MENG FAN

Role of CYBA Gene Polymorphisms in Atherosclerosis

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ACADEMIC DISSERTATION
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LIST OF ORIGINAL COMMUNICATIONS

The thesis is based on the following original communications, which are referred to in the text by their Roman numerals (I-IV).

I Fan M, Raitakari OT, Kähönen M, Juonala M, Hutri-Kähönen N, Marniemi J, Rontu R, Pörsti I, Viikari J, Lehtimäki T. CYBA C242T gene polymorphism and flow-mediated vasodilation in a population of young adults: the Cardiovascular Risk in Young Finns Study. J Hypertens. 2007;25: 1381–1387.

II Fan M, Raitakari OT, Kähönen M, Juonala M, Hutri-Kähönen N, Pörsti I, Viikari J, Lehtimäki T. The association between cigarette smoking and carotid intima-media thickness is influenced by the -930$^{A/G}$ CYBA gene polymorphism: the Cardiovascular Risk in Young Finns Study. Am J Hypertens. 2009; 22:281–287.

III Fan M, Raitakari OT, Kähönen M, Juonala M, Hutri-Kähönen N, Marniemi J, Viikari J, Lehtimäki T. CYBA C242T gene polymorphism is associated with the level of C-reactive protein in a population of young adults: the Cardiovascular Risk in Young Finns Study (manuscript submitted for publication).

IV Fan M, Kähönen M, Rontu R, Lehtinen R, Viik J, Niemi M, Nieminen T, Niemelä K, Pörsti I, Kööbi T, Turjanmaa V, Lehtimäki T. The p22phox C242T gene polymorphism is associated with a reduced risk of angiographically verified coronary artery disease in a high-risk Finnish Caucasian population. The Finnish Cardiovascular Study. Am Heart J. 2006;152:538–542.

ABBREVIATIONS

ANCOVA, analysis of covariance

AngII, angiotensin II

ANOVA, analysis of variance

AP-1, activator protein-1

BMI, body mass index

BP, blood pressure

CAD, coronary artery disease

hsCRP, Highly sensitive C-reactive protein

CYBA gene, cytochrome-β-245, α-polypeptide

DNA, deoxyribonucleic acid

EDHF, endothelium-derived hyperpolarizing factor

ERK, extracellular signal regulated kinase

FMD, flow-mediated dilatation

GPx, glutathione peroxidase

GSH peroxides, glutathione peroxides

H_2O_2, hydrogen peroxide

HDL-C, high-density lipoprotein cholesterol

HOCl, hypochlorous acid

ICAM-1, intercellular adhesion molecule-1

IKK, IκB kinase

IL, interleukin, e.g. interleukin-1

IMT, intima-media thickness

IκB, inhibitor of κB

JNK, c-jun N-terminal kinase

LDL-C, low-density lipoprotein cholesterol

LPS, lipopolysaccharide

MAPK, mitogen-activated protein kinase

MET, metabolic equivalent index

MPO, myeloperoxidase

mRNA, messenger RNA

NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase

NENO, NF-κB essential modulator

NF-κB, nuclear factor-kappa B

NIK, NF-κB inducing kinase

NO, nitric oxide

NOS, nitric oxide synthase(s)

$^{\cdot}O_2^-$, superoxide anion

$^1O_2$, singlet oxygen

O_3, ozone

$OH^{\cdot}$, hydroxyl radical

ONOO-, peroxynitrite

OR, odds ratio

PCR, polymerase chain reaction

PDGF, platelet-derived growth factor

PDGFR, platelet-derived growth factor receptor

PKG, cGMP-dependent protein kinase

Ref-1, nuclear redox factor-1

ROS, reactive oxygen species

SD, standard deviation

SEM, standard error

SHR, spontaneously hypertensive rats

SMC, smooth muscle cell

SNP, single nucleotide polymorphism

SOD, superoxide dismutase

TLR, toll-like receptor

TNF, tumour necrosis factor

TRAF6, the TNF receptor-associated factor

VCAM-1, vascular cell adhesion molecule-1

WKY, wistar kyoto rats

XO, xanthine oxidases

ABSTRACT

Background: Oxidative stress is a shared feature of different phases of atherosclerosis leading through an unfavourable risk factor profile and subclinical vascular changes to the clinical manifestations of cardiovascular disease. Not only does it play a role in a response to environmental challenge, but it may also have an effect on the process of atherosclerosis. One of the major sources of reactive oxygen species (ROS) is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which consists of several membrane-bound and cytosolic proteins. The p22phox encoded by the CYBA gene is a component of NADPH oxidase and the most essential part of its enzyme activity. Several allelic variants have been identified in the CYBA gene, and the functional effect of the C242T (rs4673), the -930 $^{A/G}$ (rs9932581) and the A640G (rs1049255) polymorphisms has been reported. However, the findings related to the associations of these polymorphisms with subclinical and clinical traits of cardiovascular disease have been conflicting.

Aims: The study investigated the possible impact of CYBA gene polymorphisms (rs4673, rs9932581, rs1049255) and their gene-environment interactions on chronic inflammatory process represented as C-reactive protein (CRP) levels, early functional and structural changes of arterial wall as well as clinical coronary heart disease (CHD) and its complications.

Subjects and Methods: Cohort studies and a nested case-control study were conducted with two Finnish populations, respectively comprising 2,283 young adults who participated in the 21-year follow-up of the Cardiovascular Risk in Young Finns Study (Studies I to III), and 402 high-risk patients selected from the participants of the Finnish Cardiovascular Study (the Study IV). Risk factor and health data were collected with questionnaires and physical examinations. Brachial arterial flow-mediated vasodilation (FMD) and carotid intima-media thickness (IMT) were assessed by ultrasound in the Cardiovascular Risk in Young Finns Study. CHD was verified by means of coronary angiography in the Finnish Cardiovascular Study. Blood and DNA samples were collected for biochemical and genetic analyses. Genotyping was performed using the 5'-nuclease assay (TaqMan).

Results: In a population-based sample of young adults, brachial artery FMD was significantly influenced by the genotype of the CYBA C242T (rs4673) polymorphism, the relationship between the C242T polymorphism and FMD being clearly present in the overweight and ever-smoking subjects but not in the normal-weight and non-smoking subjects (Study **I**). In a young Finnish population, smoking/hypertension-induced changes in carotid IMT were different across the genotypes of the CYBA -930$^{A/G}$ (rs9932581) gene polymorphism, and a linear regression analysis suggested that the smoking/hypertension-genotype interactions were associated with carotid IMT.

Moreover, a stratified analysis according to smoking/hypertension status indicated that the differences might be due to an interaction effect of smoking/hypertension with the G allele of the CYBA -930 $^{A/G}$ polymorphism among smokers or hypertensive subjects (Study **II**). In addition, the CYBA 242T allele was associated with lower CRP level, and this result was further confirmed by haplotype analysis. The carriage of haplotype 5 (rs4673**T**, rs1049255**A**, rs7195830**A**) which contained the T allele of the C242T polymorphism had the lowest level of CRP. Multivariate linear regression analysis indicated that the CYBA C242T polymorphism was an independent determinant of CRP level in the young population (Study **III**). In a high-risk Finnish population, the prevalence of the CYBA 242T allele was significantly lower among angiographically verified coronary artery disease (CAD) patients than among control subjects (Study **IV**).

Conclusions: CYBA gene polymorphisms, including the C242T (rs4673) and the -930 $^{A/G}$ (rs9932581) polymorphisms, are associated with the process of atherosclerosis from a very early stage to the clinical phase of cardiovascular diseases. The CYBA 242T allele consistently shows a protective association against the chronic inflammatory process presenting as high CRP level, impairment of endothelial function, and the development of coronary artery disease. In addition, the CYBA -930 $^{A/G}$ gene polymorphism might modify the effect of smoking/hypertension on early structural alterations of the arterial wall.

1 INTRODUCTION

Atherosclerosis is an inflammatory disease caused by multi-genetic and environmental interactions, and atherosclerotic lesions develop through a response-to-injury mechanism (Ross, 1993; Ross, 1999). Endothelial dysfunction is the earliest event of cardiovascular disease, and a chain of events will lead to several clinical and end-stage cardiovascular diseases. Although the events leading to disease progression overlap and intertwine, the modifications at any point along this chain can influence the pathophysiological process, thus altering disease progression (Dzau et al., 2006a).

Oxidative stress is a shared feature of different phases of atherosclerosis leading through risk factors and subclinical vascular changes to the clinical cardiovascular disease manifestations (Cai and Harrison, 2000; Griendling and FitzGerald, 2003a; Griendling and FitzGerald, 2003b). Most risk factors related to atherosclerosis and its complications have been found to be associated with endothelial dysfunction. Many of these risk factors – including hyperlipidemia, hypertension, diabetes, obesity and smoking – are associated with excess reactive oxygen species (ROS) production or oxidative stress (Cai and Harrison, 2000; Bonetti et al., 2003). It has been suggested that degradation of nitric oxide (NO) by increased ROS is a major mechanism underlying endothelial dysfunction. In addition to impaired endothelium-dependent relaxation, a decline in NO bioavailability is usually accompanied by many other changes in endothelial function, including altered anti-inflammatory properties (Celermajer, 1997; Landmesser et al., 2004). Activated endothelial cells have been found to be associated with a phenotype that promotes the recruitment of inflammatory cells to the sites of vascular injury, and various inflammatory molecules generated by these cells can further provoke high oxidative stress in the vasculature (Chandel et al., 2001; Bonetti et al., 2003; Park et al., 2004). Oxidative stress has been found to regulate the expression of many pro-inflammatory mediators via the activation of the transcription factor, nuclear factor-kappa B (NF-κB), therefore possibly having a pro-inflammatory effect (Li and Verma, 2002; Mogensen et al., 2003). All things considered, it seems that oxidative stress not only plays a role in a response to environmental challenge, but also affects the process of atherosclerosis. Recent evidence further suggested a role of oxidative stress in the activation of specific signalling pathways and redox-sensitive transcription factors which control several physiological responses in the vasculature, including the proliferation of smooth muscle cells (SMC), the induction of an inflammatory response, and impairment of endothelium-dependent relaxation (Griendling et al., 2000a; Ardanaz and Pagano, 2006; Clempus and Griendling, 2006; Lyle and Griendling, 2006). All of these events contribute to the pathophysiology and pathogenesis of cardiovascular disease.

