Levenson J and Simon A (1998): Evidence of carotid artery wall hypertrophy in homozygous homocystinuria. Circulation 98:2276-2281.

Meigs JB, Wilson PW, Nathan DM, D'Agostino RB, Sr., Williams K and Haffner SM (2003): Prevalence and characteristics of the metabolic syndrome in the San Antonio Heart and Framingham Offspring Studies. Diabetes 52:2160-2167.

Meyer AA, Kundt G, Steiner M, Schuff-Werner P and Kienast W (2006): Impaired flow-mediated vasodilation, carotid artery intima-media thickening, and elevated endothelial plasma markers in obese children: the impact of cardiovascular risk factors. Pediatrics 117:1560-1567.

Mitchell GF, DeStefano AL, Larson MG, Benjamin EJ, Chen MH, Vasan RS, Vita JA and Levy D (2005): Heritability and a genome-wide linkage scan for arterial stiffness, wave reflection, and mean arterial pressure: the Framingham Heart Study. Circulation 112:194-199.

Moskau S, Golla A, Grothe C, Boes M, Pohl C and Klockgether T (2005): Heritability of carotid artery atherosclerotic lesions: an ultrasound study in 154 families. Stroke 36:5-8.

Murre C, McCaw PS and Baltimore D (1989): A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. Cell 56:777-783.

Nakamura K, Inai T and Shibata Y (1999): Distribution of gap junction protein connexin 37 in smooth muscle cells of the rat trachea and pulmonary artery. Arch Histol Cytol 62:27-37.

Napoli C and Ignarro LJ (2001): Nitric oxide and atherosclerosis. Nitric Oxide 5:88-97.

Naukkarinen J, Gentile M, Soro-Paavonen A, Saarela J, Koistinen HA, Pajukanta P, Taskinen MR and Peltonen L (2005): USF1 and dyslipidemias: converging evidence for a functional intronic variant. Hum Mol Genet 14:2595-2605.

Nestel PJ, Chronopoulos A and Cehun M (2003): Arterial stiffness is rapidly induced by raising the plasma homocysteine concentration with methionine. Atherosclerosis 171:83-86.

Neunteufl T, Katzenschlager R, Hassan A, Klaar U, Schwarzacher S, Glogar D, Bauer P and Weidinger F (1997): Systemic endothelial dysfunction is related to the extent and severity of coronary artery disease. Atherosclerosis 129:111-118.

Newman WP, 3rd, Freedman DS, Voors AW, Gard PD, Srinivasan SR, Cresanta JL, Williamson GD, Webber LS and Berenson GS (1986): Relation of serum lipoprotein levels and systolic blood pressure to early atherosclerosis. The Bogalusa Heart Study. N Engl J Med 314:138-144.

Ng MC, Miyake K, So WY, Poon EW, Lam VK, Li JK, Cox NJ, Bell GI and Chan JC (2005): The linkage and association of the gene encoding upstream stimulatory factor 1 with type 2 diabetes and metabolic syndrome in the Chinese population. Diabetologia 48:2018-2024.

Nikkilä EA and Aro A (1973): Family study of serum lipids and lipoproteins in coronary heart-disease. Lancet 1:954-959.

Norja S, Nuutila L, Karhunen PJ and Goebeler S (2007): C-reactive protein in vulnerable coronary plaques. J Clin Pathol 60:545-548.

North KE, MacCluer JW, Devereux RB, Howard BV, Welty TK, Best LG, Lee ET, Fabsitz RR and Roman MJ (2002): Heritability of carotid artery structure and function: the Strong Heart Family Study. Arterioscler Thromb Vasc Biol 22:1698-1703.

Nowak M, Helleboid-Chapman A, Jakel H, Martin G, Duran-Sandoval D, Staels B, Rubin EM, Pennacchio LA, Taskinen MR, Fruchart-Najib J and Fruchart JC (2005): Insulin-mediated down-regulation of apolipoprotein A5 gene expression through the phosphatidylinositol 3-kinase pathway: role of upstream stimulatory factor. Mol Cell Biol 25:1537-1548.

Novelli G, Borgiani P, Giardina E, Mango R, Contino G, Romeo F and Mehta JL (2003): Role of genetics in prevention of coronary atherosclerosis. Curr Opin Cardiol 18:368-371.

O'Donnell CJ, Cupples LA, D'Agostino RB, Fox CS, Hoffmann U, Hwang SJ, Ingellson E, Liu C, Murabito JM, Polak JF, Wolf PA and Demissie S (2007): Genome-wide association study for subclinical atherosclerosis in major arterial territories in the NHLBI's Framingham Heart Study. BMC Med Genet 8 Suppl 1:S4.

O'Leary DH, Polak JF, Kronmal RA, Manolio TA, Burke GL and Wolfson SK, Jr. (1999): Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular Health Study Collaborative Research Group. N Engl J Med 340:14-22.

Oliver JJ and Webb DJ (2003): Noninvasive assessment of arterial stiffness and risk of atherosclerotic events. Arterioscler Thromb Vasc Biol 23:554-566.

Paffen E and DeMaat MP (2006): C-reactive protein in atherosclerosis: A causal factor? Cardiovasc Res 71:30-39.

Pajukanta P, Nuotio I, Terwilliger JD, Porkka KV, Ylitalo K, Pihlajamäki J, Suomalainen AJ, Syvänen AC, Lehtimäki T, Viikari JS, Laakso M, Taskinen MR, Ehnholm C and Peltonen L (1998): Linkage of familial combined hyperlipidaemia to chromosome 1q21-q23. Nat Genet 18:369-373.

Pajukanta P, Cargill M, Viitanen L, Nuotio I, Kareinen A, Perola M, Terwilliger JD, Kempas E, Daly M, Lilja H, Rioux JD, Brettin T, Viikari JS, Rönnemaa T, Laakso M, Lander ES and Peltonen L (2000): Two loci on chromosomes 2 and X for premature coronary heart disease identified in early- and late-settlement populations of Finland. Am J Hum Genet 67:1481-1493.

Pajukanta P, Lilja HE, Sinsheimer JS, Cantor RM, Lusis AJ, Gentile M, Duan XJ, Soro-Paavonen A, Naukkarinen J, Saarela J, Laakso M, Ehnholm C, Taskinen MR and Peltonen L (2004): Familial combined hyperlipidemia is associated with upstream transcription factor 1 (USF1). Nat Genet 36:371-376.

Pallaud C, Sass C, Zannad F, Siest G and Visvikis S (2001): APOC3, CETP, fibrinogen, and MTHFR are genetic determinants of carotid intima-media thickness in healthy men (the Stanislas cohort). Clin Genet 59:316-324.

Palmer RM, Ferrige AG and Moncada S (1987): Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 327:524-526.

Parikh A, Sochett EB, McCrindle BW, Dipchand A, Daneman A and Daneman D (2000): Carotid artery distensibility and cardiac function in adolescents with type 1 diabetes. J Pediatr 137:465-469.

Passaro A, Vanini A, Calzoni F, Alberti L, Zamboni PF, Fellin R and Solini A (2001): Plasma homocysteine, methylenetetrahydrofolate reductase mutation and carotid damage in elderly healthy women. Atherosclerosis 157:175-180.

Pastier D, Lacorte JM, Chambaz J, Cardot P and Ribeiro A (2002): Two initiator-like elements are required for the combined activation of the human apolipoprotein C-III promoter by upstream stimulatory factor and hepatic nuclear factor-4. J Biol Chem 277:15199-15206.

Patsch W and Gotto AM, Jr. (1996): Apolipoproteins: pathophysiology and clinical implications. Methods Enzymol 263:3-32.

Pedersen TR, Olsson AG, Faergeman O, Kjekshus J, Wedel H, Berg K, Wilhelmsen L, Haghfelt T, Thorgeirsson G, Pyörälä K, Miettinen T, Christophersen B, Tobert JA, Musliner TA and Cook TJ (1998): Lipoprotein changes and reduction in the incidence of major coronary heart disease events in the Scandinavian Simvastatin Survival Study (4S). Circulation 97:1453-1460.

Peltonen L, Jalanko A and Varilo T (1999): Molecular genetics of the Finnish disease heritage. Hum Mol Genet 8:1913-1923.

Pentikäinen MO, Oorni K, Ala-Korpela M and Kovanen PT (2000): Modified LDL - trigger of atherosclerosis and inflammation in the arterial intima. J Intern Med 247:359-370.

Perkins G, Goodenough D and Sosinsky G (1997): Three-dimensional structure of the gap junction connexon. Biophys J 72:533-544.

Pignoli P, Tremoli E, Poli A, Oreste P and Paoletti R (1986): Intimal plus medial thickness of the arterial wall: a direct measurement with ultrasound imaging. Circulation 74:1399-1406.

Poli A, Tremoli E, Colombo A, Sirtori M, Pignoli P and Paoletti R (1988): Ultrasonographic measurement of the common carotid artery wall thickness in hypercholesterolemic patients. A new model for the quantitation and follow-up of preclinical atherosclerosis in living human subjects. Atherosclerosis 70:253-261.

Pollex RL and Hegele RA (2007a): Genomic copy number variation and its potential role in lipoprotein and metabolic phenotypes. Curr Opin Lipidol 18:174-180.

Pollex RL and Hegele RA (2007b): Copy number variation in the human genome and its implications for cardiovascular disease. Circulation 115:3130-3138.

Portois L, Tastenoy M, Viollet B and Svoboda M (2002): Functional analysis of the glucose response element of the rat glucagon receptor gene in insulin-producing INS-1 cells. Biochim Biophys Acta 1574:175-186.

Pradhan AD, Manson JE, Rifai N, Buring JE and Ridker PM (2001): C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. Jama 286:327-334.

Providence KM, White LA, Tang J, Gonclaves J, Staiano-Coico L and Higgins PJ (2002): Epithelial monolayer wounding stimulates binding of USF-1 to an E-box motif in the plasminogen activator inhibitor type 1 gene. J Cell Sci 115:3767-3777.

Puddu P, Cravero E, Puddu GM and Muscari A (2005): Genes and atherosclerosis: at the origin of the predisposition. Int J Clin Pract 59:462-472.

Pullin CH, Wilson JF, Ashfield-Watt PA, Clark ZE, Whiting JM, Lewis MJ and McDowell IF (2002): Influence of methylenetetrahydrofolate reductase genotype, exercise and other risk factors on endothelial function in healthy individuals. Clin Sci (Lond) 102:45-50.

Putt W, Palmen J, Nicaud V, Tregouet DA, Tahri-Daizadeh N, Flavell DM, Humphries SE and Talmud PJ (2004): Variation in USF1 shows haplotype effects, gene : gene and gene : environment associations with glucose and lipid parameters in the European Atherosclerosis Research Study II. Hum Mol Genet 13:1587-1597.

Raitakari OT, Adams MR and Celermajer DS (1999): Effect of Lp(a) on the early functional and structural changes of atherosclerosis. Arterioscler Thromb Vasc Biol 19:990-995.

Raitakari OT, Juonala M, Kähönen M, Taittonen L, Laitinen T, Mäki-Torkko N, Järvisalo MJ, Uhari M, Jokinen E, Rönnemaa T, Åkerblom HK and Viikari JS (2003): Cardiovascular risk factors in childhood and carotid artery intima-media thickness in adulthood: the Cardiovascular Risk in Young Finns Study. Jama 290:2277-2283.

Raitakari OT, Juonala M and Viikari JS (2005): Obesity in childhood and vascular changes in adulthood: insights into the Cardiovascular Risk in Young Finns Study. Int J Obes (Lond) 29 Suppl 2:S101-104.

Raitakari OT, Juonala M, Rönnemaa T, Keltikangas-Järvinen L, Räsänen L, Pietikäinen M, Hutri-Kähönen N, Taittonen L, Jokinen E, Marniemi J, Jula A, Telama R, Kähönen M, Lehtimäki T, Åkerblom HK and Viikari JS (2008): Cohort Profile: The Cardiovascular Risk in Young Finns Study. Int J Epidemiol

Read ML, Clark AR and Docherty K (1993): The helix-loop-helix transcription factor USF (upstream stimulating factor) binds to a regulatory sequence of the human insulin gene enhancer. Biochem J 295 (Pt 1):233-237.

Reed KE, Westphale EM, Larson DM, Wang HZ, Veenstra RD and Beyer EC (1993): Molecular cloning and functional expression of human connexin37, an endothelial cell gap junction protein. J Clin Invest 91:997-1004.

Reiner AP, Carlson CS, Jenny NS, Durda JP, Siscovick DS, Nickerson DA and Tracy RP (2007): USF1 gene variants, cardiovascular risk, and mortality in European Americans: analysis of two US cohort studies. Arterioscler Thromb Vasc Biol 27:2736-2742.

Reneman RS, van Merode T, Hick P, Muytjens AM and Hoeks AP (1986): Age-related changes in carotid artery wall properties in men. Ultrasound Med Biol 12:465-471.

Ribeiro A, Pastier D, Kardassis D, Chambaz J and Cardot P (1999): Cooperative binding of upstream stimulatory factor and hepatic nuclear factor 4 drives the transcription of the human apolipoprotein A-II gene. J Biol Chem 274:1216-1225.

Richard G, Lin JP, Smith L, Whyte YM, Itin P, Wollina U, Epstein E, Jr., Hohl D, Giroux JM, Charnas L, Bale SJ and DiGiovanna JJ (1997): Linkage studies in erythrokeratodermias: fine mapping, genetic heterogeneity and analysis of candidate genes. J Invest Dermatol 109:666-671.

Riley WA, Freedman DS, Higgs NA, Barnes RW, Zinkgraf SA and Berenson GS (1986): Decreased arterial elasticity associated with cardiovascular disease risk factors in the young. Bogalusa Heart Study. Arteriosclerosis 6:378-386.

Riley WA, Barnes RW, Evans GW and Burke GL (1992): Ultrasonic measurement of the elastic modulus of the common carotid artery. The Atherosclerosis Risk in Communities (ARIC) Study. Stroke 23:952-956.

Risch N and Merikangas K (1996): The future of genetic studies of complex human diseases. Science 273:1516-1517.

Rosenblatt DS and Fenton WA (2001): Inherited Disorders of Folate and Cobalamin Transport and Metabolism. In: The Metabolic & Molecular Bases of Inherited Disease, pp. 3897-3933. Eds. CR Scriver, AL Beaudet, WS Sly and Valle, D, McGraw-Hill.

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

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

Ross R and Glomset JA (1976): The pathogenesis of atherosclerosis (first of two parts). N Engl J Med 295:369-377.

Saez JC, Connor JA, Spray DC and Bennett MV (1989): Hepatocyte gap junctions are permeable to the second messenger, inositol 1,4,5-trisphosphate, and to calcium ions. Proc Natl Acad Sci U S A 86:2708-2712.

Sakata T, Mannami T, Baba S, Kokubo Y, Kario K, Okamoto A, Kumeda K, Ohkura N, Katayama Y, Miyata T, Tomoike H and Kato H (2004): Potential of free-form TFPI and PAI-1 to be useful markers of early atherosclerosis in a Japanese general population (the Suita Study): association with the intimal-medial thickness of carotid arteries. Atherosclerosis 176:355-360.

Salero E, Gimenez C and Zafra F (2003): Identification of a non-canonical E-box motif as a regulatory element in the proximal promoter region of the apolipoprotein E gene. Biochem J 370:979-986.

Salomaa V, Riley W, Kark JD, Nardo C and Folsom AR (1995): Non-insulin-dependent diabetes mellitus and fasting glucose and insulin concentrations are associated with arterial stiffness indexes. The ARIC Study. Atherosclerosis Risk in Communities Study. Circulation 91:1432-1443.

Salonen R and Salonen JT (1991): Determinants of carotid intima-media thickness: a population-based ultrasonography study in eastern Finnish men. J Intern Med 229:225-231.

Samani NJ, Erdmann J, Hall AS, Hengstenberg C, Mangino M, Mayer B, Dixon RJ, Meitinger T, Braund P, Wichmann HE, Barrett JH, Konig IR, Stevens SE, Szymczak S, Tregouet DA, Iles MM, Pahlke F, Pollard H, Lieb W, Cambien F, Fischer M, Ouwehand W, Blankenberg S, Balmforth AJ, Baessler A, Ball SG, Strom TM, Braenne I, Gieger C, Deloukas P, Tobin MD, Ziegler A, Thompson JR and Schunkert H (2007): Genomewide association analysis of coronary artery disease. N Engl J Med 357:443-453.

Sarwar N, Danesh J, Eiriksdottir G, Sigurdsson G, Wareham N, Bingham S, Boekholdt SM, Khaw KT and Gudnason V (2007): Triglycerides and the risk of coronary heart disease: 10,158 incident cases among 262,525 participants in 29 Western prospective studies. Circulation 115:450-458.

Sarzani R, Brecher P and Chobanian AV (1989): Growth factor expression in aorta of normotensive and hypertensive rats. J Clin Invest 83:1404-1408.

Sawadogo M (1988): Multiple forms of the human gene-specific transcription factor USF. II. DNA binding properties and transcriptional activity of the purified HeLa USF. J Biol Chem 263:11994-12001.