One of the major sources of ROS is NADPH oxidase, which consists of several membrane-bound and cytosolic proteins. The p22phox encoded by the CYBA gene is one of the components of the NADPH oxidase (Lassegue and Clempus, 2003; Quinn and Gauss, 2004; Quinn et al., 2006; Bedard and Krause, 2007). Transfection of antisense CYBA complementary DNA into vascular SMCs has been found to lead to a decrease in the superoxide production (Ushio-Fukai et al., 1996). Thus, the p22phox could be the most essential component in the activation of NADPH oxidase. In addition, the expression of the p22phox in human atherosclerotic coronary arteries is more intense than in non-atherosclerotic arteries, indicating that the p22phox might participate in the pathophysiology and pathogenesis of atherosclerosis and associated diseases (Azumi et al., 1999). The CYBA gene is contained in a 7-kb sized genomic segment in chromosome 16q24. In the international Haplotype Map Project (www.hapmap.org) database of polymorphisms in samples of European ancestry, there are at least 9 common SNPs (minor allele frequency >1%) in this 7-kb genomic segment. Among these, the CYBA C242T gene polymorphism (rs4673), located in exon 4, is considered the most interesting because of the structural change it causes in the enzyme structure. The mutation results in an amino acid substitution (histidine to tyrosine) at position 72 of the protein (Inoue et al., 1998). The functional significance of the C242T polymorphism has been related to NADPH oxidase activity with subsequent change in the production of the superoxide anion (Guzik et al., 2000a; Wyche et al., 2004). The CYBA -930$^{A/G}$ polymorphism (rs9932581) is located at position -930$^{A/G}$ from the ATG codon in the CYBA gene promoter region (Moreno et al., 2003). The presence of the -930$^{A/G}$ polymorphism has been related to higher transcriptional activity, mRNA and protein expression of the p22phox as well as NADPH oxidase activity (Zalba et al., 2001; Moreno et al., 2003; San Jose et al., 2004). The A640G polymorphism (rs1049255) is located in the 3' untranslated region of the CYBA gene, and the studies on the functional significance of the A640G polymorphism have produced conflicting results (Wyche et al., 2004; Macias-Reyes et al., 2008; Schirmer et al., 2008). Furthermore, the association of CYBA gene polymorphisms with cardiovascular disease and its associated traits has been widely investigated, and the results are quite inconsistent (Soccio et al., 2005; San Jose et al., 2008). Differences in the genetic background of the study populations, study designs and statistic methods used might contribute to these disparate results.

In this thesis, a candidate gene approach and association analyses were used to study the possible impact of CYBA gene polymorphisms and gene-environment interactions on the early chronic inflammatory process represented by CRP level, functional and structural changes in the arterial wall, as well as clinically significant coronary artery disease (CAD). Cohort studies and a

nested case-control study were conducted with two Finnish populations, comprising 2,283 young adults who participated in the 21-year follow-up of the Cardiovascular Risk in Young Finns Study (the Study I to III), and 402 high-risk patients who were selected from the participants of the Finnish Cardiovascular Study (the Study IV).

2 REVIEW OF THE LITERATURE

2.1 Atherosclerosis

Cardiovascular disease (CVD) is the leading cause of death and poses a significant public health burden worldwide (Murray and Lopez, 1997; Lopez et al., 2006). The World Health Organization estimated that nearly 17.1 million people worldwide died of CVD in 2004, representing 30% of all global deaths (www.who.int). Atherosclerosis, the root cause for CVD, is estimated to account for over 50% of all deaths in industrialized countries (Lusis, 2000). In the following sections, the development of atherosclerosis and its common risk factors are discussed briefly.

2.1.1 Structure of the arterial wall

A normal artery consists of three concentric layers that surround the arterial lumen, each of which has a distinctive composition of cells and extracellular matrix.

The intima is the innermost layer of the arterial wall. It contains delicate connective tissue with occasional SMCs and macrophages. A monolayer of endothelial cells lines the luminal surface and forms a physical and functional barrier between flowing blood and the stroma of the arterial wall. A sheet of elastic fibres, the internal elastic lamina, separates the intima from the media layer (Stary et al., 1992).

The media is made up of synthesizing SMCs which produce collagen and contractile SMCs that are involved in the regulation of blood pressure (BP) by vasoconstriction and vasodilatation. An extracellular matrix consisting largely of elastic fibres and collagen with a lesser content of proteoglycan holds the SMCs together (Ross and Glomset, 1976).

The adventitia, the outermost layer of the arterial wall, is composed of loose connective tissue with a variety of cells including fibroblasts, SMCs, macrophages, mast cells and ganglionic cells. The outer part of this layer also contains vasa vasorum, lymphatic vessels and nerves which provide the stimulus and blood supply to the media (Ross and Glomset, 1976). The boundary between the media and adventitia is demarcated by the external elastic lamina, and the adventitia is often surrounded by a layer of adipose tissue.

2.1.2 Morphological features of atherosclerosis

Atherosclerosis is a multi-factorial disease characterized by patchy intramural thickening of the sub-intima that encroaches on the arterial lumen, and the majority of cardiovascular diseases result from the complications of atherosclerosis. Six major types of lesions reflect the early, developing and mature stage of atherosclerosis (Stary et al., 1992; Stary et al., 1994; Stary et al., 1995). The initial lesion of atherosclerosis (Type I) contains atherogenic lipoproteins, which elicit an increase in macrophages in the arterial wall, causing an adaptive intimal thickening. Macrophages accumulate lipids to form fatty streaks (Type II lesion), which are composed of layers of macrophages and SMCs, and have been found as early as during the first decade of human life. Although fatty streaks are not clinically significant, they represent the precursor of more advanced lesions. A type III lesion known as the intermediate lesion consists of scattered small extra-cellular pools of lipids that disrupt the normal unity of the intimal smooth muscle layers. These lesions may be present soon after puberty. The theroma (Type IV), considered as the first advanced lesion, is potentially symptomatic and frequent from the third decade on. It is characterized by the accumulation of lipid-rich necrotic debris and smooth muscle cells known as a lipid core. The nature of the intima above the lipid core can be changed by an increase in fibrous connective tissue, forming a fibrous cap. When a lesion with a lipid core gets a fibrous cap, the lesion is classified as type V lesion (Fibroatheroma). A type VI lesion (complicated lesion) is generally formed from a type IV and V lesion, and it is complicated with a disruption of the lesion surface, haematoma or haemorrhage and thrombotic deposits. A lesion of type V and VI composition usually begins to appear after the third decade of life. The most important clinical complication is an acute occlusion due to the formation of a thrombus or blood clot, resulting in myocardial infarction or stroke. **(Figure 1)**

2.1.3 Development of atherosclerosis

There are many hypotheses to explain the development of atherosclerosis (Stocker and Keaney, 2004). The commonly accepted one suggested by Ross is that atherosclerosis is an inflammatory disease and atherosclerotic lesions develop through a response-to-injury mechanism. In this hypothesis, endothelial dysfunction is considered the first step of atherosclerosis, and each characteristic lesion of atherosclerosis represents a different stage in a chronic inflammation process of the arterial wall (Ross and Glomset, 1976; Ross, 1993; Ross, 1999).

The endothelial dysfunction leads to compensatory responses that disturb endothelial homeostasis. Endothelial activation is associated with a phenotype that promotes the recruitment of

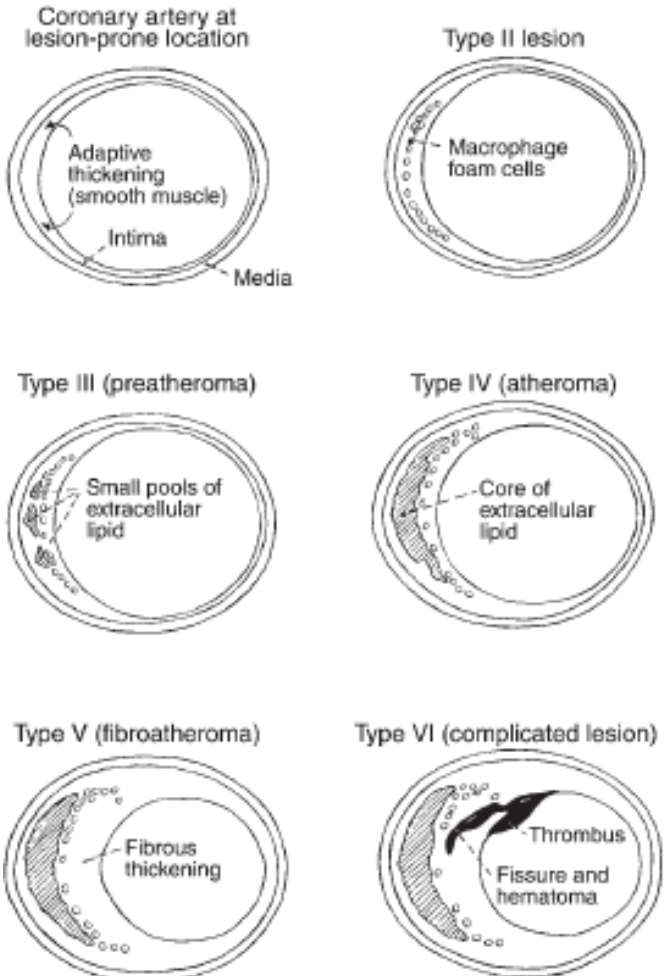


Figure 1. Cross section drawings of atherosclerotic lesions (Stary et al., 1994; Stary et al., 1995). Reproduced with permission by Wolters Kluwer Health

inflammatory cells to the sites of vascular injury, and it may also result in increased vascular permeability and intravascular thrombosis (Bonetti et al., 2003). The inflammatory responses stimulate the migration and proliferation of smooth muscle cells that become intermixed with an area of inflammation to thicken the arterial wall. If the inflammatory responses continue, increased numbers of macrophages and lymphocytes migrate into the lesions and release hydrolytic enzymes, cytokines, chemokines and growth factors, inducing further damages. Finally, cycles of accumulation of mononuclear cells, proliferation of SMCs and formation of fibrous tissue lead to advanced, complicated atherosclerotic lesions which alter the blood flow in the arteries (Ross, 1993; Ross, 1999).

2.1.4 Risk factors for atherosclerosis

Atherosclerosis is a multi-factorial disease affected by environmental as well as genetic risk factors and their interactions (Hopkins and Williams, 1981; Yusuf et al., 2004; Goldstein et al., 2006). Traditional cardiovascular risk factors comprise aging, male sex, smoking, hypertension, disturbed lipid profiles, diabetes, physical inactivity and obesity (Hopkins and Williams, 1981). More recently discovered risk factors include hemostatic factors, inflammatory markers and psychosocial factors (Jousilahti et al., 1996b; Jousilahti et al., 1999; Lusis, 2000; Fruchart et al., 2004; Nanchahal et al., 2005). Some of the risk factors such as age and sex cannot be modified by lifestyle changes. However, increased physical exercise, reduced body weight or smoking cessation may reduce the risk of atherosclerosis by changing conventional physiological or biochemical risk factors, such as hyperlipidemia as well as high blood pressure, glucose and CRP levels (Vartiainen et al., 2000). It has been observed that individuals free of the environmental risk factor burden at the age of fifty years have a significantly lower lifetime risk of CVD than those with two or more risk factors (Lloyd-Jones et al., 2006). Although these risk factors largely contribute to the risk of CVD, they cannot fully explain the development of the disease. Genetic factors may have an important role in the pathogenesis of atherosclerosis. Twin and family studies have reported an association between positive family history and mortality as well as morbidity with regard to CAD (Goldbourt and Neufeld, 1986; Jousilahti et al., 1996a; Gaeta et al., 2000). In addition, many of the established risk factors show a strong genetic component (Whitfield et al., 2004; Ingelsson et al., 2007; Keskitalo et al., 2007; Uhl et al., 2007). In recent years, more and more studies have tried to unravel the molecular basis of atherosclerosis and identify predisposing genetic factors. To date, however, the genetic traits are not available for diagnostic and prognostic purposes for cardiovascular disease in clinical routine.

2.1.5 Ultrasound studies of arteries

Ultrasound is a safe and non-invasive tool for investigating early atherosclerotic changes. The brachial artery flow-mediated dilatation and carotid artery intima-media thickness, as measured by Ultrasound, become useful parameters in assessing the predisposition to atherosclerosis in individuals with cardiovascular risk factors.

Brachial artery flow-mediated dilatation. Endothelial dysfunction is the earliest event in the development of atherosclerosis, and it is characterized by defects in the normal vascular relaxation

response to mediators, such as acetylcholine, or to increased blood flow (Corretti et al., 2002). This response is thought to be endothelium-dependent (Pohl et al., 1986; Joannides et al., 1995). The loss of endothelium-dependent responses can be measured by ultrasound studies of forearm blood flow responses (Celermajer et al., 1992). In this method, a reactive hyperemia is induced by the inflation of a pneumatic tourniquet placed around the forearm to a pressure of over 250mmHg for up to five minutes, followed by release. Increased hemodynamic shear stress during reactive hyperemia as a stimulus can provoke the endothelium to release nitric oxide with subsequent vasodilation that can be imaged and quantitated as a parameter of endothelial function (Mullen et al., 2001; Corretti et al., 2002). There is evidence that endothelial dysfunction precedes the development of clinically apparent atherosclerosis, and it has been found in symptom-free individuals with cardiovascular risk including aging, smoking, hypertension, diabetes, obesity and hyperlipidemia (Clarkson et al., 1996; Gokce et al., 2001; Engler et al., 2003; Oida et al., 2003; Järvisalo et al., 2004). Effective intervention on risk factors, such as smoking cessation and loss of body weight, may reverse the endothelial dysfunction (Lusis, 2000). In addition, it has been demonstrated that brachial artery FMD is closely correlated with coronary endothelial function, thus reflecting not only the peripheral but also the coronary circulation (Anderson et al., 1995a; Anderson et al., 1995b). Impaired brachial artery flow-mediated dilatation has been related to the prevalence of coronary atherosclerosis and independently predicted cardiovascular events in CAD patients (Neunteufl et al., 1997; Chan et al., 2003).

Carotid intima-media thickness. The intima-media thickness (IMT) is the distance between far wall lumen-intima and media-adventitia interfaces of the arterial wall. Measurement of arterial wall IMT by B-mode ultrasound was first introduced by Pignoli and colleagues, and their study of excised aorta showed close correlation between ultrasonically measured IMT and the same thickness measured by light microscopy (Pignoli et al., 1986). Because of its anatomical position, the carotid artery became the artery of choice for ultrasonic examination of IMT. Increased carotid IMT has been found to relate to many cardiovascular risk factors, such as aging, smoking, hypertension, diabetes, obesity and hyperlipidemia (Simon et al., 2002). Carotid IMT measurement also correlates with the presence of coronary atherosclerosis (Craven et al., 1990). Several larger prospective epidemiologic studies have showed that increased IMT in adults with no history of cardiovascular disease was associated with increased risk of clinical events, including myocardial infarction and stroke (Bots et al., 1997; Chambless et al., 1997; Longstreth et al., 1998; O'Leary et al., 1999; Chambless et al., 2000). Carotid IMT measurement by B-mode ultrasound has been recommended by the American Heart Association for cardiovascular evaluation to identify individuals at risk of developing cardiovascular disease.

2.2 Reactive oxygen species (ROS) and pathogenesis of atherosclerosis

2.2.1 Basic concept of ROS

ROS are a family of molecules including molecular oxygen and their derivatives produced in all aerobic cells. Many ROS possess unpaired electrons and are thus free radicals. These include molecules such as the superoxide anion ($^{\cdot}O_2^-$), hydroxyl radical ($OH^{\cdot}$), nitric oxide ($NO^{\cdot}$), and lipid radicals. Other reactive oxygen species, such as hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen ($^1O_2$), peroxynitrite (ONOO-), and hypochlorous acid (HOCL) are not free radicals per se but are biologically active and easily converted into radicals [for reviews see (Thannickal and Fanburg, 2000; Droge, 2002; Stocker and Keaney, 2004)].

Oxygen is a molecule abundant in biological systems. ROS may originate from cellular and extracellular sources, from both enzymatic and non-enzymatic paths. In biological cells, potential enzymatic sources of ROS include NADPH oxidases, xanthine oxidases (XO), nitric oxide synthases (NOS), arachidonic acid pathway enzymes lipoxygenase and cyclooxygenase, as well as myeloperoxidase (MPO). Non-enzymatically, oxygen can be converted into superoxide anion ($^{\cdot}O_2^-$) by reacting with redox active compounds such as semiubiquinone of the mitochondrial electron transport chain (Droge, 2002). **(Figure 2)**

ROS generation is generally a cascade of reactions that start with the production of $^{\cdot}O_2^-$; the $^{\cdot}O_2^-$ is therefore of critical importance among ROS. In biological systems, $^{\cdot}O_2^-$ is short-lived, and it is rapidly dismutated to H_2O_2 ($2 * 10^9\ mol/L^{-1}\ s^{-1}$), which is then converted to H_2O by either catalase or glutathione peroxidise (Valko et al., 2007). Other elements in the cascade of ROS generation include the reaction of $^{\cdot}O_2^-$, with NO to form $ONOO^-$, the peroxidase-catalyzed formation of HOCL from H_2O_2 (Hampton et al., 1998), and the iron-catalyzed Fenton reaction leading to the generation of $OH^{\cdot}$ (Stocker and Keaney, 2004; Valko et al., 2007). **(Figure 2)**