Sawadogo M and Roeder RG (1985): Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. Cell 43:165-175.

Sawadogo M, Van Dyke MW, Gregor PD and Roeder RG (1988): Multiple forms of the human gene-specific transcription factor USF. I. Complete purification and identification of USF from HeLa cell nuclei. J Biol Chem 263:11985-11993.

Sayed-Tabatabaei FA, Schut AF, Hofman A, Bertoli-Avella AM, Vergeer J, Witteman JC and van Duijn CM (2004): A study of gene--environment interaction on the gene for angiotensin converting enzyme: a combined functional and population based approach. J Med Genet 41:99-103.

Scandinavian Simvastatin Survival Study (1994): Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). Lancet 344:1383-1389.

Scanu AM, Lawn RM and Berg K (1991): Lipoprotein(a) and atherosclerosis. Ann Intern Med 115:209-218.

Schmidt VJ, Wolfle SE, Boettcher M and de Wit C (2008): Gap junctions synchronize vascular tone within the microcirculation. Pharmacol Rep 60:68-74.

Sebat J, Lakshmi B, Troge J, Alexander J, Young J, Lundin P, Maner S, Massa H, Walker M, Chi M, Navin N, Lucito R, Healy J, Hicks J, Ye K, Reiner A, Gilliam TC, Trask B, Patterson N, Zetterberg A and Wigler M (2004): Large-scale copy number polymorphism in the human genome. Science 305:525-528.

Sebat J, Lakshmi B, Malhotra D, Troge J, Lese-Martin C, Walsh T, Yamrom B, Yoon S, Krasnitz A, Kendall J, Leotta A, Pai D, Zhang R, Lee YH, Hicks J, Spence SJ, Lee AT, Puura K, Lehtimaki T, Ledbetter D, Gregersen PK, Bregman J, Sutcliffe JS, Jobanputra V, Chung W, Warburton D, King MC, Skuse D, Geschwind DH, Gilliam TC, Ye K and Wigler M (2007): Strong association of de novo copy number mutations with autism. Science 316:445-449.

Sebestjen M, Zegura B, Videcnik V and Keber I (2005): Determinants of endothelial dysfunction and carotid intima-media thickness in combined hyperlipidemia. Coron Artery Dis 16:175-180.

Seo D, Ginsburg GS and Goldschmidt-Clermont PJ (2006): Gene expression analysis of cardiovascular diseases: novel insights into biology and clinical applications. J Am Coll Cardiol 48:227-235.

Sherva R, Miller MB, Lynch AI, Devereux RB, Rao DC, Oberman A, Hopkins PN, Kitzman DW, Atwood LD and Arnett DK (2007): A whole genome scan for pulse pressure/stroke volume ratio in African Americans: the HyperGEN study. Am J Hypertens 20:398-402.

Shieh BH, Sparkes RS, Gaynor RB and Lusis AJ (1993): Localization of the gene-encoding upstream stimulatory factor (USF) to human chromosome 1q22-q23. Genomics 16:266-268.

Simon AM, Goodenough DA, Li E and Paul DL (1997): Female infertility in mice lacking connexin 37. Nature 385:525-529.

Sohl G and Willecke K (2004): Gap junctions and the connexin protein family. Cardiovasc Res 62:228-232.

Sorensen KE, Celermajer DS, Georgakopoulos D, Hatcher G, Betteridge DJ and Deanfield JE (1994): Impairment of endothelium-dependent dilation is an early event in children with familial hypercholesterolemia and is related to the lipoprotein(a) level. J Clin Invest 93:50-55.

Srinivasan SR and Berenson GS (2001): Apolipoproteins B and A-I as predictors of risk of coronary artery disease. Lancet 358:2012-2013.

Stary HC, Blankenhorn DH, Chandler AB, Glagov S, Insull W, Jr., Richardson M, Rosenfeld ME, Schaffer SA, Schwartz CJ, Wagner WD and et al. (1992): A definition of the intima of human arteries and of its atherosclerosis-prone regions. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation 85:391-405.

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

Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W, Jr., Rosenfeld ME, Schwartz CJ, Wagner WD and Wissler RW (1995): A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation 92:1355-1374.

Steinberg D and Gotto AM, Jr. (1999): Preventing coronary artery disease by lowering cholesterol levels: fifty years from bench to bedside. Jama 282:2043-2050.

Stephens M and Scheet P (2005): Accounting for decay of linkage disequilibrium in haplotype inference and missing-data imputation. Am J Hum Genet 76:449-462.

Stephens M, Smith NJ and Donnelly P (2001): A new statistical method for haplotype reconstruction from population data. Am J Hum Genet 68:978-989.

Stoop AA, Lupu F and Pannekoek H (2000): Colocalization of thrombin, PAI-1, and vitronectin in the atherosclerotic vessel wall: A potential regulatory mechanism of thrombin activity by PAI-1/vitronectin complexes. Arterioscler Thromb Vasc Biol 20:1143-1149.

Szalai AJ, Wu J, Lange EM, McCrory MA, Langefeld CD, Williams A, Zakharkin SO, George V, Allison DB, Cooper GS, Xie F, Fan Z, Edberg JC and Kimberly RP (2005): Single-nucleotide polymorphisms in the C-reactive protein (CRP) gene promoter that affect transcription factor binding, alter transcriptional activity, and associate with differences in baseline serum CRP level. J Mol Med 83:440-447.

Talmud PJ (2007): Gene-environment interaction and its impact on coronary heart disease risk. Nutr Metab Cardiovasc Dis 17:148-152.

Talmud PJ, Stephens JW, Hawe E, Demissie S, Cupples LA, Hurel SJ, Humphries SE and Ordovas JM (2005): The significant increase in cardiovascular disease risk in APOEepsilon4 carriers is evident only in men who smoke: potential relationship between reduced antioxidant status and ApoE4. Ann Hum Genet 69:613-622.

Tanaka H, Dinenno FA, Monahan KD, Clevenger CM, DeSouza CA and Seals DR (2000): Aging, habitual exercise, and dynamic arterial compliance. Circulation 102:1270-1275.

Telama R, Viikari J, Välimäki I, Siren-Tiusanen H, Åkerblom HK, Uhari M, Dahl M, Pesonen E, Lähde PL, Pietikäinen M and Suoninen P (1985): Atherosclerosis precursors in Finnish children and adolescents. X. Leisure-time physical activity. Acta Paediatr Scand Suppl 318:169-180.

Thakore AH, Guo CY, Larson MG, Corey D, Wang TJ, Vasan RS, D'Agostino RB, Sr., Lipinska I, Keaney JF, Jr., Benjamin EJ and O'Donnell C J (2007): Association of Multiple Inflammatory Markers with Carotid Intimal Medial Thickness and Stenosis (from the Framingham Heart Study). Am J Cardiol 99:1598-1602.

Tirosh A, Rudich A, Shochat T, Tekes-Manova D, Israeli E, Henkin Y, Kochba I and Shai I (2007): Changes in triglyceride levels and risk for coronary heart disease in young men. Ann Intern Med 147:377-385.

Toikka JO, Ahotupa M, Viikari JS, Niinikoski H, Taskinen M, Irjala K, Hartiala JJ and Raitakari OT (1999): Constantly low HDL-cholesterol concentration relates to endothelial dysfunction and increased in vivo LDL-oxidation in healthy young men. Atherosclerosis 147:133-138.

Tounian P, Aggoun Y, Dubern B, Varille V, Guy-Grand B, Sidi D, Girardet JP and Bonnet D (2001): Presence of increased stiffness of the common carotid artery and endothelial dysfunction in severely obese children: a prospective study. Lancet 358:1400-1404.

Tsai JC, Kuo HT, Chiu YW, Hwang SJ, Chuang HY, Chang JM, Chen HC and Lai YH (2005): Correlation of plasma homocysteine level with arterial stiffness and pulse pressure in hemodialysis patients. Atherosclerosis 182:121-127.

Tsuchiya M, Asada A, Kasahara E, Sato EF, Shindo M and Inoue M (2002): Smoking a single cigarette rapidly reduces combined concentrations of nitrate and nitrite and concentrations of antioxidants in plasma. Circulation 105:1155-1157.

Ueland PM, Refsum H, Stabler SP, Malinow MR, Andersson A and Allen RH (1993): Total homocysteine in plasma or serum: methods and clinical applications. Clin Chem 39:1764-1779.

Urbina EM, Srinivasan SR, Kieltyka RL, Tang R, Bond MG, Chen W and Berenson GS (2004): Correlates of carotid artery stiffness in young adults: The Bogalusa Heart Study. Atherosclerosis 176:157-164.

Van Camp G, Coucke P, Speleman F, Van Roy N, Beyer EC, Oostra BA and Willems PJ (1995): The gene for human gap junction protein connexin37 (GJA4) maps to chromosome 1p35.1, in the vicinity of D1S195. Genomics 30:402-403.

van der Vleuten GM, Isaacs A, Hijmans A, van Duijn CM, Stalenhoef AF and de Graaf J (2007): The involvement of upstream stimulatory factor 1 in Dutch patients with familial combined hyperlipidemia. J Lipid Res 48:193-200.

Vasan RS, Larson MG, Aragam J, Wang TJ, Mitchell GF, Kathiresan S, Newton-Cheh C, Vita JA, Keyes MJ, O'Donnell CJ, Levy D and Benjamin EJ (2007): Genome-wide association of echocardiographic dimensions, brachial artery endothelial function and treadmill exercise responses in the Framingham Heart Study. BMC Med Genet 8 Suppl 1:S2.

Vinereanu D (2006): Risk factors for atherosclerotic disease: present and future. Herz 31 Suppl 3:5-24.

Virmani R, Kolodgie FD, Burke AP, Farb A and Schwartz SM (2000): Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. Arterioscler Thromb Vasc Biol 20:1262-1275.

Visvikis-Siest S and Marteau JB (2006): Genetic variants predisposing to cardiovascular disease. Curr Opin Lipidol 17:139-151.

Wakabayashi I and Masuda H (2006): Lipoprotein (a) as a determinant of arterial stiffness in elderly patients with type 2 diabetes mellitus. Clin Chim Acta 373:127-131.

Walldius G, Jungner I, Holme I, Aastveit AH, Kolar W and Steiner E (2001): High apolipoprotein B, low apolipoprotein A-I, and improvement in the prediction of fatal myocardial infarction (AMORIS study): a prospective study. Lancet 358:2026-2033.

Wallenfeldt K, Bokemark L, Wikstrand J, Hulthe J and Fagerberg B (2004): Apolipoprotein B/apolipoprotein A-I in relation to the metabolic syndrome and change in carotid artery intima-media thickness during 3 years in middle-aged men. Stroke 35:2248-2252.

Wang Q, Rao S, Shen GQ, Li L, Moliterno DJ, Newby LK, Rogers WJ, Cannata R, Zirzow E, Elston RC and Topol EJ (2004): Premature myocardial infarction novel susceptibility locus on chromosome 1P34-36 identified by genomewide linkage analysis. Am J Hum Genet 74:262-271.

Wellcome Trust Case Control Consortium (2007): Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature 447:661-678.

Wienke A, Herskind AM, Christensen K, Skytthe A and Yashin AI (2005): The heritability of CHD mortality in danish twins after controlling for smoking and BMI. Twin Res Hum Genet 8:53-59.

Wilkinson IB, Megson IL, MacCallum T, Rooijmans DF, Johnson SM, Boyd JL, Cockcroft JR and Webb DJ (2001): Acute methionine loading does not alter arterial stiffness in humans. J Cardiovasc Pharmacol 37:1-5.

Willer CJ, Sanna S, Jackson AU, Scuteri A, Bonnycastle LL, Clarke R, Heath SC, Timpson NJ, Najjar SS, Stringham HM, Strait J, Duren WL, Maschio A, Busonero F, Mulas A, Albai G, Swift AJ, Morken MA, Narisu N, Bennett D, Parish S, Shen H, Galan P, Meneton P, Hercberg S, Zelenika D, Chen WM, Li Y, Scott LJ, Scheet PA, Sundvall J, Watanabe RM, Nagaraja R, Ebrahim S, Lawlor DA, Ben-Shlomo Y, Davey-Smith G, Shuldiner AR, Collins R, Bergman RN, Uda M, Tuomilehto J, Cao A, Collins FS, Lakatta E, Lathrop GM, Boehnke M, Schlessinger D, Mohlke KL and Abecasis GR (2008): Newly identified loci that influence lipid concentrations and risk of coronary artery disease. Nat Genet 40:161-169.

Williams C, Kingwell BA, Burke K, McPherson J and Dart AM (2005): Folic acid supplementation for 3 wk reduces pulse pressure and large artery stiffness independent of MTHFR genotype. Am J Clin Nutr 82:26-31.

Wong CW, Christen T, Roth I, Chadjichristos CE, Derouette JP, Foglia BF, Chanson M, Goodenough DA and Kwak BR (2006): Connexin37 protects against atherosclerosis by regulating monocyte adhesion. Nat Med 12:950-954.

Wong CW, Christen T, Pfenniger A, James RW and Kwak BR (2007): Do allelic variants of the connexin37 1019 gene polymorphism differentially predict for coronary artery disease and myocardial infarction? Atherosclerosis 191:355-361.

Woo KS, Chook P, Lolin YI, Cheung AS, Chan LT, Sun YY, Sanderson JE, Metreweli C and Celermajer DS (1997): Hyperhomocyst(e)inemia is a risk factor for arterial endothelial dysfunction in humans. Circulation 96:2542-2544.

Yamada Y, Izawa H, Ichihara S, Takatsu F, Ishihara H, Hirayama H, Sone T, Tanaka M and Yokota M (2002): Prediction of the risk of myocardial infarction from polymorphisms in candidate genes. N Engl J Med 347:1916-1923.

Yeh HI, Dupont E, Coppen S, Rothery S and Severs NJ (1997): Gap junction localization and connexin expression in cytochemically identified endothelial cells of arterial tissue. J Histochem Cytochem 45:539-550.

Yeh HI, Lai YJ, Chang HM, Ko YS, Severs NJ and Tsai CH (2000a): Multiple connexin expression in regenerating arterial endothelial gap junctions. Arterioscler Thromb Vasc Biol 20:1753-1762.

Yeh HI, Chang HM, Lu WW, Lee YN, Ko YS, Severs NJ and Tsai CH (2000b): Age-related alteration of gap junction distribution and connexin expression in rat aortic endothelium. J Histochem Cytochem 48:1377-1389.

Yeh HI, Chou Y, Liu HF, Chang SC and Tsai CH (2001): Connexin37 gene polymorphism and coronary artery disease in Taiwan. Int J Cardiol 81:251-255.

Yeh HI, Lu CS, Wu YJ, Chen CC, Hong RC, Ko YS, Shiao MS, Severs NJ and Tsai CH (2003): Reduced expression of endothelial connexin37 and connexin40 in hyperlipidemic mice: recovery of connexin37 after 7-day simvastatin treatment. Arterioscler Thromb Vasc Biol 23:1391-1397.

Ylä-Herttuala S, Nikkari T, Hirvonen J, Laaksonen H, Möttönen M, Pesonen E, Raekallio J and Åkerblom HK (1986): Biochemical composition of coronary arteries in Finnish children. Arteriosclerosis 6:230-236.

Ylä-Herttuala S, Palinski W, Rosenfeld ME, Steinberg D and Witztum JL (1990): Lipoproteins in normal and atherosclerotic aorta. Eur Heart J 11 Suppl E:88-99.

Zahler S, Hoffmann A, Gloe T and Pohl U (2003): Gap-junctional coupling between neutrophils and endothelial cells: a novel modulator of transendothelial migration. J Leukoc Biol 73:118-126.

Zdravkovic S, Wienke A, Pedersen NL, Marenberg ME, Yashin AI and De Faire U (2002): Heritability of death from coronary heart disease: a 36-year follow-up of 20 966 Swedish twins. J Intern Med 252:247-254.

Zeiher AM, Drexler H, Wollschlager H and Just H (1991): Modulation of coronary vasomotor tone in humans. Progressive endothelial dysfunction with different early stages of coronary atherosclerosis. Circulation 83:391-401.

9.1 Electronic database information

The HapMap Project
http://www.hapmap.org (10.7.2008)

National Human Genome Research Institute, National Institutes of Health:
http://www.genome.gov/page.cfm?pageID=26525384 (21.5.2008)

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Associations between connexin37 gene polymorphism and markers of subclinical atherosclerosis: The Cardiovascular Risk in Young Finns study

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Abstract

Objective: Connexin37 (cx37) C1019T polymorphism has been shown to associate with coronary artery disease in different populations. We investigated whether this polymorphism associates with carotid artery intima–media thickness (IMT), carotid artery compliance (CAC) and brachial artery flow mediated dilatation (FMD) – i.e., early ultrasound markers of subclinical atherosclerosis – in a clinically healthy population of young Finnish adults.

Methods and results: 1440 individuals from the Cardiovascular Risk in Young Finns study were genotyped and studied using cardiovascular risk factor and ultrasound data obtained in 2001. In linear regression models, no significant association between the cx37 polymorphism and carotid IMT, CAC or brachial artery FMD (ANOVA, $p = 0.159$, 0.151 and 0.547), respectively, was found in the whole population or in women and men separately.