ROS exist in biological cells and tissues in low concentrations. Their concentrations are determined by the balance between their rates of production and their rates of clearance by various antioxidant compounds and enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (Droge, 2002; Li and Shah, 2004; Stocker and Keaney, 2004). **(Figure 2)**

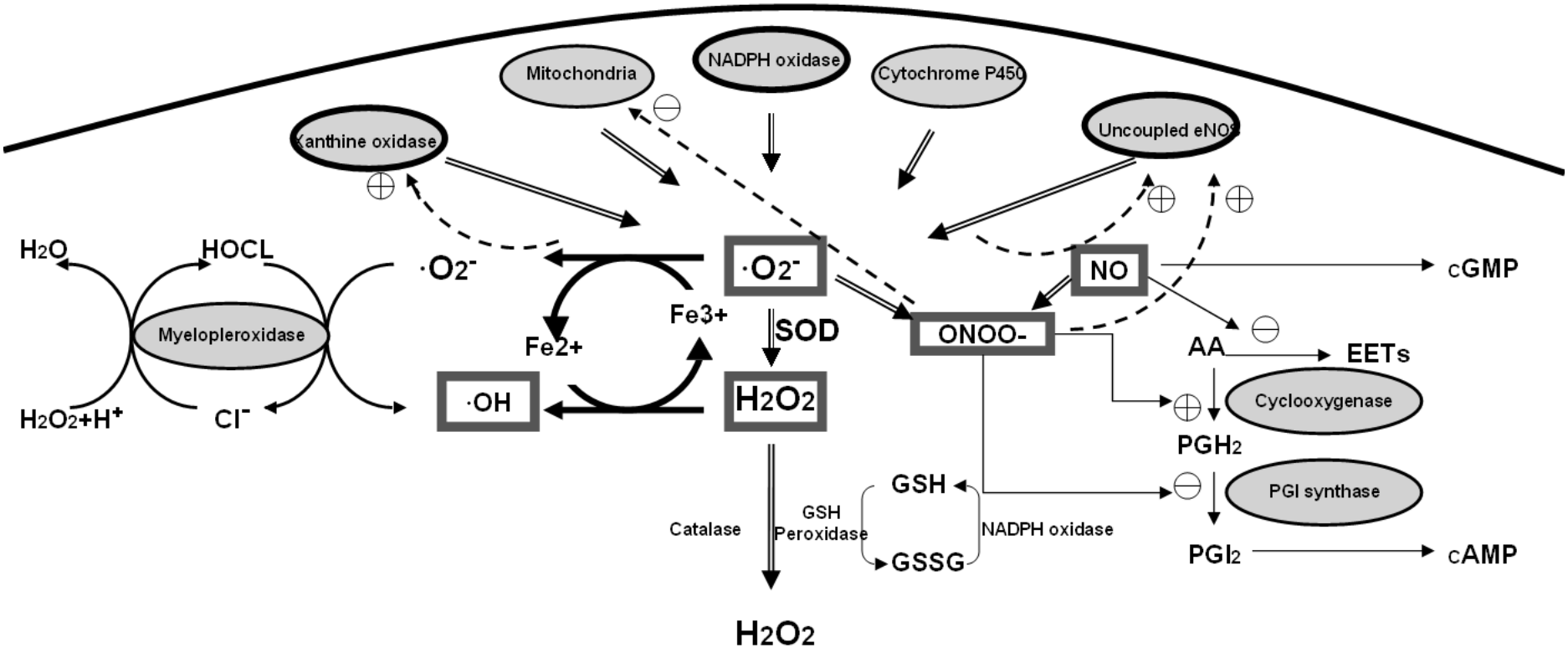


Figure 2. Schematic diagram showing the sources of ROS and the signalling pathways in endothelial cells. Modified from Li et al. 2004.

Under physiological conditions, ROS influence many physiological processes including host defence, hormone biosynthesis, fertilization and cellular signalling. However, excessive production of ROS, outstripping endogenous antioxidant systems, has been implicated in processes in which they oxidize biological macromolecules, such as DNA, proteins, lipids, and carbohydrates. The term oxidative stress describes a condition in which there is an imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage. Oxidative stress has also been implicated in a large number of human diseases including atherosclerosis and cancer as well as the aging process (Droge, 2002; Taniyama and Griendling, 2003; Li and Shah, 2004).

2.2.2 ROS and endothelial dysfunction

The endothelium is a single layer of cells that lines the inner surface of all blood vessels. In normal healthy circumstances, the vascular endothelium serves not only as a passive barrier between flowing blood and the vascular wall but acts as a vital regulator to maintain vascular homeostasis (Gimbrone, 1995).

A healthy endothelium senses mechanical and hormonal stimuli. In response, it synthesizes and releases various vasoactive factors (Luscher and Barton, 1997; Vapaatalo and Mervaala, 2001). The controlling of vascular tone is accomplished by the release of numerous vasodilator and vasoconstrictor substances. The vasodilator substances produced by the endothelium include NO, prostacyclin, various endothelium-derived hyperpolarizing factors (EDHF) and C-type natriuretic peptide. The endothelium also produces vasoconstrictor substances, such as endothelin-1, angiotensin II (Ang II), thromboxane A_2 and ROS. Inflammatory modulators of the endothelium include NO, intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), E-selectin and NF-κB. The modulation of hemostasis includes the release of plasminogen activator, tissue factor inhibitor, the von Willebrand factor, NO, prostacyclin, thromboxane A_2, plasminogen-activator inhibitor-1, and fibrinogen. The normal functions of the endothelium are mediated through the regulated synthesis and release of these vasoactive endogenous compounds, and thereafter maintain the equilibrium between vasodilatation and vasoconstriction, the inhibition and promotion of the proliferation and migration of smooth muscle cells, the prevention and stimulation of the adhesion and aggregation of platelets, as well as thrombogenesis and fibrinolysis (Vapaatalo and Mervaala, 2001; Verma and Anderson, 2002; Vanhoutte, 2003; Nadar et al., 2004).

Endothelial function has been assessed primarily in terms of endothelium-dependent vasomotion, largely based on the assumption that impaired endothelium-dependent vasorelaxation reflects alterations of other functions of the endothelium as well. The term endothelial dysfunction

has been used to refer to an impairment of endothelium-dependent vasorelaxation caused by a reduction in the amount of bioavailable NO in the vascular wall, and several other pathological conditions, including altered anticoagulant and anti-inflammatory properties of the endothelium, impaired modulation of vascular growth and dysregulation of vascular remodelling (Celermajer, 1997; Landmesser et al., 2004). All these changes have been associated with the development of atherosclerosis (Bonetti et al., 2003). In humans, endothelial dysfunction can be assessed biochemically by measuring different markers (e.g., adhesion molecules, cytokines, and prostanoids) in the blood, or functionally by measuring endothelium-dependent vasodilatation in vitro (isolated arteries) and in vivo either in response to agonists or to changes in blood flow in the forearm, coronary or peripheral circulation.

Possible causes of endothelial dysfunction include mechanical stress, several forms of dyslipidemia, hypertension, diabetes, smoking, obesity, genetic alterations and combinations of these and other factors. Although the pathophysiology of endothelial dysfunction is complex and involves multiple mechanisms, a large body of evidence indicates that exceeding generation of ROS both within endothelial cells and in adjacent cells has a major role in the genesis of endothelial activation and dysfunction (Li and Shah, 2004). The potential sources of ROS are implicated in endothelial physiology and pathophysiology, including the mitochondrial electron transport chain, XO, cytochrome P-450 enzymes, uncoupled NOSs, the phagocytic MPO system and NADPH oxidases (Li and Shah, 2004; Ray and Shah, 2005). Of these sources within the vasculature, a family of multi-subunit NADPH oxidases appears to be a predominant contributor of endothelial $^{\cdot}O_2^-$.

The negatively charged $^{\cdot}O_2^-$ radical is unstable in an aqueous solution (half-life of a few seconds); the interaction between $^{\cdot}O_2^-$ and NO occurs at an extremely rapid rate of $6.7 * 10^9$ mol/L^{-1} s^{-1}. This is 3(/three) times faster than the reaction rate for $^{\cdot}O_2^-$ with SOD (Thomson et al., 1995). Under physiological conditions, endogenous anti-oxidative defence systems minimize this interaction and maintain a tenuous balance between $^{\cdot}O_2^-$ and NO. However, exceeding $^{\cdot}O_2^-$ generation may out-compete SOD and then inhibit the biological activity of endothelium-derived NO, accompanied by many alterations in endothelial function that further increase the propensity for vasoconstriction, cellular proliferation, inflammation and thrombosis in the vascular wall. Moreover, increased $^{\cdot}O_2^-$ reacts with NO to form a highly reactive molecule, $ONOO^-$. This reaction not only directly results in a decline in NO bioavailability, but the resulting product, $ONOO^-$, uncouples eNOS, inefficiently activating the guanylyl cyclase – $ONOO^-$ also enhances oxidative

stress by inhibiting superoxide dismutases, inactivates the prostacyclin synthase by tyrosine nitration and reduces the EDHF component, all of which contribute to the impairment of endothelium-derived vasodilation (Wolin et al., 2002; Stocker and Keaney, 2004; Feletou and Vanhoutte, 2006). In the absence of immediately accessible NO, most biological effects of $\cdot O_2^-$ are likely to be secondary to H_2O_2 production, and $\cdot O_2^-$ therefore usually serves as a precursor for H_2O_2. H_2O_2 is a weak oxidizing agent and poorly reactive, and it plays a particularly important role in signal transduction (Griendling and Harrison, 1999; Rhee, 1999; Buetler et al., 2004). Recent studies indicate that H_2O_2 released from the endothelium after conversion from $\cdot O_2^-$ appears to be an EDHF component, contributing to the regulation of vascular tone in certain vascular beds (Li and Shah, 2004; Shimokawa and Matoba, 2004; Graier and Hecker, 2008). In addition, increased $\cdot O_2^-$ also causes a reduction of Fe^{3+} to Fe^{2+} with subsequent release of intracellular Fe^{2+}stores, and the liberated Fe^{2+} can react with H_2O_2 to form another highly reactive molecule, the hydroxyl radical ($OH^{\cdot}$). Both $OH^{\cdot}$ and $ONOO^-$can oxidize proteins, DNA and lipids, contributing to endothelial cell dysfunction and eventual cell death (Wolin et al., 2002).