Conclusions: The connexin37 C1019T polymorphism is not related with markers of subclinical atherosclerosis in young adults.

Keywords: Connexin37; Polymorphism; Intima–media thickness; Carotid artery compliance; Flow mediated dilatation

1. Introduction

Carotid artery intima–media thickness (IMT) is a measure of preclinical atherosclerosis that correlates with risk factors for coronary artery disease [1–3]. Elevated IMT has been reported in children exposed to atherosclerotic risk factors [4,5], and exposure to risk factors during childhood has been shown to predict IMT in adulthood [6–8]. Heredity has been shown to have an important role in explaining the variability of IMT regardless of traditional cardiovascular risk factors [1,9,10].

Decreased arterial elasticity, measured as carotid artery compliance (CAC), predicts cardiovascular events in high risk people [11] and relates to cardiovascular risk factors [12,13]. Flow mediated dilatation (FMD) of the brachial artery can be measured by ultrasound and is used to study early functional changes in the endothelium [14]. Impaired

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FMD is considered as a key early event in atherosclerosis [14,15].

The pathophysiological process of atherosclerosis it has been thought, in part, to result from inappropriate interactions between endothelial and smooth muscle cells of vascular walls [16]. These interactions occur mainly through humoral ways [17], but they may also be mediated by cell-to-cell interactions through gap junctions. Gap junctions are channels connecting adjacent cells, thus allowing exchange of small molecules [18], such as ions and second messengers [18]. The gap junction channels are constructed from proteins called connexins [18]. Thus far about 20 connexin isoforms have been identified in mammals. Vascular cells express mainly connexin37, connexin40 and connexin43 [19–22]. Atherogenic processes may relate to changes in connexin37 expression such as regeneration after injury [23], ageing [24] and hyperlipidemia [25]. Also, there are reports on homocysteine affecting the expression of connexins either directly [26] or via affecting the secretion of tumour necrosis factor-α (TNF-α) or other cytokines [27] known to affect the gap junctional intercellular communication [28].

It has also been suggested that intercellular communication via gap junctions may be a factor in atherogenesis because it has been observed that cells in atheromas express vascular wall connexins differentially [29]. One animal study, where vasodilatation was induced by acetylcholine in mouse arterioles that supply skeletal muscle, concluded that vasodilatation was conducted along the endothelium and that cx37 was one of the major component forming gap junctions between arteriolar endothelial cells [30]. A C to T substitution at nucleotide 1019 in the connexin37 gene, which changes a proline to serine at amino acid 319 (Pro319Ser) has been shown to associate with coronary artery disease. The C allele has been associated with carotid artery atherosclerotic plaques [31], both alleles C and T have been shown to have an association with coronary artery atherosclerosis [32,33] and the T allele has shown an association with risk of myocardial infarction (MI) [34,35]. To our knowledge there are no published reports on the relationship between the cx37 polymorphism and CAC or brachial artery FMD.

The purpose of this study was to investigate whether connexin37 C1019T polymorphism is associated with early markers of atherosclerosis, including carotid artery IMT, CAC and brachial artery FMD in a population based sample of young adults.

2. Methods

2.1. Study population

Genetic analysis was performed on a representative sample of 1440 individuals from an ongoing population based prospective cohort study, The Cardiovascular Risk in Young Finns. Description of the cohort has been published previously [7,36,37]. At the beginning of the study in 1980, 3596 3–18 year-old children and adolescents from the 4320 randomly chosen individuals from the national register participated. The latest follow up was done in 2001 and included 2283 study subjects (63.5% of the original cohort). The subjects were between the ages of 24 and 39 years. Participants gave written consent and the local ethics committees approved the study.

2.2. Physical and biochemical measurements

Blood pressure (BP) was measured using a random zero sphygmomanometer. Mean BP was calculated using measured systolic (SP) and diastolic (DP) blood pressure values; mean $BP = DP + 1/3(SP - DP)$. Information regarding cardiovascular risk factors was collected with a standardized questionnaire. Body mass index (BMI) was calculated using height and weight data [$BMI = kg/m^2$].

Serum lipids, apolipoprotein A-1 (apoA-1), apolipoprotein B (apoB), C-reactive protein (CRP) and homocysteine concentrations were determined from venous blood samples drawn after 12 h of fasting. All determinations were done in duplicate in the same laboratory. Standard enzymatic methods were used for serum total cholesterol, triglycerides and high-density lipoprotein (HDL) cholesterol. Serum apoA-1 and apoB were analysed immunoturbidometrically. Low-density lipoprotein (LDL) cholesterol concentration was calculated by the Friedewald formula. Sensitive CRP was measured using a latex turbidimetric immunoassay (Wako Chemicals GmbH, Neuss, Germany). The lower detection limit for the assay was 0.06 mg/l. Homocysteine concentrations were measured with a microparticle enzyme immunoassay kit (Abbott Laboratories, Tokyo, Japan). Details of these methods have been described previously [7,37,38].

2.3. Ultrasound measurements

IMT of the left common carotid artery was determined using Sequoia 512 ultrasound mainframes (Acuson, CA, USA) with 13.0 MHz linear array transducers. Details of the whole procedure have been described elsewhere [7]. In short, the image of left carotid artery posterior (far) wall was scanned following a standard protocol. A magnified image from the angle showing the greatest distance between the lumen–intima interface and the media–adventitia interface was recorded. A moving scan with the duration of 5 s, which included the beginning of the carotid bifurcation and the common carotid artery was recorded and stored in digital format on optical discs for subsequent off-line analysis. Analysis was done by a single reader blinded to the subjects' details. The analysis was performed using ultrasonic calipers. From the clip image, the best quality end-diastolic frame was selected. From this image, at least four measurements of the common carotid far wall were taken approximately 10 mm proximal to the bifurcation to derive maximal carotid IMT. To assess reproducibility of IMT measurements, we re-examined 60

subjects 3 months after the initial visit (2.5% random sample). The between visit coefficient of variation of IMT measurements was 6.4%.

Assessment of CAC was made by selecting the best quality cardiac cycle from the 5-s clip images. The diameter of the common carotid artery was measured at least twice in end-diastole and end-systole. The mean of the measurements was used as the end-diastolic and the end-systolic diameters. Ultrasound and concomitant brachial blood pressure measurements were used to calculate CAC = $([D_s - D_d]/D_d)/(P_s - P_d)$, where D_s is the systolic diameter, D_d the diastolic diameter, P_s the systolic blood pressure and P_d is the diastolic blood pressure. The between-visit coefficient of variation was 2.7% for diastolic carotid diameter and 16.3% for CAC. [12]

Brachial artery FMD was measured by ultrasound. The left brachial artery was measured at rest and during reactive hyperaemia which was induced by a tourniquet placed on the forearm and a pressure of 250 mmHg held for 4.5 min. Arterial diameter was measured three times at three time points (40, 60 and 80 s) and the maximum FMD was calculated from the average of the three measurements at each time point. The vessel diameter during reactive hyperaemia was expressed as relative to the resting scan (100%). The between-visit coefficient of variation was 26% for FMD [38].

2.4. DNA purification and genotyping

DNA was purified from samples of whole blood white cells, which had been stored frozen at −20 °C. Purification was performed using the Quiagen QIAmp DNA Blood Mini Kit (Quiagen Inc., Hilden Germany) according to the manufacturer's protocol.

Nucleotide sequences for the primers and allele specific probes were deduced from published sequences in the Gene Bank database. Primer and probe design was done in conjugation with Applied Biosystems using the Assays by Design service. Genotyping was performed by the 5′ nuclease assay for allelic discrimination [39] using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). A 25 μl reaction volume was used in the polymerase chain reactions (PCR) containing genomic DNA, 1× Universal Master Mix, 900 nM of each primer and 200 nM of each probe. The reactions were performed in 96-well plates following a standard protocol for TaqMan MGB probes. After completing the PCR reactions allele specific fluorescence was measured and the genotypes CC, CT and TT were deduced by the allelic discrimination analysis module.

2.5. Statistical analysis

Continuous variables between the cx37 genotypes in men and women were compared by one-way analysis of variance (ANOVA). The χ^2-test was used for comparing categorical variables between the genotypes.

Linear regression models for IMT, CAC and FMD as continuous dependent variables were done using covariates: cx37 genotype, age, sex, BMI, current smoking, mean BP, total cholesterol, HDL, LDL, triglycerides, CPR, homocysteine, apoA-1 and apoB. However, highly correlated variables like LDL cholesterol and apoB and HDL and apoA-1 were not included in the same models.

Analyses were done using statistical software SPSS (Version 14.0, SPSS Inc., Chicago, IL, USA). A p-value of less than 0.05 was considered significant.

3. Results

The prevalence of the cx37 genotypes were CC 33.8%, CT 49.8% and TT 16.4%. There were no significant differences in age or sex among the different genotype groups (Tables 1 and 2). The genotype distribution was in Hardy–Weinberg equilibrium. The 1440 subject study group was half male and half female, $n = 720$ in both groups. The 401 (28.6%) subjects were current smokers and 1000 (71.4%) subjects were non-smokers or ex-smokers or smoked <1 cigarette/week. There were 39 missing values for smoking.

In the whole population, there was a significant difference in BMI values between the cx37 genotype groups (ANOVA, $p = 0.029$) shown in Table 1. In women, total cholesterol was lowest in the CC group ($p = 0.042$) and in men the CT group had lowest BMI and CRP values (p-values 0.028 and 0.023, respectively) as compared to other genotypes (Table 2). After correction for multiple comparisons the p-values (ANOVA) for total cholesterol in women and BMI and CRP for men did not remain significant. No other associations between the cx37 genotypes and other studied variables were found. In men there was a trend for a higher IMT value in the CC genotype group, but the difference was not statistically significant ($p = 0.059$). No significant associations were found between the cx37 C1017T genotypes and carotid IMT, CAC or brachial artery FMD in the whole population (Table 1) or in females or males separately (Table 2).

In a recessive model for the C allele there were no significant differences between the genotypes in the whole population. In women, total cholesterol was lower in the CC genotype ($p = 0.043$) and in men the T-allele carriers had lower CRP and IMT values (p-values 0.033 and 0.017, respectively). With 1440 subjects our study had over 90% power to detect 0.016 mm difference in IMT related to the gene effect at the $p > 0.05$ level.

4. Discussion

The relation between cx37 C1019T polymorphism and markers of subclinical atherosclerosis CAC and FMD have not been previously studied in a healthy population of young adults. Earlier studies on the biology of the cx37 gene [22,30] suggest that cx37 C1019T polymorphism may play a role in early atherosclerosis. In an animal model, it was suggested that induced cx37 expression may be an indicator of vascular

Table 1
Clinical characteristics and biochemical parameters according to the connexin37 C1019>T polymorphism genotypes

Variable (unit)	Connexin 37 genotype			*P*
	CC	CT	TT	
Subjects (men)	486 (241)	717 (350)	237 (129)	0.317
Subjects (smokers)	475 (249)	697 (377)	229 (128)	0.673
Age (years)	32.2 ± 5.0	31.7 ± 4.9	31.8 ± 5.2	0.232
Body mass index (kg/m^2)	25.2 ± 4.7	24.9 ± 4.2	25.8 ± 4.6	0.029
Mean BP (mmHg)	89.7 ± 10.2	89.5 ± 10.1	91.2 ± 10.7	0.089
Mean IMT (mm)	0.587 ± 0.098	0.576 ± 0.092	0.578 ± 0.083	0.159
Maximal FMD (%)	8.07 ± 4.44	8.13 ± 4.44	7.75 ± 4.38	0.547
CAC (mm/mmHg)	2.12 ± 0.73	2.17 ± 0.75	2.07 ± 0.72	0.151
Total cholesterol (mmol/l)	5.15 ± 0.95	5.18 ± 0.98	5.16 ± 1.06	0.829
LDL cholesterol (mmol/l)	3.28 ± 0.84	3.29 ± 0.85	3.26 ± 0.96	0.888
HDL cholesterol (mmol/l)	1.26 ± 0.31	1.30 ± 0.31	1.27 ± 0.32	0.083
Triglycerides (mmol/l)	1.34 ± 0.79	1.32 ± 0.88	1.40 ± 0.83	0.502
C-reactive protein (mg/l)	2.12 ± 5.08	1.73 ± 3.06	2.04 ± 4.17	0.222
Homocysteine (μmol/l)	10.10 ± 4.51	9.87 ± 3.49	9.80 ± 3.35	0.521
Apolipoprotein A-1 (g/l)	1.48 ± 0.25	1.51 ± 0.26	1.49 ± 0.25	0.200
Apolipoprotein B (g/l)	1.08 ± 0.26	1.07 ± 0.27	1.08 ± 0.26	0.698

Values are means ± standard deviations. *Statistics*: Analysis of variance for continuous variables, χ^2-test for categorical variables. Due to skewed distribution homocysteine, triglycerides and C-reactive protein were logarithmically transformed before analysis. BP, blood pressure; IMT, carotid artery intima–media thickness; FMD, brachial artery flow mediated dilatation; CAC, carotid artery compliance; LDL, low-density lipoprotein; HDL, high density lipoprotein.

smooth muscle cells responding to haemodynamic changes (shear stress), and that there may be involvement of cx37 in vasodilatory responses of vessels [22]. In another animal model, it was shown that vasodilatation is conducted along the endothelial layer and that cx37 is one of the primary components in gap junctions between endothelial cells [30]. Considering the above, the polymorphism could contribute to functional or structural alterations in the vascular wall. However, in this study on a healthy young adult population we found no association between the cx37 C1019T polymorphism and early vascular changes associated with atherosclerosis.

Previous studies have associated the C1019 polymorphism with carotid IMT in a Swedish male population [31], and with coronary artery disease (CAD) in a Taiwanese population [32]. In two Japanese studies, the 1019T polymorphism associated with CAD in high risk men [33]. And in the other study [34] the 1019T polymorphism was associated with risk of myocardial infarction (MI). In a Caucasian population from Sicily also the T polymorphism was associated with MI [35] and in a recent Swiss study the T polymorphism was an independent predictor of CAD [40]. These previous studies are all on subjects with an advanced phenotype of atherosclerosis or end point phenomenon. We found no significant evidence

Table 2
Clinical characteristics and biochemical parameters according to the connexins37 C1019>T polymorphism genotypes in women and men

Variable (unit)	Connexin 37 genotypes in women			*P* value	Connexin37 genotypes in men			*P* value
	CC (*n* = 245)	CT (*n* = 367)	TT (*n* = 108)		CC (*n* = 241)	CT (*n* = 350)	TT (*n* = 129)	
Subjects (smokers)	240 (107)	359 (180)	105 (48)	0.377	235 (142)	338 (197)	124 (80)	0.476
Age (years)	32.1 ± 5.2	31.7 ± 4.8	31.8 ± 5.2	0.692	32.3 ± 4.9	31.6 ± 5.1	31.9 ± 5.3	0.294
Body mass index (kg/m^2)	24.2 ± 4.7	24.5 ± 4.5	25.2 ± 4.6	0.178	26.11 ± 4.46	25.3 ± 3.8	26.2 ± 4.5	0.028
Mean BP (mmHg)	86.1 ± 9.2	86.5 ± 8.7	87.1 ± 10.5	0.654	93.5 ± 9.7	92.7 ± 10.3	94.6 ± 9.8	0.184
Mean IMT (mm)	0.569 ± 0.087	0.568 ± 0.084	0.569 ± 0.075	0.967	0.604 ± 0.106	0.585 ± 0.099	0.586 ± 0.089	0.059
Maximal FMD (%)	9.02 ± 4.56	8.88 ± 4.49	8.70 ± 4.36	0.826	7.08 ± 4.08	7.28 ± 4.22	6.89 ± 4.24	0.664
CAC (mm/mmHg)	2.27 ± 0.80	2.29 ± 0.79	2.20 ± 0.77	0.565	1.97 ± 0.63	2.05 ± 0.68	1.97 ± 0.67	0.245
Total cholesterol (mmol/l)	4.99 ± 0.91	5.18 ± 0.94	5.03 ± 1.01	0.042	5.30 ± 0.95	5.19 ± 1.02	5.27 ± 1.10	0.348
LDL cholesterol (mmol/l)	3.10 ± 0.74	3.22 ± 0.79	3.11 ± 0.83	0.155	3.47 ± 0.89	3.37 ± 0.91	3.39 ± 1.04	0.447
HDL cholesterol (mmol/l)	1.37 ± 0.29	1.42 ± 0.30	1.38 ± 0.32	0.118	1.15 ± 0.29	1.17 ± 0.27	1.17 ± 0.28	0.541
Triglycerides (mmol/l)	1.16 ± 0.58	1.21 ± 0.85	1.18 ± 0.63	0.876	1.53 ± 0.92	1.44 ± 0.89	1.58 ± 0.93	0.074
C-reactive protein (mg/l)	2.22 ± 5.21	2.08 ± 3.18	2.67 ± 5.46	0.440	2.02 ± 4.94	1.37 ± 2.89	1.51 ± 2.54	0.023
Homocysteine (μmol/l)	8.94 ± 3.73	9.07 ± 3.23	8.79 ± 2.49	0.743	11.26 ± 4.92	10.72 ± 3.55	10.64 ± 3.74	0.293
Apolipoprotein A-1 (g/l)	1.56 ± 0.26	1.59 ± 0.27	1.56 ± 0.27	0.197	1.40 ± 0.20	1.41 ± 0.20	1.43 ± 0.22	0.467
Apolipoprotein B (g/l)	0.99 ± 0.24	1.03 ± 0.24	1.00 ± 0.26	0.241	1.16 ± 0.26	1.11 ± 0.28	1.15 ± 0.24	0.059

Values are means ± standard deviations. Data on smoking are missing from 16 women and 23 men. *Statistics*: Analysis of variance for continuous variables. χ^2-Test was used for categorical variables. Due to skewed distribution homocysteine, triglycerides and C-reactive protein were logarithmically transformed before analysis. BP, blood pressure; IMT, carotid artery intima–media thickness; FMD, brachial artery flow mediated dilatation; CAC, carotid artery compliance; LDL, low-density lipoprotein, HDL, high density lipoprotein.

associating the cx37 polymorphism and IMT in a healthy population, but the possibility remains that an association between the CC genotype and IMT may exist..Taking into consideration the long lag time between an increase in IMT and occurrence of MI it is obvious that pathological, genetic and lifetime risk factor burdens behind the development of these vascular phenotypes would vary at different time points.