2.2.3 ROS and vascular smooth muscle cell proliferation

In the process of atherosclerosis, SMC proliferation and migration into the intima start in the early stages of lesion formation (Stary et al., 1992; Ross, 1993; Stary et al., 1994). Vascular SMC proliferation thus leads to intimal thickening, which indicates some degree of atherosclerosis progression. The intima-medial thickness (IMT) of the carotid artery observed in ultrasonography has been widely used to assess this stage of atherosclerosis. In addition, vascular disorders such as restenosis after angioplasty have a significant proliferative component, resulting from SMC and/or fibroblast migration and multiplication in the intima.

Several growth factors and hormones as well as mechanical stress cause vascular smooth muscle cells to proliferate and migrate, and most of these factors initially activate the membrane receptors such as the tyrosine kinase-coupled receptors (e.g. platelet-derived growth factor, epidermal growth factor, and fibroblast growth factor) or the heptahelical G protein-coupled receptors (e.g. angiotensin II and thrombin). Receptor binding in turn starts a cascade of growth signalling transduction reactions that activate specific genes and trigger a variety of key biochemical timing devices (Seger and Krebs, 1995; Schmitz and Berk, 1997). There are many growth-related signalling events that together regulate cellular proliferation and migration. A classical signal-transduction pathway in cellular growth, transformation and de-differentiation is the

mitogen-activated protein kinase (MAPK) signalling cascades (Davis, 1993; Seger and Krebs, 1995; Schmitz and Berk, 1997; Yang et al., 2003). A core triple kinase module consisting of MAP3Ks, MAP2Ks and MAPKs is a feature of MAPK cascades. MAPKs can activate directly transcriptional targets, such as the AP-1 transcription factor, or this can occur via the indicated downstream protein kinases. **(Figure 3)** Protein phosphorylation plays a diverse role in signal transduction, leading to changes in gene expression. Target proteins including receptors, enzymes and transcription factors can either be activated or inactivated by the state of phosphorylation of their specific amino acid

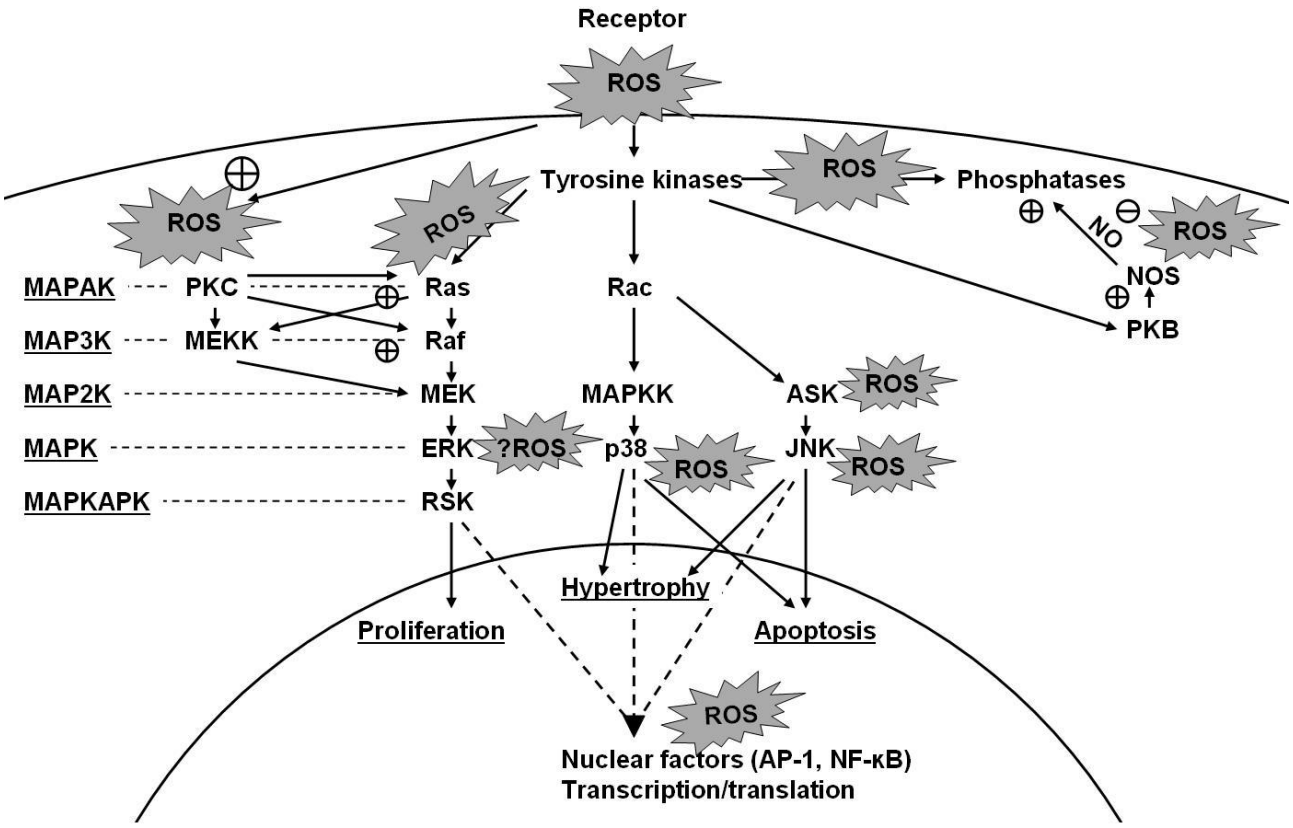


Figure 3. Schematic diagram showing ROS and the potential targets in the MAPKs pathway in vascular smooth muscle cells. The major groups of MAPKs in mammalian cells include extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38MAPK. The AP-1 transcription factor, which controls expression of the growth-related genes, comprises as a heterodimer of Fos and Jun proteins, which are protein products of the c-fos and c-jun genes. Up to six tiers in a cascade contribute to the amplification and specificity of the transmitted signals. Modified from Wolin et al. 2002.

residues. The state of phosphorylation is dictated by the activity of protein kinases (tyrosine or serine/threonine kinases) and phosphatase (protein tyrosine phophatases) (Suzuki et al., 1997; Enslen and Davis, 2001). Accumulating evidence indicates that the contribution of phosphotyrosine protein tyrosine phophatases to the control of the cell phosphorylation state is as relevant as phosphotyrosine protein kinases.

Early works by Rao et al. showed that ROS, such as $^{\cdot}O_2^-$, H_2O_2 and $OH^{\cdot}$, stimulated an increase in the number of vascular smooth muscle cells, DNA synthesis, as well as c-Jun and c-fos mRNA expression (Rao and Berk, 1992; Rao et al., 1993a; Rao et al., 1993b). In contrast, NO inhibited SMC growth in vitro and vivo, suggesting a role for NO as a regulator against smooth muscle cell proliferation (Moncada and Higgs, 1993; Lloyd-Jones and Bloch, 1996; Sarkar and Webb, 1998). In addition, a role for ROS in SMC growth was further supported by the findings that exogenous H_2O_2 or chemical agents that generated ROS induced tyrosine phosphorylation, mitogen-activated protein kinase (MAPK) stimulation, DNA synthesis and cell growth (Baas and Berk, 1995; Sundaresan et al., 1995). A few studies have showed that H_2O_2 may also be synthesized endogenously in SMCs as a response to the activation by hormones (e.g. Ang II) or growth factors (e.g. PDGF) (Griendling et al., 1994; Sundaresan et al., 1995). It has been reported that Ang II-induced epidermal growth factor receptor (EGFR) and the platelet-derived growth factor receptor (PDGFR) phosphorylation are both mediated through NADPH oxidase-derived $^{\cdot}O_2^-$ (Griendling et al., 1994; Zafari et al., 1998; Heeneman et al., 2000; Ushio-Fukai et al., 2001; Szocs et al., 2002; Ardanaz and Pagano, 2006). Because the short half-life of $^{\cdot}O_2^-$ limits its likelihood of serving a paracrine role in the vasculature, its more stable metabolite, H_2O_2, activates the intrinsic receptor kinase and its binding with the protein complex (Rao, 1996; Ardanaz and Pagano, 2006), or modulates signalling molecules that, in turn, transactivate the receptor (Heeneman et al., 2000; Ushio-Fukai et al., 2001; Ardanaz and Pagano, 2006). Because the profile of signalling pathways affected by ROS is similar to that activated by hormones and growth factors, many studies have documented a role of ROS as a second messenger in the control of vascular smooth muscle cell proliferation (Griendling and Ushio-Fukai, 1998; Weber et al., 2004; Clempus and Griendling, 2006). Not only are receptor-associated signals modulated by ROS, but those further downstream that activate several regulatory molecules in the cytoplasm and in the nucleus are modified as well (Lyle and Griendling, 2006). (**Figure 3)**

2.2.4 ROS and inflammation

Atherosclerosis has been widely considered as a chronic inflammatory process of the arterial wall, and the lesions develop as a result of inflammatory stimuli, subsequent release of various cytokines, proliferation of smooth muscle cells, synthesis of connective tissue matrix, and accumulation of macrophages and lipids (Ross, 1999; Libby, 2002). The orchestration of these processes is performed by blood and arterial cells including endothelial cells, smooth muscle cells, platelets, lymphocytes, monocytes and macrophages. In a mutually interactive manner, these cells generate various molecules including cytokines, growth factors, eicosanoids and proteases which provoke an inflammatory condition in the vasculature. Meanwhile, the inflammatory process produces a larger amount of ROS or an oxidative stress condition.

At early stages of atherosclerosis, the inflammatory phenotype is characterized by increased basal levels of leukocyte rolling, adherence and emigration, platelet-endothelial cell adhesion, as well as enhanced ROS production by endothelial cells (Ross, 1999; Lusis, 2000; Libby, 2002). The use of leukocyte count, C-reactive protein (CRP), interleukin (IL)-18, IL-6 and soluble CD40 etc. as inflammatory markers, has become a useful tool for researchers and clinicians (Pearson et al., 2003; Packard and Libby, 2008). **(Table 1)** Many epidemiological studies using circulating inflammation markers have found that the established risk factors, including smoking, obesity, aging, hypertension, diabetes and hyperlipidemia, are predictive of circulating inflammatory markers such as CRP or soluble intercellular adhesion molecule-1(Rohde et al., 1999; Libby and Ridker, 2004).

Table 1. Inflammation markers for research and potential clinical use. Modified from Pearson et al 2003.

Inflammatory markers	Stability	Assay availability	WHO standards available	Coefficient of Variation
Adhesion molecules	Unstable	Limited	No	Under 15%
Cytokines	Unstable	Few	Yes	Under 15%
Fibrinogen	Unstable	Many	Yes	Under 8%
Serum amyloid A	Stable	One	Yes	Under 9%
hs CRP	Stable	Many	Yes	Under 10%
WBC count	Stable	Many	Yes	Under 3%

2.2.4.1 Triggers of endogenous ROS generation

Pro-inflammatory cytokines and LPS (lipopolysaccharide) stimulation can result in the activation of NF- κB, a transcription factor which regulates a large number of pro-inflammatory gene expressions. These stimuli can also trigger the formation of ROS (Legrand-Poels et al., 1997; Bonizzi et al., 1999; Chandel et al., 2001). **(Figure 4)** The role of ROS in NF- κB activation by inflammatory cytokines and LPS has been extensively studied. Recent studies indicate that early ROS production after these stimuli serves as a key messenger for subsequent NF-κB activation.

IL-1β. IL-1β is a potent pro-inflammatory cytokine, and its binding with the type 1 IL-1 receptor initiates the formation of an intracellular receptor-associated protein complex responsible for transducing receptor signals (O'Neill and Greene, 1998). This process has been found to trigger NADPH dependent $\cdot O_2^-$ production (Bonizzi et al., 1999; Li and Engelhardt, 2006; Li et al., 2006). Spontaneous dismutation of $\cdot O_2^-$ to H_2O_2 increases local H_2O_2, which facilitates the redox-dependent association of TRAF6, a member of the TNF receptor-associated factor family of adaptor proteins, with the receptor complex. **(Figure 4)** The interaction of TRAF6 with the receptor complex leads to the activation of downstream IκB kinases (IKK) and NF-κB (Li et al., 2006). In addition, it has also been suggested that H_2O_2 might regulate NIK in IL-1β -mediated induction of NF- κB (Li and Engelhardt, 2006).

LPS. A recent study by Park et al. suggested that NADPH oxidase was the source of ROS upon LPS stimulation. Direct interaction of TLR4 with Nox 4, a subunit of NADPH oxidase, might be involved in LPS-induced NF- κB activation (Park et al., 2004). However, whether this local ROS production triggers the activation of TRAF6, as with the IL-1β signalling pathway above, is currently unknown.

TNF-α. Like IL-1β, TNF-α is a potent pro-inflammatory cytokine, and the signalling pathway that leads to NF-κB activation is now well established (Devin et al., 2000; Li and Verma, 2002). Although antioxidants have been reported to inhibit TNF-induced NF-κB activation, the molecular mechanisms are poorly understood (Frey et al., 2002; Garg and Aggarwal, 2002; Li et al., 2002).

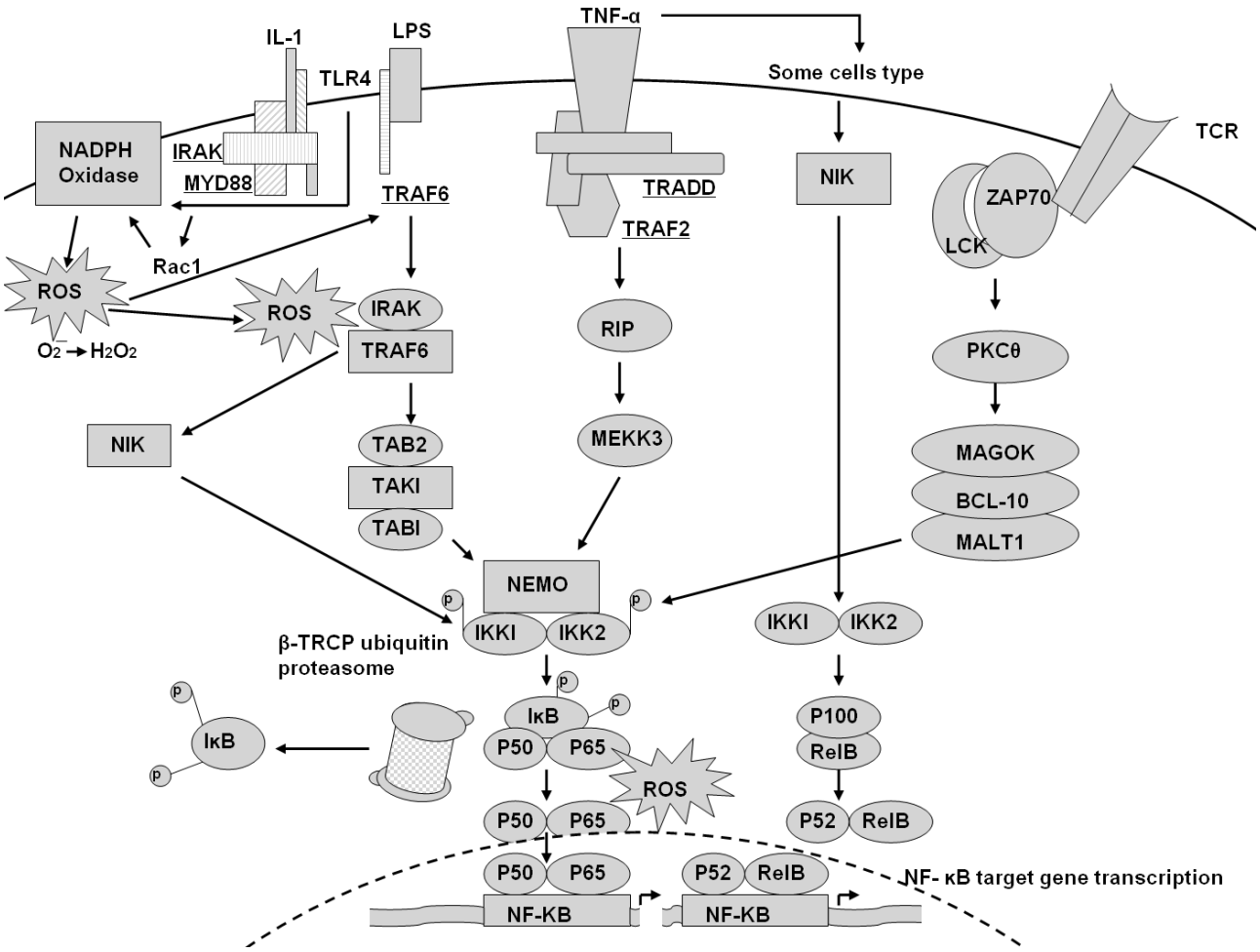


Figure 4. Schematic diagram showing NF-kB activating pathway by pro-inflammatory cytokines and LPS (lipopolysaccharide) stimulation. NF-κB is the general name for a family of transcription factors consisting of 5 members: p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2). The most abundant complex, often referred to as 'NF-κB', is p65/p50. The NF-κB dimer exists in the cytoplasm in an inactive form associated with regulatory proteins called inhibitors of κB (IκBs). The IκB kinase (IKK) complex is composed of two kinases (IKK1and IKK2) and the regulatory protein NEMO. The NF-κB inducing kinase (NIK) is involved in an alternative pathway, the NEMO-independent NF-κB activation pathway (Yamaoka et al., 1998; Li and Verma, 2002; Hayden and Ghosh, 2004; Yamamoto and Gaynor, 2004).