In conclusion, studying the connexin37 C1019T polymorphism in regard to early markers of functional or structural changes caused by atherosclerosis there does not seem to be an association with carotid artery CAC or brachial FMD, but we cannot rule out a possibility of an association with carotid IMT.

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References

[1] Fox CS, Polak JF, Chazaro I, et al. Genetic and environmental contributions to atherosclerosis phenotypes in men and women: heritability of carotid intima–media thickness in the Framingham Heart Study. Stroke 2003;34:397–401.
[2] Espeland MA, Tang R, Terry JG, Davis DH, Mercuri M, Crouse 3rd JR. Associations of risk factors with segment-specific intimal–medial thickness of the extracranial carotid artery. Stroke 1999;30:1047–55.
[3] Folsom AR, Eckfeldt JH, Weitzman S, et al. Relation of carotid artery wall thickness to diabetes mellitus, fasting glucose and insulin, body size, and physical activity. Atherosclerosis Risk in Communities (ARIC) Study Investigators. Stroke 1994;25:66–73.
[4] Järvisalo MJ, Jartti L, Näntö-Salonen K, et al. Increased aortic intima–media thickness: a marker of preclinical atherosclerosis in high-risk children. Circulation 2001;104:2943–7.
[5] Pauciullo P, Iannuzzi A, Sartorio R, et al. Increased intima–media thickness of the common carotid artery in hypercholesterolemic children. Arterioscler Thromb 1994;14:1075–9.
[6] Davis PH, Dawson JD, Riley WA, Lauer RM. Carotid intimal–medial thickness is related to cardiovascular risk factors measured from childhood through middle age: the Muscatine Study. Circulation 2001;104: 2815–9.
[7] Raitakari OT, Juonala M, Kähönen M, et al. Cardiovascular risk factors in childhood and carotid artery intima–media thickness in adulthood: the Cardiovascular Risk in Young Finns Study. JAMA 2003;290:2277–83.
[8] Li S, Chen W, Srinivasan SR, et al. Childhood cardiovascular risk factors and carotid vascular changes in adulthood: the Bogalusa Heart Study. JAMA 2003;290:2271–6.
[9] Cuomo S, Guarini P, Gaeta G, et al. Increased carotid intima–media thickness in children-adolescents, and young adults with a parental history of premature myocardial infarction. Eur Heart J 2002;23:1345–50.
[10] Jousilahti P, Puska P, Vartiainen E, Pekkanen J, Tuomilehto J. Parental history of premature coronary heart disease: an independent risk factor of myocardial infarction. J Clin Epidemiol 1996;49:497–503.
[11] Blacher J, Pannier B, Guerin AP, Marchais SJ, Safar ME, London GM. Carotid arterial stiffness as a predictor of cardiovascular and all-cause mortality in end-stage renal disease. Hypertension 1998;32:570–4.
[12] Juonala M, Järvisalo MJ, Mäki-Torkko N, Kähönen M, Viikari JS, Raitakari OT. Risk factors identified in childhood and decreased carotid artery elasticity in adulthood: the Cardiovascular Risk in Young Finns Study. Circulation 2005;112:1486–93.
[13] Ferreira I, Henry RM, Twisk JW, van Mechelen W, Kemper HC, Stehouwer CD. The metabolic syndrome, cardiopulmonary fitness, and subcutaneous trunk fat as independent determinants of arterial stiffness: the Amsterdam Growth and Health Longitudinal Study. Arch Intern Med 2005;165:875–82.
[14] Celermajer DS, Sorensen KE, Gooch VM, et al. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. Lancet 1992;340:1111–5.
[15] Neunteufl T, Katzenschlager R, Hassan A, et al. Systemic endothelial dysfunction is related to the extent and severity of coronary artery disease. Atherosclerosis 1997;129:111–8.
[16] Sosa-Melgarejo JA, Berry CL. Myoendothelial contacts in arteriolosclerosis. J Pathol 1992;167:235–9.
[17] Griendling KK, Alexander RW. Endothelial control of the cardiovascular system: recent advances. FASEB J 1996;10:283–92.
[18] Goodenough DA, Goliger JA, Paul DL. Connexins, connexons, and intercellular communication. Annu Rev Biochem 1996;65:475–502.
[19] Bruzzone R, Haefliger JA, Gimlich RL, Paul DL. Connexin40, a component of gap junctions in vascular endothelium, is restricted in its ability to interact with other connexins. Mol Biol Cell 1993;4:7–20.
[20] Gabriels JE, Paul DL. Connexin43 is highly localized to sites of disturbed flow in rat aortic endothelium but connexin37 and connexin40 are more uniformly distributed. Circ Res 1998;83:636–43.
[21] Yeh HI, Dupont E, Coppen S, Rothery S, Severs NJ. Gap junction localization and connexin expression in cytochemically identified endothelial cells of arterial tissue. J Histochem Cytochem 1997;45:539–50.
[22] Cai WJ, Koltai S, Kocsis E, Scholz D, Schaper W, Schaper J. Connexin37, not Cx40 and Cx43, is induced in vascular smooth muscle cells during coronary arteriogenesis. J Mol Cell Cardiol 2001;33:957–67.
[23] Yeh HI, Lai YJ, Chang HM, Ko YS, Severs NJ, Tsai CH. Multiple connexin expression in regenerating arterial endothelial gap junctions. Arterioscler Thromb Vasc Biol 2000;20:1753–62.
[24] Yeh HI, Chang HM, Lu WW, et al. Age-related alteration of gap junction distribution and connexin expression in rat aortic endothelium. J Histochem Cytochem 2000;48:1377–89.
[25] Yeh HI, Lu CS, Wu YJ, et al. Reduced expression of endothelial connexin37 and connexin40 in hyperlipidemic mice: recovery of connexin37 after 7-day simvastatin treatment. Arterioscler Thromb Vasc Biol 2003;23:1391–7.
[26] Li H, Brodsky S, Kumari S, et al. Paradoxical overexpression and translocation of connexin43 in homocysteine-treated endothelial cells. Am J Physiol Heart Circ Physiol 2002;282:H2124–33.
[27] van Rijen HV, van Kempen MJ, Postma S, Jongsma HJ. Tumour necrosis factor alpha alters the expression of connexin43, connexin40, and connexin37 in human umbilical vein endothelial cells. Cytokine 1998;10:258–64.

[28] Hu J, Cotgreave IA. Differential regulation of gap junctions by proinflammatory mediators in vitro. J Clin Invest 1997;99:2312–6.
[29] Kwak BR, Mulhaupt F, Veillard N, Gros DB, Mach F. Altered pattern of vascular connexin expression in atherosclerotic plaques. Arterioscler Thromb Vasc Biol 2002;22:225–30.
[30] Looft-Wilson RC, Payne GW, Segal SS. Connexin expression and conducted vasodilation along arteriolar endothelium in mouse skeletal muscle. J Appl Physiol 2004;97:1152–8.
[31] Boerma M, Forsberg L, Van Zeijl L, et al. A genetic polymorphism in connexin 37 as a prognostic marker for atherosclerotic plaque development. J Intern Med 1999;246:211–8.
[32] Yeh HI, Chou Y, Liu HF, Chang SC, Tsai CH. Connexin37 gene polymorphism and coronary artery disease in Taiwan. Int J Cardiol 2001;81:251–5.
[33] Hirashiki A, Yamada Y, Murase Y, et al. Association of gene polymorphisms with coronary artery disease in low- or high-risk subjects defined by conventional risk factors. J Am Coll Cardiol 2003;42: 1429–37.
[34] Yamada Y, Izawa H, Ichihara S, et al. Prediction of the risk of myocardial infarction from polymorphisms in candidate genes. N Engl J Med 2002;347:1916–23.
[35] Listi F, Candore G, Lio D, et al. Association between C1019T polymorphism of connexin37 and acute myocardial infarction: a study in patients from sicily. Int J Cardiol 2005;102:269–71.
[36] Åkerblom HK, Viikari J, Uhari M, et al. Atherosclerosis precursors in Finnish children and adolescents. I. General description of the cross-sectional study of 1980, and an account of the children's and families' state of health. Acta Paediatr Scand Suppl 1985;318:49–63.
[37] Juonala M, Viikari JS, Hutri-Kähönen N, et al. The 21-year follow-up of the Cardiovascular Risk in Young Finns Study: risk factor levels, secular trends and east–west difference. J Intern Med 2004;255: 457–68.
[38] Juonala M, Viikari JS, Laitinen T, et al. Interrelations between brachial endothelial function and carotid intima–media thickness in young adults: the cardiovascular risk in young Finns study. Circulation 2004;110:2918–23.
[39] Livak KJ. Allelic discrimination using fluorogenic probes and the 5' nuclease assay. Genet Anal 1999;14:143–9.
[40] Wong CW, Christen T, Pfenniger A, James RW, Kwak BR. Do allelic variants of the connexin37 1019 gene polymorphism differentially predict for coronary artery disease and myocardial infarction? Atherosclerosis 2007;191:355–61.

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The influence of smoking and homocysteine on subclinical atherosclerosis is modified by the connexin37 C1019T polymorphism – The Cardiovascular Risk in Young Finns Study

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Abstract

Background: A polymorphism C1019T on the connexin37 (*Cx37*) gene has been found to associate with coronary artery disease. There are conflicting results on which allele confers risk, and the possibility of interactions between the polymorphism and risk factors has been raised. In this study, we examined interactions between the *Cx37* polymorphism and common risk factors and their associations to early vascular parameters of atherosclerosis: carotid artery intima-media thickness (IMT), and carotid artery compliance (CAC) and brachial artery flow mediated dilatation (FMD).
Methods: A population of 1440 healthy young adults from the Cardiovascular Risk in Young Finns Study was studied. The subjects were genotyped and their cardiovascular risk factor and ultrasound data gathered in 2001 were used for the statistical analyses.
Results: In the whole population, homocysteine in subjects with the TT genotype was found to be associated with higher FMD values (p for interaction 0.038) and remained so in three different adjusted models (p for interaction 0.022–0.038). In women with the CC genotype, smoking was found to be associated with higher FMD values and the smoking-by-genotype interaction remained significant in three adjusted models (p for interaction 0.001–0.041). In women with TT genotype, the effect of smoking was opposite, i.e., FMD values for smokers were lower compared to non-smokers. In men, physical activity interacted with *Cx37* on CAC in the CT and TT genotypes (p for interaction 0.011). No significant interactions were found to predict IMT.
Conclusions: The effect of smoking and homocysteine levels on arterial endothelial functions and elasticity were modified by the allelic variation of the *Cx37* gene. These data suggest that variation in the connexin gene may modify effects risk factors have on vascular function.
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Keywords: atherosclerosis; brachial artery flow mediated dilatation; carotid artery compliance; connexin37; interaction; intima-media thickness; polymorphism.

Introduction

Atherosclerosis is a multifactorial and polygenic disorder that can lead to coronary artery disease (CAD) and myocardial infarction (MI). Traditional risk factors include smoking, hypertension, hypercholesterolemia and diabetes (1). Some of the risk factors have their own genetic determinants, but also other genetic patterns have been shown to predict morbidity or endpoints of this disease (2). Candidate gene analyses have identified many polymorphisms that associate with or predispose to atherosclerosis, CAD or MI (3, 4). One such polymorphism is the C1019T polymorphism of the connexin37 (*Cx37*) gene (3, 5). The connexin37 functions as a structural protein in gap junctions (6). Previous studies have shown that *Cx37* is expressed in endothelial cells (7) and monocyte/macrophages (8). The Cx37 protein has been shown to be involved in regeneration after injury (9) and ageing (10), which may play roles in the pathogenesis of atherosclerosis. Further, the hemichannels forming gap junctions have been suggested to play a role in the initiation of plaque development by regulating ATP-dependent monocyte adhesion (8). The C allele of the C1019T polymorphism has been associated

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with carotid artery atherosclerotic plaques (5) and both CAD and MI (11). Both C and T alleles have been associated with CAD (4, 12), and the T allele has been associated with the risk of MI (3, 13, 14).

Previous studies have shown that vascular parameters indicating subclinical atherosclerosis are influenced by a positive family history of atherosclerosis in healthy subjects. Family history of stroke or MI has been associated with common carotid artery intima-media thickness (IMT) independently of conventional risk factors (15) and family history of CAD has been associated with impaired brachial artery flow mediated dilatation (FMD) (16). Therefore, genetics may play a role in disease progression. These effects may be direct or indirect. Genes may modify the effects of risk factors so that carriers of different alleles may have different phenotypes. Even in healthy subjects, the predetermined genotype determines the influence that a certain risk factor has on an individual's phenotype.

We previously studied whether the *Cx37* polymorphism is associated with IMT, carotid artery compliance (CAC) or FMD and found no significant differences between the genotypes (17). However, there was a slight increase in IMT values in men with the CC genotype, and the possible role of the polymorphism or its interactions with major risk factors could not be ruled out. The conflicting results of previous studies on *Cx37* and atherosclerosis have been thought to arise for different reasons and one is the interactions of genes with either other genes or environmental factors (11). To our knowledge, there are no published reports on environmental factors interacting with the *Cx37* polymorphism in association with IMT, CAC or FMD.

We examined the possibility that the effect of major cardiovascular risk factors on the vascular parameters may be different in the different *Cx37* genotypes. We investigated whether common risk variables, such as age, smoking, physical activity, blood pressure, obesity, high homocysteine, total cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides and C-reactive protein (CRP) or low high-density lipoprotein (HDL) cholesterol interact with the *Cx37* C1019T polymorphism and whether the possible interactions associate with early markers of atherosclerosis, including IMT, CAC or FMD in a population-based sample of young adults. This type of explorative study approach has the potential to generate a new hypothesis that needs to be replicated in other cohorts.

Materials and methods

Study population

The population for genetic analysis was a representative sample of 1440 individuals of the ongoing Cardiovascular Risk in Young Finns Study. The cohort has been described previously (18–20). Briefly, in 1980 3596 children and adolescents between the ages of 3 and 18 years from 4320 randomly chosen individuals from the national register participated in the study. The latest follow-up study was carried out in 2001 and it included 2283 subjects (63.5% of the original cohort). The study subjects were between the ages of 24 and 39 years. Participants gave their written consent and the study was approved by the local Ethics Committees.

General information

Information regarding smoking habits, educational status and physical activity were collected with a questionnaire. Smoking habits in this study were determined as a two-valued variable: smokers were current smokers and non-smokers had never smoked or were ex-smokers. Physical activity of the subjects was determined as described previously, a calculated metabolic equivalent (MET) index for physical activity was used in this study (21).

Physical and biochemical measurements

Body mass index (BMI) was calculated using height and weight data (BMI = kg/m^2). Blood pressure (BP) was measured using a random zero sphygmomanometer. Mean BP was calculated using the values for systolic (SP) and diastolic (DP) blood pressure in the formula: mean BP = DP + 1/3(SP–DP).

Serum lipids, sensitive CRP and homocysteine were determined from venous blood samples drawn after a 12-h fast. All blood analyses were carried out in duplicate in the same laboratory. Standard enzymatic methods were used for serum total cholesterol, triglycerides and HDL cholesterol. The Friedewald formula was used to calculate the concentration of LDL cholesterol. A latex turbidimetric immunoassay was used to measure sensitive CRP (Wako Chemicals GmbH, Neuss, Germany). The lower detection limit for the assay was 0.06 mg/L. Homocysteine concentrations were measured with a microparticle enzyme immunoassay kit (Abbott Laboratories, Tokyo, Japan). Details of these methods have been described previously (18, 20, 22)

DNA purification and genotyping

DNA purification was performed using the Quiagen QIAmp DNA Blood Mini Kit (Quiagen Inc., Hilden, Germany) according to the manufacturer's protocol. DNA was purified from samples of whole blood white cells, which had been stored frozen at –20°C.

Published sequences in the Gene Bank database were used to deduce the nucleotide sequences for the primers and allele specific probes. Primer and probe design was carried out in conjugation with Applied Biosystems using the Assays by Design service. The 5′ nuclease assay for allelic discrimination (23) using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was used for genotyping. Polymerase chain reactions (PCRs) were carried out in a 25-μL reaction volume containing genomic DNA, 1× Universal Master Mix, 900 nM of each primer and 200 nM of each probe. The reactions were performed in 96-well plates following a standard protocol for TaqMan MGB probes. Allele specific fluorescence was measured after the PCRs and the genotypes CC, CT and TT were deduced by the allelic discrimination analysis module. Random duplicates were used as a quality control.

Ultrasound measurements

The left common carotid artery IMT was determined using Sequoia 512 ultrasound mainframes (Acuson, Mountain View, CA, USA) with 13.0 MHz linear array transducers. Details of the procedure have been described previously (18). In brief, the image of the left carotid artery posterior (far) wall was scanned following a standard protocol. A magnified image from the angle showing the greatest distance between the lumen-intima interface and the media-adventi-

tia interface was recorded. A moving scan of 5 s, which included the beginning of the carotid bifurcation and the common carotid artery was recorded and stored in digital format on optical disks for subsequent off-line analysis. Analysis was carried out by a single reader blinded to the subjects' details. Ultrasonic calipers were used to perform the analysis. The best quality end-diastolic frame was selected from the clip image. To derive maximal carotid IMT, at least four measurements of the common carotid far wall were taken approximately 10 mm proximal to the bifurcation. To assess reproducibility of IMT measurements, 60 subjects were re-examined 3 months after the initial visit (2.5% random sample). The between-visit coefficient of variation of IMT measurements was 6.4%.

To assess CAC, the best quality cardiac cycle from the 5-s clip images was selected. The diameter of the common carotid artery was measured in end-diastole and end-systole at least twice. The mean of the measurements was used as the end-diastolic and the end-systolic diameters. Ultrasound and concomitant brachial blood pressure measurements were used to calculate $CAC = ([D_s - D_d]/D_d)/(P_s - P_d)$, where D_s is the systolic diameter; D_d the diastolic diameter; P_s the systolic blood pressure and P_d the diastolic blood pressure. The between-visit coefficient of variation was 2.7% for the diastolic carotid diameter and 16.3% for CAC (24).

Ultrasound was used to measure the brachial artery FMD. The left brachial artery was first measured at rest and then during reactive hyperaemia, which was induced by a tourniquet placed on the forearm and a pressure of 250 mm Hg held for 4.5 min. In total, three measurements of the arterial diameter were performed at end-diastole at a fixed distance from an anatomic marker at rest (baseline) and 40, 60 and 80 s after cuff release. The vessel diameter in scans after reactive hyperaemia was expressed as the percentage relative to the resting scan (100%). The average of three measurements at each time point was used to derive the maximum FMD (the greatest value between 40 and 80 s). The between-visit coefficient of variation for the baseline brachial diameter was 3.2% and 26.0% for FMD (22).

Statistical analysis

Continuous variables between the *Cx37* genotypes in men and women were compared by one-way analysis of variance, and the χ^2-test was used for comparing categorical variables between the genotypes. Study characteristics of this population have been published previously (17).

Interactions between the *Cx37* genotype and risk factors (age, BMI, current smoking, physical activity, mean BP, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, CRP and homocysteine) on IMT, CAC and FMD were analysed for the whole population, and men and women separately using analysis of co-variance.

Linear regression models were used to study the associations between risk factors and vascular parameters. Several differently adjusted models were used for these analyses. The variables used included age, sex, brachial artery diameter, current smoking, physical activity, BMI, mean BP, HDL cholesterol and LDL cholesterol. Variables with skewed distribution were logarithmically transformed before analyses, i.e., homocysteine, triglycerides and CRP. The analyses were carried out with SPSS statistical software (version 15.0, SPSS Inc., Chicago, IL, USA). A p-value of less than 0.05 was considered statistically significant.

Results

In the study population, the prevalence of the *Cx37* genotypes was 33.8% for CC, 49.8% for CT and 16.4% for TT. The genotype distribution was in Hardy-Weinberg equilibrium. There were no significant differences in age or sex between the different genotype groups. Characteristics are summarised in Table 1. In all subjects, there was a significant difference in BMI values between the *Cx37* genotype groups ($p = 0.029$). In men, there was a significant difference in BMI and CRP between the genotypes (p-values 0.028 and 0.023, respectively). In women, there was a significant difference between the genotypes in total cholesterol ($p = 0.042$). Values for FMD were available in 1321 cases, for CAC in 1433 cases and for the physical activity index in 1179 cases. Smoking information was missing in 39 cases. The study population has been described previously (17).

Results of significant interactions between the *Cx37* genotype and risk factors on IMT, CAC and FMD in the whole population and divided by sex are shown in Table 2. In total, 11 variables were tested, and at a

Table 1 Characteristics of the study population according to the connexin37 (*Cx37*) C1019T polymorphism genotypes.

Variable, unit	Connexin37 genotype		
	CC	CT	TT
Subjects (men)	486 (241)	717 (350)	237 (129)
Non smokers/smokers	297/178	455/242	153/76
Age, years	32.2±5.0	31.7±4.9	31.8±5.2
Body mass index, kg/m^2	25.2±4.7	24.9±4.2	25.8±4.6
Mean blood pressure, mm Hg	89.7±10.2	89.5±10.1	91.2±10.7
Mean IMT, mm	0.587±0.098	0.576±0.092	0.578±0.083
Maximal FMD, %	8.07±4.44	8.13±4.44	7.75±4.38
CAC, %/10 mm Hg	2.12±0.73	2.17±0.75	2.07±0.72
Total cholesterol, mmol/L	5.15±0.95	5.18±0.98	5.16±1.06
LDL cholesterol, mmol/L	3.28±0.84	3.29±0.85	3.26±0.96
HDL cholesterol, mmol/L	1.26±0.31	1.30±0.31	1.27±0.32
Triglycerides, mmol/L	1.34±0.79	1.32±0.88	1.40±0.83
C-reactive protein, mg/L	2.12±5.08	1.73±3.06	2.04±4.17
Homocysteine, μmol/L	10.10±4.51	9.87±3.49	9.80±3.35
Physical activity index, % (range)	18 (0–113)	16 (0–80)	15 (0–70)

Values are means±standard deviations. IMT, carotid artery intima-media thickness; FMD, brachial artery flow mediated dilatation; CAC, carotid artery compliance; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

Table 2 Connexin37 (*Cx37*) genotype interactions with risk factors predicting early vascular signs of atherosclerosis.

	Interaction	IMT p-value	CAC p-value	FMD p-value
Whole population	*Cx37**Hcy	0.810	0.956	0.038
Men	*Cx37**MET	0.318	0.011	0.649
Women	*Cx37**smoking	0.655	0.522	0.004

Statistics: the interactions were analysed by analyses of covariance. The variables included in the interaction analyses were homocysteine (Hcy), C-reactive protein, triglycerides, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, current smoking (smoking), age, body mass index, mean blood pressure and physical activity (MET). Due to skewed distribution, homocysteine, triglycerides and CRP were logarithmically transformed before analysis. Only results for variables with significant findings are presented. IMT, intima-media thickness; CAC, carotid artery compliance; FMD, flow mediated dilatation.

significance level of 0.05 there is a possibility that 5% of the results are randomly positive.

The interaction effect between homocysteine and *Cx37* on FMD, shown in Table 3, was significant in the whole population and showed that higher levels of homocysteine were associated with higher FMD values in subjects with the TT genotype in three different adjusted models (p for interaction 0.022–0.038). In the unadjusted model, however, the interaction was significant in the CT genotype and the association was opposite, in this group a higher homocysteine value was associated with lower FMD values (p for interaction 0.038).

A significant interaction effect between smoking and *Cx37* on FMD was found in women , as shown in Table 4. The results indicated that in smokers with the CC genotype, FMD values are significantly increased compared to non-smokers. This was found in the unadjusted model and in three different adjusted models (p for interaction from 0.001 to 0.041). In the TT genotype, the effect was opposite, i.e., smokers had lower FMD values compared to non-smokers. Figure 1 illustrates the differences in FMD values between smokers and non-smokers in the three genotypes.

In men, there was a significant interaction effect between physical activity and *Cx37* on CAC (p = 0.011) both in the CT and TT genotypes. The effect did not remain significant in the adjusted models (Table 5). In Figure 2, the values for CAC are shown in different

Table 3 The relationship between homocysteine predicting brachial artery flow mediated dilatation (FMD) in connexin37 (*Cx37*) genotype groups in the whole population.

	CC (n = 446)			CT (n = 661)			TT (n = 214)			p for interaction
	β	SE	p	β	SE	p	β	SE	p	
Model 1	−0.591	0.643	0.358	−1.713	0.570	0.003	1.263	1.035	0.224	0.038
Model 2	0.620	0.643	0.335	−0.940	0.575	0.103	2.504	1.020	0.015	0.022
Model 3	0.505	0.645	0.435	−0.896	0.589	0.129	2.414	1.056	0.023	0.038
Model 4	0.345	0.739	0.641	−0.793	0.637	0.215	2.273	1.090	0.039	0.093
Model 5	0.576	0.632	0.362	−0.916	0.566	0.106	2.467	1.012	0.016	0.025
Model 6	0.443	0.736	0.548	−0.872	0.649	0.180	2.192	1.138	0.056	0.088

Model 1, no adjustment; Model 2, adjusted for age and sex and brachial artery diameter; Model 3, adjusted for age, sex, brachial artery diameter and current smoking; Model 4, adjusted for age, brachial artery diameter and physical activity; Model 5, adjusted for age, sex, brachial artery diameter, BMI, mean BP, HDL-C and LDL-C. Model 6, adjusted for all the above. Statistics: multivariate models. Analysis of covariance was used to analyse *Cx37* genotype interactions with homocysteine on FMD. Adjusted with risk factors as shown in Models 2–6. β, unstandardised regression coefficient, values are regression coefficients (expressed in %) for a 1-unit change in serum homocysteine; SE, standard error; BMI, body mass index; BP, blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

Table 4 The relationship between smoking (no/yes) predicting brachial artery flow mediated dilatation (FMD) in connexin37 (*Cx37*) genotype groups in women.

	CC (n = 228)			CT (n = 350)			TT (n = 102)			p for interaction
	β	SE	p	β	SE	p	β	SE	p	
Model 1	2.129	0.651	0.001	−0.139	0.531	0.794	−1.452	1.057	0.173	0.004
Model 2	2.200	0.623	0.001	−0.124	0.511	0.808	−1.933	1.009	0.058	0.001
Model 3	1.754	0.762	0.023	0.099	0.598	0.868	−1.349	1.154	0.247	0.059
Model 4	2.211	0.626	0.001	0.044	0.498	0.929	−2.349	1.017	0.023	0.001
Model 5	1.789	0.776	0.022	0.218	0.585	0.709	−2.433	1.209	0.049	0.041

Model 1, no adjustment; Model 2, adjusted for age, brachial artery diameter; Model 3, adjusted for age, brachial artery diameter and physical activity; Model 4, adjusted for age, brachial artery diameter, BMI, mean BP, HDL-C and LDL-C; Model 5, adjusted for all the above. Statistics: multivariate models. Analysis of covariance was used to analyse *Cx37* genotype interactions with smoking on FMD. Adjusted with risk factors as shown in Models 2–5. β, unstandardised regression coefficients, values are regression coefficients (expressed in %) for the absence/presence of smoking; SE, standard error; BMI, body mass index; BP, blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

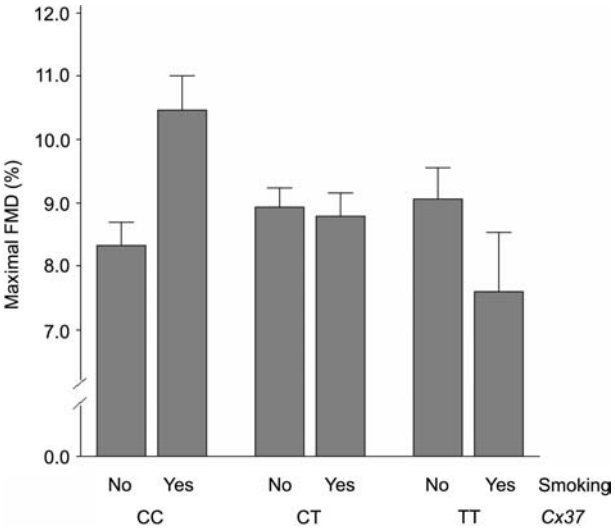

Figure 1 The mean values for brachial artery flow mediated dilatation (FMD) in the different connexin37 (*Cx37*) genotypes in women divided by smoking habits.

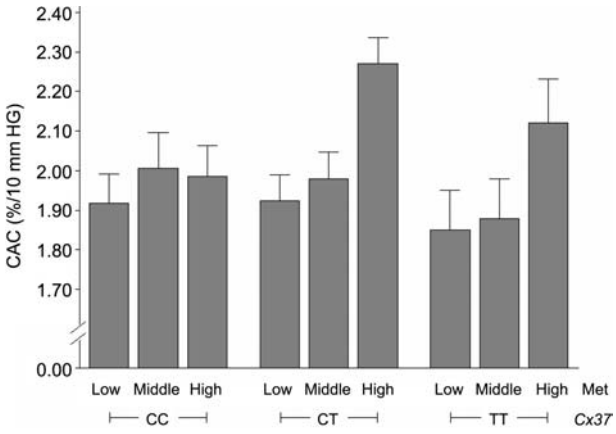

Figure 2 The mean values for carotid artery compliance (CAC) in the different connexin37 (*Cx37*) genotypes in men divided by tertiles of the physical activity index (MET).

genotype groups according to tertiles of the physical activity index. We did not find any significant interactions between genotype and risk factors predicting IMT.

Discussion

This study examined the possible effects of the *Cx37* C1019T polymorphism on different risk factors for atherosclerosis in association with early subclinical markers IMT, FMD or CAC. We found that in female smokers, the effect of smoking seems to be modulated by the polymorphism in association with FMD. Also, the interaction term remained significant in most of the different multivariate models. In women, smoking was indirectly associated with FMD in subjects with the TT genotype, but directly in subjects with the CC genotype. We also found a significant interaction between homocysteine and FMD in three different multivariate models for the TT genotype. Lastly, physical activity seemed to predict CAC in men with the CT and TT genotypes, but not in men with the CC genotype. However, the interaction term was significant only in the unadjusted model.

As our approach was exploratory with the aim to generate a new hypothesis, we chose not to apply conventional multiple comparison methods, as these techniques may negate the value of the information in data with the risk of missing important findings (25). We do, however, acknowledge that some of the study observations may be the result of chance alone.

There are no previous studies on possible interactions of atherosclerosis risk factors with the *Cx37* polymorphism on IMT, CAC and FMD. Smoking is well known to cause vascular dysfunction and the effect is mediated by impaired nitric oxide (NO) production (26, 27). The mechanism of how the interaction between smoking and the *Cx37* polymorphism affects the endothelium may lie in the expression of different amino acids encoded by the polymorphism and this might affect the biophysical properties of the gap junction. Previously, it has been shown that NO inhibits the transfer of molecules through gap junctions by influencing the *Cx37* in cell culture (28). This raises the question of whether the polymorphism can affect the effect that smoking has on FMD by influencing the gap junctional intercellular communication in women homozygous for either the C or T allele of the *Cx37* polymorphism. In view of our results, the effects of smoking may be more harmful on the endo-

Table 5 The relationship between physical activity predicting carotid artery compliance (CAC) in connexin37 (*Cx37*) genotype groups in men.

	CC (n=237)			CT (n=349)			TT (n=129)			p for interaction
	β	SE	p	β	SE	p	β	SE	p	
Model 1	−1.7E−005	0.002	0.994	0.009	0.002	<0.001	0.008	0.004	0.033	0.011
Model 2	0.001	0.002	0.813	0.007	0.002	0.002	0.005	0.004	0.151	0.102
Model 3	0.000	0.002	0.847	0.008	0.002	<0.001	0.006	0.004	0.124	0.062
Model 4	0.001	0.002	0.598	0.006	0.002	0.004	0.005	0.004	0.171	0.310
Model 5	0.001	0.002	0.617	0.008	0.002	<0.001	0.005	0.004	0.147	0.234

Model 1, no adjustment; Model 2, adjusted for age; Model 3, adjusted for age and current smoking; Model 4, adjusted for age, BMI, mean BP, HDL-C, LDL-C; Model 5, adjusted for all the above. Statistics: multivariate models. Analysis of covariance was used to analyse *Cx37* genotype interactions with physical activity on CAC. Adjusted with risk factors as shown in Models 2–5. β, unstandardised regression coefficients, values are regression coefficients (expressed in %) for a 1-unit change in the metabolic index of physical activity. SE, standard error; BMI, body mass index; BP, blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

thelial function in women with the TT genotype than in the other genotypes. In addition, paradoxically, smoking was associated with improved FMD in women with the CC genotype.