2.2.4.2 ROS and cellular adhesion molecules

Under normal physiological conditions, the endothelial monolayer in contact with flowing blood resists the adhesion of leukocytes. However, the attachment of blood leukocytes to endothelial cells

has been observed after pro-inflammatory stimuli, including an atherogenetic diet, hypercholesterolemia, obesity, hyperglycaemia, insulin resistance, hypertension and smoking (Lusis, 2000; Libby and Theroux, 2005; Lamon and Hajjar, 2008). It has been recognized that leukocytes are recruited to the intima at the sites of lesion predilection by a highly coordinated and well-regulated process that involves the expression and activation of adhesion molecules on endothelial cells and circulating leukocytes.

Cellular adhesion molecules are a diverse group of surface proteins that have been categorized into selectins, integrins and immunoglobulin superfamily members (Stocker and Keaney, 2004). The activity of cellular adhesion molecules is regulated by distinct mechanisms. Histological studies demonstrated increased endothelial expression of VCAM-1 and ICAM-1 in developing and established atherosclerotic lesions (Poston et al., 1992; Li et al., 1993). VCAM-1 seems to be a key inducible endothelium-expressed adhesion molecule that mediates monocyte recruitment to early lesions in experimental animal models of atherogenesis. In contrast, ICAM-1 has been implicated in more advanced atherosclerotic lesions (Cybulsky and Gimbrone, 1991; Nakashima et al., 1998; Collins et al., 2000; Cybulsky et al., 2001). In addition, mice lacking ICAM-1, P-selectin and $\beta_2$integrins were less likely to develop atherosclerotic lesions than wild-type mice, suggesting that adhesion molecules modulated inflammatory responses in atherosclerosis (Stocker and Keaney, 2004).

Oxidative events have been implicated in the regulation of cellular adhesion molecules on both the endothelium and circulating blood cells. Oxidized LDL or its oxidized fatty acids have been found to enhance the VCAM-1 gene expression induced by cytokines (Khan et al., 1995). Even in the absence of cytokines, human arterial endothelial cells exposed to oxidized LDL alone expressed ICAM-1, VCAM-1 and E-selectin (Amberger et al., 1997). In addition, a wide range of antioxidants, such as the thiol N-acetylcysteine, the metal-chelator pyrrolidine dithiocarbamate (Marui et al., 1993) and a glutathione peroxidase mimic, were found to inhibit cytokine-induced endothelial cell adhesion molecule expression (d'Alessio et al., 1998; Anderson et al., 1999), providing evidence that cellular oxidative events could modulate the expression of adhesion molecules on the endothelium.

NADPH-dependent $\cdot O_2^-$ generation appears critical to TNF-α-induced up-regulation of VCAM-1, IAM-1 and E-selectin, as it could be suppressed by SOD (Tummala et al., 2000; Chen et al., 2003b). Similarly, O_2^- has been implicated in the up-regulation of ICAM-1 in endothelial cells by oscillatory shear stress (Hwang et al., 2003). Although NADPH-dependent O_2^- has been found to regulate cytokine-induced adhesion molecules, the study by Tummala et al. also showed that

intracellular O_2^- generation was not associated with TNF-α induced NF-κB binding activity and NF-κB-driven promoters. The authors suggested that there were at least two distinct pathways involved in VCAM-1 and ICAM-1 gene expression, and NADPH-dependent O_2^- might modulate gene expression through redox-sensitive mechanisms (Tummala et al., 2000).

Cytokine-induced cell adhesion molecules including VCAM-1, ICAM-1 and E-selectin can be regulated at the level of gene transcription, and it requires binding of the transcription factor NF-κB to the regulatory region within the promoters of each of these genes (Collins et al., 1995). NF-κB is a redox-sensitive transcription factor (Janssen-Heininger et al., 2000). The involvement of ROS in eliciting NF-κB activation and adhesion molecule expression has been underscored by a number studies. In many ways, H_2O_2 is the ROS most suited for signalling function. Early studies showed that exposure of endothelial cells to H_2O_2 increased surface expression of adhesion molecules such as ICAM-1 and P-selectin (Patel et al., 1991; Bradley et al., 1993). Activation of NF-κB by H_2O_2 has been found responsible for TNF-α induction of ICAM-1 and VCAM-1 as well as angiotensin II-induced up-regulation of VCAM-1 (Pueyo et al., 2000; True et al., 2000; Chen et al., 2003a). Moreover, endogenously produced H_2O_2 after a brief ischemic episode has been found to induce the up-regulation of ICAM-1 and MCP-1 through NF-κB activation (Lakshminarayanan et al., 2001; Ziegelstein et al., 2004).

2.2.4.3 ROS and NF-κB activation

NF-κB is one of the pivotal regulators of pro-inflammatory gene expression, and it plays a central role in inflammatory response in atherosclerosis by inducing the transcription of pro-inflammatory cytokines, chemokines, adhesion molecules, matrix metalloproteinases (MMPs), cyclo-oxygenase 2 (COX2) and inducible nitric oxide synthase (iNOS) (Tak and Firestein, 2001; Li and Verma, 2002; Pashkow et al., 2008). Activated NF-κB has been identified in endothelial cells, macrophages and smooth muscle cells of human atherosclerotic lesions (Brand et al., 1996; Bourcier et al., 1997; Monaco et al., 2004).

A wide range of stimuli, including pro-inflammatory cytokines, stress signals and pathogens can activate the cells through their respective receptors (e.g. Toll/IL-1, TNF, and T cell receptor), resulting in the activation of different signal transduction cascades, which will eventually activate the IκB kinase (IKK) complex (Yamaoka et al., 1998). IKK activation can initiate the phosphorylation of IκB, resulting in the ubiquitination and degradation of IκB by the proteasome. This process can release NF- B dimers from the cytoplasmic NF-κB-IκB complex, allowing them to

translocate to the nucleus (Chen et al., 1995; Thompson et al., 1995n294). **(Figure 4)** Once in the nucleus, NF-κB can bind to κB enhancer elements on specific genes promoting transcription. The number of genes described to be regulated by NF- κB is over 160, whereas the number of factors shown to induce the NF-κB activation pathway is even higher (de Winther et al., 2005). Besides the classical activation mentioned above, a novel NEMO-independent NF-κB activation pathway has been described. This alternative pathway involves NF-κB inducing kinase (NIK) activation of IKK1, and leads to the phosphorylation and processing of p100, generating p52/RelB. It is induced by the B-cell activating factor, lymphotoxin β, CD40 ligand, and infection by HTLV or EBV (Li and Karin, 1999). However, these stimuli also activate the classical pathway mentioned above. **(Figure 4)**

NF-κB is a redox-sensitive transcription factor, and the redox status of the cell is important in the regulation of NF-κB activity (Janssen-Heininger et al., 2000). It has been reported that the activation of NF-κB through the release and degradation of IκBα occurs under oxidative conditions in some cells, whereas the binding of activated and translocated NF-κB to DNA has been shown to be dependent of the reducing conditions (Schreck et al., 1991; Hayashi et al., 1993; Anderson et al., 1994; Ginn-Pease and Whisler, 1998). A broad range of antioxidants, such as pyrrolidine dithiocarbamate, N-acetylcysteine and glutathione, as well as over-expression of antioxidant enzymes such as SMD-Mg, glutathione peroxidase and thioredoxin peroxidase, has been reported to inhibit NF-κB activation in various cells (Flohe et al., 1997; Li and Karin, 1999; Garg and Aggarwal, 2002; Brigelius-Flohe et al., 2004; Ginnan et al., 2008). Although accumulating evidence supports a role for ROS in activating NF-κB, the oxidant-sensitive molecular targets that regulate NF-κB activation remain to be elucidated.

2.3 Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase

The NADPH oxidase is a group of plasma membrane-associated enzymes found in a variety of cells of mesodermal origin, and its sole function is to generate ROS. Fifty years ago, Sbarra and Karnovsky first reported the existence of such an enzyme in neutrophils. Since then, NADPH oxidase has been studied extensively in phagocytes such as neutrophilic and eosinophilic granulocytes, monocytes and macrophages. Exposure of these cells to stimuli has been found to activate a respiratory burst caused by an activation of the plasma membrane-bound NADPH oxidase. Recently, it has become clear that all cell types in the vascular wall also produce O_2^-,

mostly via an enzyme similar to the leukocyte NADPH oxidase (Babior, 1999; Lassegue and Clempus, 2003; Quinn et al., 2006; Bedard and Krause, 2007).