High levels of homocysteine have previously been shown to be a risk factor for endothelial dysfunction (29, 30). The results of this study showed that in the TT genotype homocysteine was associated with higher FMD values when adjusted with other risk factors. This may be an indication of genotype modulating the effect of a risk factor. However, we cannot predict the outcome for this population in the case of higher homocysteine values. Our population had mostly normal levels of homocysteine, as in our data only 5.6% of the population could be considered to have high total homocysteine, i.e., values above the 15 μmol/L level. Interestingly, in the unadjusted model the interaction was not significant for the TT genotype, but became so in different adjusted models. The overall significance of this result remains unclear, as there are also many other interactions with different genetic and environmental risk factors. The significance of this particular finding requires further studies.

Regarding physical activity, it has previously been shown that subjects who are physically more active have better arterial compliance, and that an exercise intervention can increase arterial elasticity in those who do not habitually exercise (31). We found a clear association between physical activity and CAC only in men with the CT genotype. One explanation for this observation may be positive heterosis, where subjects heterozygous for a specific allele show a significantly greater effect for the studied trait than do homozygous subjects for either allele (32).

In a recent study, it was shown that monocytes expressing serine on codon 319 (1019T) of the *Cx37* protein are more adhesive to the endothelium than those expressing proline (8). Potentially, this could have atherogenic effects and previous studies have associated the T allele with MI (3, 13). We had previously found that men with the CC genotype have a trend for higher IMT values than the other genotypes and hypothesised that a gene-environment interaction might be an explanation for the observation (17). In the present analysis, however, we could not demonstrate any gene-risk factor interactions to associate with IMT.

In summary, we found that the associations of smoking and homocysteine on FMD, and the association of physical activity on CAC may be modulated by the *Cx37* polymorphism. The significance of these results needs to be confirmed in other populations.

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References

1. Gensini GF, Comeglio M, Colella A. Classical risk factors and emerging elements in the risk profile for coronary artery disease. Eur Heart J 1998;19(Suppl A):A53–61.
2. Marenberg ME, Risch N, Berkman LF, Floderus B, de Faire U. Genetic susceptibility to death from coronary heart disease in a study of twins. N Engl J Med 1994; 330:1041–6.
3. Yamada Y, Izawa H, Ichihara S, Takatsu F, Ishihara H, Hirayama H, et al. Prediction of the risk of myocardial infarction from polymorphisms in candidate genes. N Engl J Med 2002;347:1916–23.
4. Hirashiki A, Yamada Y, Murase Y, Suzuki Y, Kataoka H, Morimoto Y, et al. Association of gene polymorphisms with coronary artery disease in low- or high-risk subjects defined by conventional risk factors. J Am Coll Cardiol 2003;42:1429–37.
5. Boerma M, Forsberg L, Van Zeijl L, Morgenstern R, De Faire U, Lemne C, et al. A genetic polymorphism in connexin 37 as a prognostic marker for atherosclerotic plaque development. J Intern Med 1999;246:211–8.
6. Goodenough DA, Goliger JA, Paul DL. Connexins, connexons, and intercellular communication. Annu Rev Biochem 1996;65:475–502.
7. Yeh HI, Dupont E, Coppen S, Rothery S, Severs NJ. Gap junction localization and connexin expression in cytochemically identified endothelial cells of arterial tissue. J Histochem Cytochem 1997;45:539–50.
8. Wong CW, Christen T, Roth I, Chadjichristos CE, Derouette JP, Foglia BF, et al. Connexin37 protects against atherosclerosis by regulating monocyte adhesion. Nat Med 2006;12:950–4.
9. Yeh HI, Lai YJ, Chang HM, Ko YS, Severs NJ, Tsai CH. Multiple connexin expression in regenerating arterial endothelial gap junctions. Arterioscler Thromb Vasc Biol 2000;20:1753–62.
10. Yeh HI, Chang HM, Lu WW, Lee YN, Ko YS, Severs NJ, et al. Age-related alteration of gap junction distribution and connexin expression in rat aortic endothelium. J Histochem Cytochem 2000;48:1377–89.
11. Wong CW, Christen T, Pfenniger A, James RW, Kwak BR. Do allelic variants of the connexin37 1019 gene polymorphism differentially predict for coronary artery disease and myocardial infarction? Atherosclerosis 2007; 191:355–61.
12. Yeh HI, Chou Y, Liu HF, Chang SC, Tsai CH. Connexin37 gene polymorphism and coronary artery disease in Taiwan. Int J Cardiol 2001;81:251–5.
13. Listi F, Candore G, Lio D, Russo M, Colonna-Romano G, Caruso M, et al. Association between C1019T polymorphism of connexin37 and acute myocardial infarction: a study in patients from Sicily. Int J Cardiol 2005;102: 269–71.
14. Listi F, Candore G, Balistreri CR, Caruso M, Incalcaterra E, Hoffmann E, et al. Connexin37 1019 gene polymorphism in myocardial infarction patients and centenarians. Atherosclerosis 2007;191:460–1.
15. Jerrard-Dunne P, Markus HS, Steckel DA, Buehler A, von Kegler S, Sitzer M. Early carotid atherosclerosis and family history of vascular disease: specific effects on arterial sites have implications for genetic studies. Arterioscler Thromb Vasc Biol 2003;23:302–6.
16. Clarkson P, Celermajer DS, Powe AJ, Donald AE, Henry RM, Deanfield JE. Endothelium-dependent dilatation is impaired in young healthy subjects with a family history of premature coronary disease. Circulation 1997;96: 3378–83.

17. Collings A, Islam MS, Juonala M, Rontu R, Kähönen M, Hutri-Kähönen N, et al. Associations between connexin37 gene polymorphism and markers of subclinical atherosclerosis: the Cardiovascular Risk in Young Finns study. Atherosclerosis 2007;195:379–84.
18. Raitakari OT, Juonala M, Kähönen M, Taittonen L, Laitinen T, Mäki-Torkko N, et al. Cardiovascular risk factors in childhood and carotid artery intima-media thickness in adulthood: the Cardiovascular Risk in Young Finns Study. J Am Med Assoc 2003;290:2277–83.
19. Åkerblom HK, Viikari J, Uhari M, Räsänen L, Byckling T, Louhivuori K, et al. Atherosclerosis precursors in Finnish children and adolescents. I. General description of the cross-sectional study of 1980, and an account of the children's and families' state of health. Acta Paediatr Scand Suppl 1985;318:49–63.
20. Juonala M, Viikari JS, Hutri-Kähönen N, Pietikäinen M, Jokinen E, Taittonen L, et al. The 21-year follow-up of the Cardiovascular Risk in Young Finns Study: risk factor levels, secular trends and east-west difference. J Intern Med 2004;255:457–68.
21. Raitakari M, Mansikkaniemi K, Marniemi J, Viikari JS, Raitakari OT. Distribution and determinants of serum high-sensitive C-reactive protein in a population of young adults: the Cardiovascular Risk in Young Finns Study. J Intern Med 2005;258:428–34.
22. Juonala M, Viikari JS, Laitinen T, Marniemi J, Helenius H, Rönnemaa T, et al. Interrelations between brachial endothelial function and carotid intima-media thickness in young adults: the Cardiovascular Risk in Young Finns Study. Circulation 2004;110:2918–23.
23. Livak KJ. Allelic discrimination using fluorogenic probes and the 5′ nuclease assay. Genet Anal 1999;14:143–9.
24. Juonala M, Järvisalo MJ, Mäki-Torkko N, Kähönen M, Viikari JS, Raitakari OT. Risk factors identified in childhood and decreased carotid artery elasticity in adulthood: the Cardiovascular Risk in Young Finns Study. Circulation 2005;112:1486–93.
25. Rothman KJ. No adjustments are needed for multiple comparisons. Epidemiology 1990;1:43–6.
26. Tsuchiya M, Asada A, Kasahara E, Sato EF, Shindo M, Inoue M. Smoking a single cigarette rapidly reduces combined concentrations of nitrate and nitrite and concentrations of antioxidants in plasma. Circulation 2002; 105:1155–7.
27. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 1987;327:524–6.
28. Bilsborough W, Green DJ, Mamotte CD, van Bockxmeer FM, O'Driscoll GJ, Taylor RR. Endothelial nitric oxide synthase gene polymorphism, homocysteine, cholesterol and vascular endothelial function. Atherosclerosis 2003;169:131–8.
29. Woo KS, Chook P, Lolin YI, Cheung AS, Chan LT, Sun YY, et al. Hyperhomocyst(e)inemia is a risk factor for arterial endothelial dysfunction in humans. Circulation 1997;96:2542–4.
30. Kanani PM, Sinkey CA, Browning RL, Allaman M, Knapp HR, Haynes WG. Role of oxidant stress in endothelial dysfunction produced by experimental hyperhomocyst(e)inemia in humans. Circulation 1999;100:1161–8.
31. Tanaka H, Dinenno FA, Monahan KD, Clevenger CM, DeSouza CA, Seals DR. Aging, habitual exercise, and dynamic arterial compliance. Circulation 2000;102: 1270–5.
32. Comings DE, MacMurray JP. Molecular heterosis: a review. Mol Genet Metab 2000;71:19–31.

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ORIGINAL ARTICLE

Associations of methylenetetrahydrofolate reductase C677T polymorphism with markers of subclinical atherosclerosis: The Cardiovascular Risk in Young Finns Study

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Objective. To study whether the methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism or serum homocysteine concentration is associated with carotid artery intima media thickness (IMT), carotid artery compliance (CAC) or brachial artery flow mediated dilatation (FMD) in a healthy Finnish adult population. ***Methods.*** Cross-sectional data obtained in 2001 for the Cardiovascular Risk in Young Finns Study were used. Carotid artery IMT, CAC and brachial FMD were measured by ultrasound and serum homocysteine concentrations using a commercial immunoassay kit. We studied 1,440 subjects (aged 24–39 years). Genotyping was performed using the 5′ nuclease TaqMan assay. ***Results.*** Homocysteine values differed between genotypes in women and men (ANOVA, $p<0.001$ for both sex groups): the genotype raised values in the order of CC, CT, TT. There was a significant difference in CAC values between the MTHFR genotypes in men (ANOVA, $p=0.008$), and the CC genotype had the lowest values. In multivariate linear regression analysis adjusted for other major coronary risk factors (e.g. age, smoking, body mass index, systolic blood pressure, C-reactive protein), the association remained significant ($R^2=25.8$ %, beta=0.091; $p=0.02$). Homocysteine level was directly associated with CAC in the whole population ($R^2=18.0$ %, beta=0.012; $p=0.014$) and in women ($R^2=9.3$%, beta=0.02; $p=0.013$), but not in men ($R^2=15.2$ %, beta=0.004; $p=0.444$). We found no association between homocysteine level or the MTHFR polymorphism and carotid IMT or brachial artery FMD. ***Conclusions.*** The findings suggest that the MTHFR polymorphism does not influence IMT or FMD, but that the T allele may have an effect on CAC in men.

Keywords: Atherosclerosis; homocysteine; methylenetetrahydrofolate reductase; SNP

Introduction

Atherosclerosis is a multifactorial process that develops silently for decades before clinical manifestations appear. Elevated carotid artery intima media thickness (IMT) has been associated with cardiovascular risk factors [1] and has been shown to predict future cardiovascular events, including myocardial infarction and stroke [2,3]. Arterial elasticity has also been suggested to be an independent predictor for cardiovascular events in high-risk individuals [4,5], and is associated with several cardiovascular risk factors [6–8]. Elasticity can be assessed by measuring carotid artery compliance (CAC), which indicates the ability of the arterial wall to expand in response to pulse pressure [9]. Flow-mediated dilatation (FMD) of the brachial artery can be measured by ultrasound and is used to study early functional changes in the endothelium [10]. Impaired FMD reflects endothelial dysfunction, which may represent an early event in atherosclerosis [11].

Elevated levels of serum homocysteine have been reported to associate with an increased risk of coronary artery disease, stroke and peripheral vascular disease [12]. A mutation in the methylenetetrahydrofolate reductase (MTHFR) gene C677T has been found [13] and those individuals with the TT genotype have higher total homocysteine levels compared to the other genotypes [14,15]. Results regarding the association between the polymorphism and IMT are controversial, with both positive [16] and negative [17,18] results being reported. In a recent review, it was concluded that most studies have demonstrated elevated IMT in those with the TT genotype of the MTHFR polymorphism [19].

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In a previous article studying the correlation between plasma homocysteine levels and arterial stiffness of the brachial artery ($n=109$), the MTHFR polymorphism was not found to be a significant factor predicting arterial stiffness [20]. In another previous report where arterial stiffness (systemic arterial compliance) was studied in relation to the MTHFR C677T genotype, during folic acid supplementation (study group $n=41$) the arterial stiffness response was found to be independent of the MTHFR genotype [21]. To our knowledge, there are no previous publications on the relationship between the MTHFR polymorphism and CAC.

FMD in relation to the MTHFR polymorphism has been studied in two relatively small populations of healthy adults. In a study of 126 subjects, the MTHFR C677T genotype did not influence FMD [22]. In another study ($n=53$), the MTHFR 677T allele carriers did not differ significantly in their FMD levels compared to the CC genotype [23].

As earlier studies have been based on relatively small populations, our objective is to look for the MTHFR C667T polymorphism in a large population-based sample of healthy Finns and jointly to analyse whether there is an association between the polymorphism or serum homocysteine and measured early markers of atherosclerosis: carotid artery IMT, CAC and brachial artery FMD.

Material and methods

Subjects and clinical characteristics

The study population is a sample of 1,440 study subjects from the ongoing prospective cohort study entitled The Cardiovascular Risk in Young Finns. Descriptions of the study population have been published earlier [24–26]. Briefly, 4,320 randomly chosen children and adolescents of ages 3, 6, 9, 12, 15 and 18 years were invited to participate in the study in 1980; a total of 3,596 participated. In 2001, at the latest follow-up, 2,228 of the original subjects, now between 24 and 39 years of age, participated. For economic reasons, the study group was limited to 1,440 individuals randomly selected from all subjects with complete follow-up data. The participants signed a consent form and the study was approved by local ethics committees.

Participants filled in a standardized questionnaire assessing general cardiovascular risk factors. Body mass index (BMI) was calculated using height and weight data (BMI=kg/m^2). Blood pressure (BP) was measured with a random zero sphygmomanometer. The average of three measurements was used in the statistical analysis. Smoking habits were characterized as a two-valued variable: smokers were current or ex-smokers; non-smokers had never smoked. In 39 cases, values for smoking were not available.

Biochemical parameters

Fasting blood samples were collected for the analysis of C-reactive protein (CRP), glucose, insulin, homocysteine, serum lipids, apolipoprotein A-I (apoA-1) and apolipoprotein B (apoB). Analyses of the different parameters were conducted in duplicate. Serum total cholesterol, triglycerides and high-density lipoprotein (HDL) cholesterol analyses were done using standardized enzymatic methods, and apoA-I and apoB by immunoturbidimetry, as described previously [24,25]. The Friedewald formula was used to calculate the concentration of low-density lipoprotein (LDL) cholesterol. Sensitive CRP was measured with a latex turbidimetric immunoassay (Wako Chemicals GmbH, Nuess, Germany), the lower detection limit being 0.06 mg/L. Glucose was measured enzymatically (glucose dehydrogenase; Olympus Diagnostica GmbH, Hamburg, Germany). Microparticle enzyme immunoassay kits were used to measure insulin (Abbott Laboratories, Diagnostic Division, Dainabot) and homocysteine (Imx assay; Abbott Laboratories, Tokyo, Japan) [11].

MTHFR 677 C>T (Ala222Val) genotyping

Genomic DNA was extracted by BioRobot M48 Workstation in accordance with the manufacturer's instructions (Qiagen Inc., Hilden, Germany). DNA samples were genotyped by employing the 5′ nuclease assay in combination with specific fluorogenic TaqMan MGB probes, using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, Calif., USA). The nucleotide sequences of primers and allele-specific wild-type and variant probes, labelled with reporter dyes, were deduced from sequences deposited in the GenBank database and synthesized in conjugation with Applied Biosystems using the Assays-by-Design tool. PCR reactions containing genomic DNA, 1× Universal PCR Master Mix, 900 nM of each primer and 200 nM of each probe, were performed in 96-well plates in a total volume of 25 μL, in accordance with the standard protocol. The endpoint reading of the fluorescence generated from each probe during the PCR amplification was measured using the allelic discrimination analysis module, resulting in clear identification of three genotypes [27].

Carotid artery measurements

IMT of the left common carotid artery was measured using Sequoia 512 ultrasound mainframes (Acuson, Calif., USA) with 13.0 MHz linear array transducers. Details of the procedure have been described previously [11,24]. Briefly, a standard protocol was used to scan the posterior wall of the left carotid artery. A 5-second moving scan was recorded and stored in digital form for subsequent off-line analysis. A single reader, blinded to the subjects' details, analysed the images. A second analyser reanalysed 113 of the scans to assess reproducibility. The between-observer coefficient of variation was 5.2 %.

To assess CAC, the best-quality cardiac cycle was selected from the 5-second clip images. The common carotid diameter was measured at least twice in end-diastole and end-systole, respectively. The mean of the measurements was used as the end-diastolic and the end-systolic diameter. Ultrasound and concomitant brachial blood pressure measurements were used to calculate $CAC=([D_s-D_d]/D_d)/(P_s-P_d)$, where D_s is the systolic diameter, D_d the diastolic diameter, P_s the systolic blood pressure and P_d the diastolic blood pressure. The between-visit coefficient of variation was 2.7 % for diastolic carotid diameter and 16.3 % for CAC [7].

FMD was measured by ultrasound. The left brachial artery diameter was measured at rest and during reactive hyperaemia, which was induced by placing a tourniquet on the forearm and holding a pressure of 250 mmHg for 4.5 min. Arterial diameter was measured three times at three time-points (40, 60 and 80 s), and the maximum FMD was derived from the average of the three measurements at each time-point. The vessel diameter during reactive hyperaemia was expressed as relative to the resting scan (100 %). The between-visit coefficient of variation was 26 % for FMD [11].

Statistical analysis

Continuous variables between the MTHFR genotypes in men and women were compared by one way analysis of variance (ANOVA; Table I). Associations between selected atherosclerotic risk factors and measured parameters IMT, CAC, FMD and homocysteine were analysed using a multivariate linear regression model. Homocysteine was not normally distributed and was used as a dependent variable after a logarithmical transformation in statistical analyses (results showed as crude values). The variables used in each model were selected after stepwise analyses (data not shown), and as sex was a significant variable the analyses were conducted for the entire population as well as for men and women separately. The final linear regression models for IMT, CAC and FMD as dependent variables used the following covariates: sex, age, current smoking, BMI, MTHFR, systolic blood pressure, CRP, glucose, insulin, triglycerides, apoA-1, apoB and

Table I. Clinical characteristics and biochemical parameters of the study population according to methylenetetrahydrofolate reductase (MTHFR) gene 677C>T polymorphism genotype. The values are expressed as mean and standard deviation (SD).

	MTHFR genotype in women						MTHFR genotype in men					
	CC (n=419)		CT (n=264)		TT (n=37)		CC (n=416)		CT (n=263)		TT (n=41)	
Characteristic	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Age (years)	31.9	4.9	31.8	5.1	32.0	5.4	32.2	5.1	31.5	4.9	31.1	5.1
Body mass index (kg/m^2)	24.6	4.7	24.4	4.7	24.0	3.9	25.8	4.1	25.7	4.2	25.9	4.4
Systolic BP (mmHg)	116	12	116	13	114	11	129	13	130	15	130	11
Mean IMT (mm)	0.566	0.082	0.569	0.083	0.579	0.098	0.594	0.094	0.588	0.106	0.585	0.115
Maximal FMD (%)	8.77	4.43	9.12	4.61	8.65	4.29	7.11	4.25	7.11	4.07	7.63	4.03
CAC (%/10 mmHg)	2.26	0.79	2.27	0.79	2.41	0.83	*1.95	0.65	2.11	0.67	2.07	0.69
Total cholesterol (mmol/L)	5.11	0.95	5.05	0.92	5.15	1.02	5.27	1.02	5.16	1.00	5.34	0.90
LDL cholesterol (mmol/L)	3.17	0.77	3.13	0.78	3.25	0.86	3.42	0.96	3.36	0.89	3.48	0.73
HDL cholesterol (mmol/L)	1.40	0.30	1.38	0.29	1.36	0.28	1.18	0.29	1.14	0.26	1.18	0.23
Triglycerides (mmol/L)	1.18	0.82	1.17	0.60	1.20	0.44	1.49	0.93	1.48	0.85	1.52	0.95
C-reactive protein (mg/L)	2.3	4.1	2.1	4.7	2.7	4.7	1.6	3.1	1.7	4.6	1.2	2.1
Homocysteine (μmol/L)	**8.43	2.30	9.29	3.62	12.97	6.35	**10.04	2.27	10.93	3.85	19.06	8.62
Insulin (mU/L)	7.84	5.11	7.98	5.91	7.32	4.93	7.29	4.62	7.51	4.71	7.51	4.99
Glucose (mmol/L)	4.9	0.7	4.9	0.8	4.9	0.4	5.2	0.5	5.3	1.3	5.2	0.4
Apolipoprotein A-1 (g/L)	1.58	0.27	1.57	0.26	1.55	0.24	1.41	0.21	1.39	0.19	1.43	0.18
Apolipoprotein B (g/L)	1.01	0.23	1.00	0.24	1.02	0.25	1.13	0.27	1.12	0.26	1.14	0.24

n=number of subjects; BP=blood pressure; IMT=carotid artery intima media thickness; FMD=brachial artery flow-mediated dilatation; CAC=Carotid artery compliance; LDL=low density lipoprotein; HDL=high density lipoprotein. Statistics: Analysis of variance between genotypes *p=0.008, **p<0.001. There were no statistically significant genotype differences in the other variables given in the table.

homocysteine. As lipoproteins HDL and apoA-1 as well as LDL and apoB, are dependent on each other, we ran two linear regression models; one with apoA-1 and apoB, the other with HDL and LDL (data not shown). Results are shown as regression coefficients and standard errors. *P*-values of <0.05 were considered statistically significant. Analyses were performed using the statistical software SPSS version 14.0 (SPSS Inc., Chicago, Ill., USA).

Results

The frequencies for the MTHFR C677T genotype were: 835 (58 %), 527 (36.6 %) and 78 (5.4 %) for CC, CT and TT genotypes, respectively. The genotype distribution was in Hardy-Weinberg equilibrium.

Comparison between the MTHFR genotypes showed a significant difference in homocysteine values of men ($p<0.001$) and women ($p<0.001$), where the TT genotype had a higher mean value compared to the other genotypes (Table I). There was a significant difference in CAC between the genotypes in men ($p=0.008$), the CC genotype having the lowest value. Other ultrasound variables did not differ significantly between genotypes. Descriptive characteristics of the study population are shown in Table I.

Associations between risk factors and homocysteine

After initial analyses and stepwise regression modelling, the variables for the final linear models were selected as shown in Tables II–V. In the final multivariate model, factors associating with homocysteine level in all subjects included sex (beta=1.615; $p<0.001$), with men having higher values, as well as MTHFR genotype (beta=2.098; $p<0.001$), apoA-1 (beta=−1.754; $p<0.001$) and glucose (beta=−0.284; $p=0.006$), as demonstrated in Table II. In sex-stratified analysis, the MTHFR genotype remained significant for both sexes (beta=1.483; $p<0.001$ and beta=2.707; $p<0.001$ for women and men, respectively). The genotype elevated homocysteine values in the order CC, CT and TT. ApoA-1 remained a significant correlate of homocysteine values in women (beta=−1.819; $p<0.001$) and glucose in men (beta=−0.260; $p<0.001$). For a model in which apoA-1 and apoB were replaced with HDL and LDL, the adjusted R^2 values were 17.6 % ($p<0.001$), 8.6 % ($p<0.001$) and 15.8 % ($p<0.001$) for all subjects, women and men, respectively.

Associations between risk factors and carotid artery IMT

Table III gives associations between risk factors and IMT. The adjusted R^2 for the model was 12.8 % ($p<0.001$). In all subjects, the significant predictors of IMT were age (beta=0.005; $p<0.001$), BMI (beta=0.002; $p<0.001$) and systolic BP (beta=0.000; $p=0.044$). Age remained a significant correlate in the sex stratified analysis, but there was a different trend in other factors between men and women. In women ($R^2=11.9$ %, $p<0.001$), triglycerides (beta=0.012; $p=0.023$) and apoA-1 (beta=−0.035; $p=0.004$) became significant. In men ($R^2=13.3$ %, $p<0.001$ for the model), BMI (beta=0.005; $p<0.001$) remained significant in addition to CRP (beta=0.002; $p=0.048$), glucose (beta=0.011; $p=0.046$) and insulin (beta=−0.003; $p=0.002$) becoming significant correlates of IMT. The MTHFR genotype or homocysteine did not associate with IMT in either the whole population or in the groups divided by sex. In the

Table II. Multivariate linear regression model for the relationship between atherosclerosis risk factors and serum homocysteine (μmol/L) (dependent) in the whole population and divided by gender.

	All subjects			Women			Men		
Explanatory variable	Beta	SE	*p*-value	Beta	SE	*p*-value	Beta	SE	*p*-value
Sex (female/male)	1.615	0.214	<0.001	–	–	–	–	–	–
Age (years)	0.007	0.020	0.268	0.026	0.025	0.359	−0.012	0.031	0.792
Current smoking (no/yes)	0.209	0.190	0.268	0.109	0.242	0.321	0.364	0.294	0.547
Body mass index (kg/m^2)	0.013	0.027	0.548	0.007	0.032	0.526	0.019	0.045	0.939
MTHFR (CC>CT>TT)	2.098	0.157	<0.001	1.483	0.202	<0.001	2.707	0.239	<0.001
C-reactive protein (mg/L)	−0.022	0.024	0.444	−0.023	0.029	0.475	−0.012	0.040	0.971
Glucose (mmol/L)	−0.284	0.119	0.006	−0.326	0.166	0.050	−0.260	0.174	0.047
Insulin (mU/L)	0.005	0.023	0.548	0.029	0.026	0.386	−0.032	0.040	0.939
Triglycerides (mmol/L)	−0.008	0.151	0.270	−0.241	0.209	0.059	0.195	0.220	0.629
Apolipoprotein A-1 (g/L)	−1.754	0.409	<0.001	−1.819	0.485	<0.001	−1.415	0.729	0.113
Apolipoprotein B (g/L)	0.459	0.500	0.231	0.654	0.658	0.226	0.384	0.752	0.535

Beta=unstandardized regression coefficient; SE=standard error; MTHFR=methylenetetrahydrofolate reductase. Statistics: Adjusted $R^2=18$ % ($p<0.001$) for the total population model and 9.3 % ($p<0.001$) and 15.8 % ($p<0.001$) for women and men, respectively; model with crude values. *P*-values are from a model where homocysteine was logarithmically transformed due to a skewed distribution.

Table III. Multivariate linear regression model for the relationship between atherosclerosis risk factors and mean carotid artery intima media thickness (mm) (dependent) in the whole population and divided by gender.

	All subjects			Women			Men		
Explanatory variable	Beta	SE	*p*-value	Beta	SE	*p*-value	Beta	SE	*p*-value
Sex (female/male)	0.008	0.006	0.170	–	–	–	–	–	–
Age (years)	0.005	0.000	<0.001	0.005	0.001	<0.001	0.004	0.001	<0.001
Current smoking (no/yes)	0.007	0.005	0.155	0.005	0.006	0.360	0.006	0.007	0.391
Body mass index (kg/m^2)	0.002	0.001	0.001	0.001	0.001	0.378	0.005	0.001	<0.001
MTHFR (CC>CT>TT)	0.001	0.004	0.854	0.006	0.005	0.235	−0.006	0.006	0.369
Systolic blood pressure (mmHg)	<0.001	0.000	0.044	<0.001	0.000	0.135	<0.001	0.000	0.176
C-reactive protein (mg/L)	0.001	0.001	0.206	4.2E-005	0.001	0.954	0.002	0.001	0.048
Glucose (mmol/L)	0.006	0.003	0.066	0.001	0.004	0.803	0.011	0.005	0.046
Insulin (mU/L)	−0.001	0.001	0.106	<0.001	0.001	0.570	−0.003	0.001	0.002
Triglycerides (mmol/L)	0.004	0.004	0.345	0.012	0.005	0.023	−0.003	0.005	0.617
Apolipoprotein A-1 (g/L)	−0.015	0.010	0.133	−0.035	0.012	0.004	0.013	0.018	0.472
Apolipoprotein B (g/L)	0.003	0.012	0.804	−0.014	0.016	0.402	0.024	0.019	0.205
Homocysteine (μmol/L)	<0.001	0.001	0.670	−0.001	0.001	0.173	0.001	0.001	0.584

Abbreviations: Beta=unstandardized regression coefficient; SE=standard error; MTHFR=methylenetetrahydrofolate reductase. Statistics: Adjusted R^2=12.8 % ($p<0.001$) for the total population model and 1.9 % ($p<0.001$) and 13.3 % ($p<0.001$) for women and men, respectively.

model which employed HDL and LDL cholesterol instead of apoA-1 and apoB, the R^2 was 12.7 %, $p<0.001$ for the whole population and 11.0 %, $p<0.001$, and 13.4 %, $p<0.001$, for women and men, respectively. Neither MTHFR genotype nor homocysteine was a significant predictor of IMT in this model.

Associations between risk factors and carotid artery CAC

Factors associating with CAC in the whole population (R^2=27.5%, $p<0.001$) included age (beta=−0.034; $p<0.001$), systolic BP (beta=−0.020; $p<0.001$), insulin (beta=−0.011; p=0.010), apoB (beta=−0.273; p=0.003) and homocysteine (beta=0.012; p=0.014) (Table IV). Stratified by sex, the model's R^2 values were 26.1 % ($p<0.001$) and 25.8 % ($p<0.001$), for women and men, respectively. Age and systolic BP remained significant in both groups and apoA-1 and homocysteine in women. In men, however, systolic BP and insulin remained significant, and the MTHFR genotype was a significant predictor of CAC (beta=0.091; p=0.02). In men, the genotype was associated with increasing CAC values in the order of CC, CT and TT. Replacing apoA-1 and apoB with HDL and LDL cholesterol values, the R^2 for the total population was

Table IV. Multivariate linear regression model for the relationship between atherosclerosis risk factors and carotid artery compliance (%/10 mmHg) (dependent) in the whole population and divided by gender.

	All subjects			Women			Men		
Explanatory variable	Beta	SE	*p*-value	Beta	SE	*p*-value	Beta	SE	*p*-value
Sex (female/male)	−0.007	0.043	0.866	–	–	–	–	–	–
Age (years)	−0.034	0.004	<0.001	−0.037	0.005	<0.001	−0.036	0.005	<0.001
Current smoking (no/yes)	0.046	0.034	0.180	0.039	0.052	0.451	0.048	0.044	0.277
Body mass index (kg/m^2)	−0.002	0.005	0.627	0.001	0.007	0.937	−0.003	0.007	0.640
MTHFR (CC>CT>TT)	0.038	0.030	0.210	−0.010	0.045	0.832	0.091	0.039	0.020
Systolic blood pressure (mmHg)	−0.020	0.001	<0.001	−0.025	0.002	<0.001	−0.017	0.002	<0.001
C-reactive protein (mg/L)	−0.004	0.004	0.390	−0.004	0.006	0.552	−0.002	0.006	0.748
Glucose (mmol/L)	0.031	0.025	0.202	0.037	0.036	0.380	0.022	0.033	0.498
Insulin (mU/L)	−0.011	0.004	0.010	−0.009	0.006	0.115	−0.014	0.006	0.024
Triglycerides (mmol/L)	0.035	0.027	0.202	0.045	0.045	0.314	0.025	0.033	0.446
Apolipoprotein A-1 (g/L)	−0.035	0.075	0.634	−0.102	0.106	0.334	0.149	0.110	0.178
Apolipoprotein B (g/L)	−0.273	0.090	0.003	−0.452	0.141	0.001	−0.099	0.114	0.386
Homocysteine (μmol/L)	0.012	0.005	0.014	0.020	0.008	0.013	0.004	0.006	0.444

Beta=unstandardized regression coefficient; SE=standard error; MTHFR=methylenetetrahydrofolate reductase. Statistics: Adjusted R^2=27.5 % ($p<0.001$) for the total population model and 26.1 % ($p<0.001$) and 25.8 %, ($p<0.001$) for women and men, respectively.

Table V. Multivariate linear regression model for the relationship between atherosclerosis risk factors and maximal brachial artery flow-mediated dilatation (%) (dependent) in the whole population and divided by gender.

	All subjects			Women			Men		
Explanatory variable	Beta	SE	*p*-value	Beta	SE	*p*-value	Beta	SE	*p*-value
Sex (female/male)	−1.485	0.310	<0.001	–	–	–	–	–	–
Age (years)	0.017	0.025	0.508	0.025	0.036	0.476	0.005	0.036	0.882
Current smoking (no/yes)	0.243	0.245	0.322	0.650	0.351	0.064	−0.201	0.345	0.559
Body mass index (kg/m^2)	0.155	0.036	<0.001	0.169	0.048	<0.001	0.127	0.056	0.023
MTHFR (CC>CT>TT)	0.240	0.215	0.265	0.329	0.304	0.279	0.074	0.306	0.809
Systolic blood pressure (mmHg)	−0.019	0.010	0.069	−0.011	0.016	0.478	−0.026	0.014	0.065
C-reactive protein (mg/L)	0.004	0.031	0.892	−0.019	0.043	0.656	0.036	0.046	0.425
Glucose (mmol/L)	−0.227	0.175	0.196	−0.181	0.239	0.448	−0.289	0.264	0.275
Insulin (mU/L)	0.008	0.030	0.789	−0.016	0.038	0.672	0.056	0.060	0.258
Triglycerides (mmol/L)	0.111	0.190	0.559	0.185	0.297	0.534	0.033	0.250	0.894
Apolipoprotein A-1 (g/L)	1.037	0.538	0.054	1.059	0.720	0.142	1.115	0.869	0.200
Apolipoprotein B (g/L)	0.234	0.642	0.715	0.265	0.939	0.778	0.302	0.886	0.733
Homocysteine (μmol/L)	−0.006	0.034	0.852	−0.053	0.054	0.329	0.035	0.044	0.435

Beta=unstandardized regression coefficient; SE=standard error; MTHFR=methylenetetrahydrofolate reductase. Statistics: Adjusted R^2=5.8 % ($p<0.001$) for the total population model and 2.5 % ($p=0.004$) and 1.1 % ($p=0.09$) for women and men, respectively.

27.1 % (p<0.001), and 25.4 % ($p<0.001$) and 26.1 % ($p<0.001$), for women and men, respectively.

Associations between risk factors and carotid artery FMD

Results of the multivariate linear model for risk factors associated with FMD are given in Table V. The R^2 was 5.8 % ($p<0.001$) for the whole population and 2.5 % ($p=0.004$) and 1.1 % ($p=0.09$) for women and men, respectively. Sex and BMI were significant factors in the whole population: men had lower FMD values (beta=−1.485; $p<0.001$) and BMI was directly related to FMD (beta=0.155; $p<0.001$). In sex-stratified analysis, BMI was the only significant correlate in both women (beta=0.169; $p<0.001$) and men (beta=0.127; $p=0.023$). MTHFR genotype and homocysteine were not related to FMD.

Discussion

In the present study, we found no association between homocysteine or the MTHFR C677T polymorphism and carotid artery IMT or brachial artery FMD in a population-based sample of young Finns. There was an expected difference in homocysteine values between the MTHFR genotype groups in both sexes, the homocysteine values increasing in the order of CC, CT and TT. Homocysteine was directly associated with CAC in the whole population and remained a significant predictor of CAC in women. A novel finding was that the MTHFR polymorphism was a significant predictor of CAC in men, the CC genotype having the lowest values. At the same time, the CC homozygous men had the lowest homocysteine values and as there was no significant association in the linear model between homocysteine and CAC, this may indicate that the MTHFR to CAC association is independent of serum homocysteine values.

Homocysteine, MTHFR polymorphism and IMT

Elevated homocysteine values have been associated with developing atherosclerosis, measured by increased values of IMT [17,18]. In this study, however, we did not find a significant association between homocysteine levels and IMT. In a recent review, the conclusion of eight (independent) studies was that the association between total homocysteine and IMT is weak or absent in the general population [19], and the results of the present study support these previous findings. Furthermore, we did not specifically look for an association between hyperhomocysteinaemia and IMT, as in our data only 5.6 % of the population could be considered to have high total homocysteine, i.e. values above 15 μmol/L [28].

There is a report associating the MTHFR TT genotype with an increased IMT in an elderly Japanese population [16], and it has been found to be a risk factor for carotid stenosis in Japanese women [29]. Also, the polymorphism has been associated with IMT in a French male population [30] and reported to be a risk factor for IMT in healthy elderly Italian women [31]. However, a number of studies have come to the opposite conclusion. In a Canadian population of various ethnic groups, the MTHFR TT genotype was not associated with IMT [18]. In Italian non-insulin-dependent

diabetes mellitus patients, the MTHFR polymorphism did not predict higher IMT values [32]; the same results have been reported in Australian and Dutch populations [17,33]. In the studies where a positive association was found, the study populations were slightly older compared to our population. Age of the population plays a significant role; in our study, age was the most significant factor for IMT.

In light of many previous studies, one might draw the conclusion that the TT genotype may be a factor worth considering in the pathogenesis of coronary artery disease. However, a recent meta-analysis on the MTHFR C677T polymorphism and coronary heart disease did not find evidence to support such an association [34]. On the other hand, another meta-analysis reported a greater risk for stroke in the TT genotype versus CC [35]. These findings do not contradict, as the phenotype of atherosclerosis during the course of the disease is affected by cumulative effects of many risks, including various genetic factors.

Homocysteine, MTHFR polymorphism and CAC

The MTHFR polymorphism had a significant association with CAC in men. It seems that the CC genotype in men has the lowest CAC values and the lowest homocysteine values. This may seem contradictory. Interestingly, however, in the linear model the association of homocysteine with CAC was not significant in the male population. This finding suggests that the association between the MTHFR genotype and CAC may not be dependent on the homocysteine level. Arterial stiffness has been shown to predict cardiovascular mortality in end-stage renal disease [5] or primary coronary events in hypertensive patients [4]. In previous studies, homocysteine has been associated with arterial stiffness. Homocysteine was a main factor influencing lower limb pulse wave velocity in a population of endstage renal disease patients [5]. In another study, homocysteine levels correlated strongly with aortic stiffness in hypertensive patients [36]. In two studies in which homocysteine levels were raised by a methionine load, the first found no association with arterial stiffness (measured by pulse wave) [37], whereas the second study described a homocysteine-related increase in arterial stiffness in the central arterial circulation [38]. In light of these findings, it could be hypothesized that the MTHFR TT genotype, which is associated with higher homocysteine levels, would associate with CAC or arterial stiffness, but then an association with homocysteine would also be expected. Our finding was the opposite of those mentioned above, i.e. higher homocysteine values associated with better elasticity of the carotid artery in the present population. The reason for this is not clear, and we do not have an explanation for the possible mechanism behind it. There may be a difference in the study material: our population is healthy and does not suffer from any chronic illness, and also generally did not have homocysteine values considered higher than normal. There was a trend for men with higher homocysteine values and in the TT genotype the difference was more pronounced. The TT genotype groups are quite small, and a few higher values can raise the mean value. As the result suggests the association to be independent of homocysteine values, we do not consider this a significant fact. One previous study also found a similar controversial result [39]. In this study, associations between MTHFR genotypes and the number of gaps in the internal elastic lamina (IEL) were studied in arterial walls. The TT genotype carriers, who have higher homocysteine values, had fewer defects in the IEL compared to the CC group; this was an unexpected result. Homocysteine values in blood do not necessarily compare with the concentrations in the vascular wall, in or between cells. It has also been suggested that cells may respond in different ways to changes in the levels of intracellular and extracellular homocysteine [40]. So, it may be that the effects of homocysteine on the vascular wall in the pathogenesis of atherosclerosis are a result of a more complex mechanism and not necessarily a straightforward cause of high circulatory homocysteine. Furthermore, there may be other factors that influence homocysteine values or vessel elasticity, such as environmental factors, other genetic polymorphisms, gene-environment or gene–gene interactions. As an unexpected result, however, we cannot rule out the possibility of a chance finding.

The present results suggest that the association between MTHFR and CAC is independent of homocysteine levels. There are only a few studies that have examined the MTHFR polymorphism in relation to arterial stiffness. Tsai et al. found no association between the MTHFR T allele and arterial stiffness in hemodialysis patients in Taiwan. The study population was divided according to the homocysteine values, and there was a significant correlation with homocysteine and arterial stiffness [20], but the T allele frequencies in the groups did not differ significantly. In an Australian paper studying the effect of folate supplementation on blood pressure and large artery stiffness in healthy or slightly hypertensive patients, the responses of these parameters were found to be independent of MTHFR genotype [21]. To our knowledge, the

results of the present study are the first reporting of an association with MTHFR genotype and CAC.

Limitations

The study population was fairly young, ages between 24 and 39 years, and in such an age group of healthy people one would not expect to find clear manifestations of atherosclerosis. Age is a significant predictive factor for vascular parameters. However, the study was on subclinical atherosclerosis and we wanted to see if subtle changes could be seen in our young adult population. The novel results we found were for the TT genotype, and only 5.4 % (n=78) of the study population carried this genotype. This is in line with other Finnish populations. The small size of the subgroup gives less credibility to the results.

In conclusion, we found no association between the MTHFR C667T polymorphism or homocysteine and IMT or FMD in a Finnish population of healthy men and women, but the T allele did have an independent association with higher CAC, a marker of arterial elasticity, in men. More studies are required to determine whether this result can be duplicated with other populations and to further investigate the finding in order to explain the possible underlying mechanism.

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References

[1] Heiss G, Sharrett AR, Barnes R, Chambless LE, Szklo M, Alzola C. Carotid atherosclerosis measured by B-mode ultrasound in populations: associations with cardiovascular risk factors in the ARIC study. Am J Epidemiol 1991;134:250–6.

[2] O'Leary DH, Polak JF, Kronmal RA, Manolio TA, Burke GL, Wolfson SK, Jr.. Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular Health Study Collaborative Research Group. N Engl J Med 1999;340:14–22.

[3] Chambless LE, Folsom AR, Clegg LX, et al. Carotid wall thickness is predictive of incident clinical stroke: The Atherosclerosis Risk in Communities (ARIC) study. Am J Epidemiol 2000;151:478–87.

[4] Boutouyrie P, Tropeano AI, Asmar R, et al. Aortic stiffness is an independent predictor of primary coronary events in hypertensive patients: A longitudinal study. Hypertension 2002;39:10–15.

[5] Blacher J, Pannier B, Guerin AP, Marchais SJ, Safar ME, London GM. Carotid arterial stiffness as a predictor of cardiovascular and all-cause mortality in end-stage renal disease. Hypertension 1998;32:570–4.

[6] Tounian P, Aggoun Y, Dubern B, et al. Presence of increased stiffness of the common carotid artery and endothelial dysfunction in severely obese children: a prospective study. Lancet 2001;358:1400–4.

[7] Juonala M, Järvisalo MJ, Mäki-Torkko N, Kähönen M, Viikari JS, Raitakari OT. Risk factors identified in childhood and decreased carotid artery elasticity in adulthood: The Cardiovascular Risk in Young Finns Study. Circulation 2005;112:1486–93.

[8] Ferreira I, Henry RM, Twisk JW, van Mechelen W, Kemper HC, Stehouwer CD. The metabolic syndrome, cardiopulmonary fitness, and subcutaneous trunk fat as independent determinants of arterial stiffness: The Amsterdam Growth and Health Longitudinal Study. Arch Intern Med 2005;165:875–82.

[9] Riley WA, Barnes RW, Evans GW, Burke GL. Ultrasonic measurement of the elastic modulus of the common carotid artery. The Atherosclerosis Risk in Communities (ARIC) Study. Stroke 1992;23:952–6.

[10] Celermajer DS, Sorensen KE, Gooch VM, et al. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. Lancet 1992;340:1111–15.

[11] Juonala M, Viikari JS, Laitinen T, et al. Interrelations between brachial endothelial function and carotid intima-media thickness in young adults: The Cardiovascular Risk in Young Finns Study. Circulation 2004;110:2918–23.

[12] Homocysteine and risk of ischemic heart disease and stroke: A meta-analysis. J Am Med Assoc 2002;288:2015–22.

[13] Kang SS, Wong PW, Zhou JM, et al. Thermolabile methylenetetrahydrofolate reductase in patients with coronary artery disease. Metabolism 1988;37:611–13.

[14] Klerk M, Verhoef P, Clarke R, Blom HJ, Kok FJ, Schouten EG. MTHFR 677C–>T polymorphism and risk of coronary heart disease: A meta-analysis. J Am Med Assoc 2002;288: 2023–31.

[15] Brattström L, Wilcken DE, Ohrvik J, Brudin L. Common methylenetetrahydrofolate reductase gene mutation leads to hyperhomocysteinemia but not to vascular disease: The result of a meta-analysis. Circulation 1998;98:2520–6.

[16] Kawamoto R, Kohara K, Tabara Y, et al. An association of 5,10-methylenetetrahydrofolate reductase (MTHFR) gene polymorphism and common carotid atherosclerosis. J Hum Genet 2001;46:506–10.

[17] McQuillan BM, Beilby JP, Nidorf M, Thompson PL, Hung J. Hyperhomocysteinemia but not the C677T mutation of methylenetetrahydrofolate reductase is an independent risk determinant of carotid wall thickening. The Perth Carotid Ultrasound Disease Assessment Study (CUDAS). Circulation 1999;99:2383–8.

[18] Kelemen LE, Anand SS, Hegele RA, et al. Associations of plasma homocysteine and the methylenetetrahydrofolate

reductase C677T polymorphism with carotid intima media thickness among South Asian, Chinese and European Canadians. Atherosclerosis 2004;176:361–70.

[19] Durga J, Verhoef P, Bots ML, Schouten E. Homocysteine and carotid intima-media thickness: A critical appraisal of the evidence. Atherosclerosis 2004;176:1–19.

[20] Tsai JC, Kuo HT, Chiu YW, et al. Correlation of plasma homocysteine level with arterial stiffness and pulse pressure in hemodialysis patients. Atherosclerosis 2005;182:121–7.

[21] Williams C, Kingwell BA, Burke K, McPherson J, Dart AM. Folic acid supplementation for 3 wk reduces pulse pressure and large artery stiffness independent of MTHFR genotype. Am J Clin Nutr 2005;82:26–31.

[22] Pullin CH, Wilson JF, Ashfield-Watt PA, et al. Influence of methylenetetrahydrofolate reductase genotype, exercise and other risk factors on endothelial function in healthy individuals. Clin Sci (Lond) 2002;102:45–50.

[23] Imamura A, Okumura K, Matsui H, et al. Endothelial nitric oxide synthase and methylenetetrahydrofolate reductase gene polymorphisms are associated with endothelial dysfunction in young, healthy men. Can J Cardiol 2004;20:1229–34.

[24] Raitakari OT, Juonala M, Kähönen M, et al. Cardiovascular risk factors in childhood and carotid artery intima-media thickness in adulthood: The Cardiovascular Risk in Young Finns Study. J Am Med Assoc 2003;290:2277–83.

[25] Juonala M, Viikari JS, Hutri-Kähönen N, et al. The 21-year follow-up of the Cardiovascular Risk in Young Finns Study: Risk factor levels, secular trends and east–west difference. J Intern Med 2004;255:457–68.

[26] Åkerblom HK, Viikari J, Uhari M, et al. Atherosclerosis precursors in Finnish children and adolescents. I. General description of the cross-sectional study of 1980, and an account of the children's and families' state of health. Acta Paediatr Scand Suppl 1985;318:49–63.

[27] Livak KJ. Allelic discrimination using fluorogenic probes and the 5′ nuclease assay. Genet Anal 1999;14:143–9.

[28] Ueland PM, Refsum H, Stabler SP, Malinow MR, Andersson A, Allen RH. Total homocysteine in plasma or serum: Methods and clinical applications. Clin Chem 1993;39: 1764–79.

[29] Inamoto N, Katsuya T, Kokubo Y, et al. Association of methylenetetrahydrofolate reductase gene polymorphism with carotid atherosclerosis depending on smoking status in a Japanese general population. Stroke 2003;34:1628–33.

[30] Pallaud C, Sass C, Zannad F, Siest G, Visvikis S. APOC3, CETP, fibrinogen, and MTHFR are genetic determinants of carotid intima-media thickness in healthy men (the Stanislas cohort). Clin Genet 2001;59:316–24.

[31] Passaro A, Vanini A, Calzoni F, et al. Plasma homocysteine, methylenetetrahydrofolate reductase mutation and carotid damage in elderly healthy women. Atherosclerosis 2001;157: 175–80.

[32] Mazza A, Motti C, Nulli A, et al. Serum homocysteine, MTHFR gene polymorphism, and carotid intimal-medial thickness in NIDDM subjects. J Thromb Thrombolysis 1999;8:207–12.

[33] Durga J, Bots ML, Schouten EG, Kok FJ, Verhoef P. Low concentrations of folate, not hyperhomocysteinemia, are associated with carotid intima-media thickness. Atherosclerosis 2005;179:285–92.

[34] Lewis SJ, Ebrahim S, Davey Smith G. Meta-analysis of MTHFR 677C–>T polymorphism and coronary heart disease: Does totality of evidence support causal role for homocysteine and preventive potential of folate? Br Med J 2005;331:1053.

[35] Casas JP, Bautista LE, Smeeth L, Sharma P, Hingorani AD. Homocysteine and stroke: Evidence on a causal link from Mendelian randomisation. Lancet 2005;365:224–32.

[36] Bortolotto LA, Safar ME, Billaud E, et al. Plasma homocysteine, aortic stiffness, and renal function in hypertensive patients. Hypertension 1999;34:837–42.

[37] Wilkinson IB, Megson IL, MacCallum T, et al. Acute methionine loading does not alter arterial stiffness in humans. J Cardiovasc Pharmacol 2001;37:1–5.

[38] Nestel PJ, Chronopoulos A, Cehun M. Arterial stiffness is rapidly induced by raising the plasma homocysteine concentration with methionine. Atherosclerosis 2003;171:83–6.

[39] Hämelahti P, Järvinen O, Sisto T, et al. Methylenetetrahydrofolate reductase gene C677T mutation is related to the defects in the internal elastic lamina of the artery wall. Eur J Clin Invest 2002;32:869–73.

[40] Hultberg B, Andersson A, Isaksson A. Higher export rate of homocysteine in a human endothelial cell line than in other human cell lines. Biochim Biophys Acta 1998;1448:61–9.