2.3.1 Structure of NADPH

In phagocytes, the core NADPH oxidase consists of 4 major subunits: a plasma membrane spanning cytochrome b558 composed of a large subunit gp91phox (Nox2) and a smaller p22phox subunit, and 2 cytosolic components, p47phox and p67phox. Additional components of the enzyme include p40phox and the small G proteins Rac (Rac1/2) and Rap1A. **(Figure 5)** Rac1/2 participates in the assembly of the active complex. The p40phox and Rap1A have also been shown to play a role in regulating enzyme activity; however, their specific functions are still not well understood (Babior, 1999; Griendling et al., 2000b; Bokoch and Knaus, 2003; Lassegue and Clempus, 2003; Quinn and Gauss, 2004; Quinn et al., 2006). The structural features of non-phagocyte NADPH oxidase seem

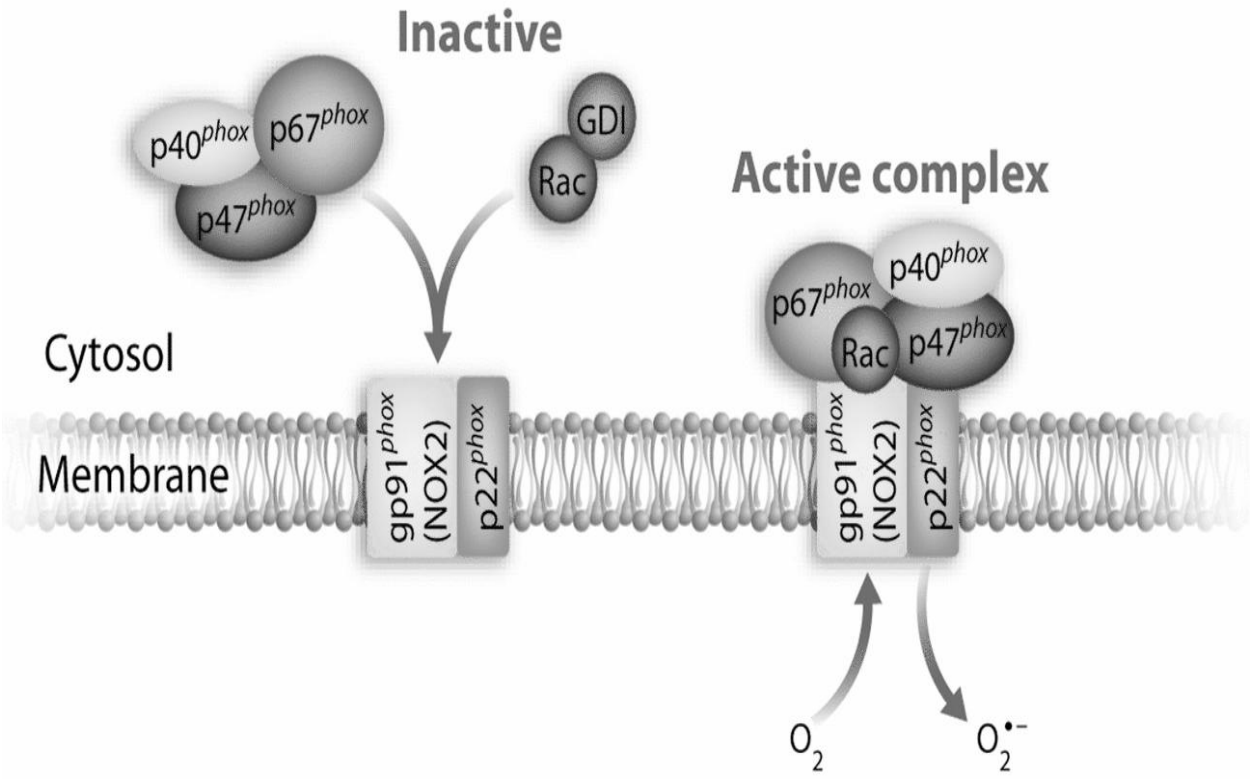


Figure 5. Schematic diagram showing NADPH oxidase activation (Quinn et al., 2006). Reproduced with permission provided by Portland Press LTD.

similar or even identical with those of their phagocyte counterparts. However, it has been discovered that the subunit gp91phox is only one member (Nox2) of a new family of gp91phox homologous proteins termed Nox in non-phagocyte. The mammalian Nox homologues have been identified in many cells and tissues **(Table 2)** (Ray and Shah, 2005; Zalba et al., 2005b; Quinn et al., 2006; Bedard and Krause, 2007; Cave, 2009).

In phagocytes, gp91phox (Nox2) and p22phox are essential subunits of cytochrome b558, and the absence of either protein results in a non-functional NADPH oxidase (Parkos et al., 1989). Similar to gp91phox, Nox1 and Nox4 can couple directly with p22phox to form a superoxide generating NADPH oxidase in a variety of cells and tissues. The activity of Nox1 and Nox2 can be regulated by the association with cytosolic subunits. By contrast, Nox4 may only interact with p22phox for full activity (Ambasta et al., 2004). Although p22phox is similarly expressed in phagocyte and non-phagocyte cells, distinct gp91phox (Nox2) homologues are present in a variety of non-phagocyte cells. All three layers of the vessel wall express Nox family members, but different Nox proteins seem to have different cellular locations. Nox4 are expressed in all vascular cells, whereas gp91phox (Nox2) expression predominates in the endothelium and the adventitia in conduit vessels (Chen and Keaney, 2004). The vascular expression of Nox1 is low, and a role for Nox1 in vascular $^{\cdot}O_2^-$ generation has mainly been demonstrated in vascular smooth muscle cells (Chen and Keaney, 2004; Brandes and Kreuzer, 2005; Bedard and Krause, 2007). As a consequence of the heterogeneity in vascular NADPH oxidase expression, O_2^- generation in response to identical stimuli might vary between cell types.

Table 2. The expression of the major components of NADPH oxidase

Components	Chromosome Location	Tissues or cell types
Nox 1	Xq22	Vascular smooth muscle cells, endothelial cells, fibroblasts, gastric pit and colon epithelium
Nox2	Xp21.1	Neutrophils, lymphocytes, macrophages, endothelial cells, fibroblasts, vascular smooth muscle cells, cardiomyocytes, neurons, lung and kidney
Nox3	6q25.1-26	Fetal tissue and inner ear
Nox4	11q14.2-q21	Fetal tissue, kidney, heart, lung, pancreas, placenta, ovary, testis, osteoclasts and vascular smooth muscle cells
Nox5	15q22.31	Fetal tissue, ovary, testis, mammary glands, placenta, lymphocytes, spleen, pancreas, stomach and vascular smooth muscle cells
DUOX1	15q21	Thyroid, salivary glands, colon, rectum, bronchi and cerebellum
DUOX2	15q15.3-q21	Thyroid, salivary glands, colon, rectum, bronchi, pancreas and prostate
p22phox	16q24	Lymphocytes, Neutrophils, macrophages, endothelial cells, vascular smooth muscle cells, fibroblasts, osteoclasts, kidney, liver, lung, spleen, pancreas, testis, placenta, ovary, neurons and eye
p47phox	7q11.23	Lymphocytes, Neutrophils, macrophages, vascular smooth muscle cells, endothelial cells, fibroblasts, osteoclasts, kidney, liver, lung, spleen, pancreas, testis, placenta, ovary, neurons and eye
p67phox	1q25	Lymphocytes, Neutrophils, macrophages, vascular smooth muscle cells, endothelial cells, fibroblasts, osteoclasts, kidney, liver, lung, spleen, pancreas, testis, placenta, ovary, neurons and eye
p40phox	22q1,3.1	Lymphocytes, Neutrophils, macrophages, vascular smooth muscle cells, endothelial cells and fibroblasts
Rac	12q13.12	Lymphocytes, Neutrophils, macrophages, vascular smooth muscle cells and endothelial cells

2.3.2 Activation of NADPH oxidases

Although the structure is similar, the activity of phagocyte NADPH oxidase differs from the non-phagocyte NADPH oxidases. Under resting conditions, the leukocyte NADPH oxidase is inactive. A variety of stimuli, such as cytokines, chemokines, microbes, viruses and other foreign antigens, can trigger the respiratory burst of cells to produce a large amount of NADPH-dependent superoxide (Carlson et al., 2004). Activation of the NADPH oxidase proceeds through a series of transitory interactions between the cytosolic phox proteins and the membrane-bound flavocytochrome b558. The p47phox is the subunit chiefly responsible for transporting the cytosolic complex from the cytosol to the membrane during the activation of NADPH oxidase. The small GTPase Rac is also critical for oxidase activation, and its membrane binding is independent of other cytosolic phox proteins. The translocation of Rac2, p47phox and p67phox to the plasma membrane where p67phox interacts via its activated domain with the membrane bound subunits pg91 phox results in the functional NADPH oxidase **(Figure 5)** (Babior, 1999; Vignais, 2002; Brandes and Kreuzer, 2005; Quinn et al., 2006).

In contrast to the leukocytes, in un-stimulated cardiovascular cells, vascular NADPH oxidases continuously produce low levels of intracellular O_2^- and its ROS derivatives such as H_2O_2. The rate of O_2^- production in vascular cells is only 1%–10% of that in the leukocytes. Results from a large number of studies have suggested that at least part of NADPH oxidase is preassembled in vascular cells and likely responsible for constitutive activity, and another part is activated by the translocation of cytosolic subunits in a manner similar to leukocyte NADPH oxidase (Lassegue and Clempus, 2003). Cardiovascular NADPH oxidase activation could be regulated by a large number of activators of G-protein-coupled receptors, agonists on tyrosine kinase receptors, cytokines, chemokines and mechanical stimuli (Brandes and Kreuzer, 2005). The regulation of enzyme activity in cardiovascular cells occurs on at least two levels. Firstly, the activation of NADPH oxidase can be mediated by intracellular second messengers including calcium. Secondly, enzyme activity can also be modulated by the up-regulation of the component mRNAs (Griendling et al., 2000b).

2.3.3 NADPH oxidase and atherosclerosis

During the past half century, numerous studies have demonstrated that the phagocyte NADPH oxidase plays a crucial role in the non-specific host defence against foreign antigens by producing a

large amount of superoxide. Defects in gp91phox, p22phox, p47phox or p67phox lead to a rare genetic disorder known as CGD (chronic granulomatous disease). As a result, patients with CGD experience severe recurrent infections due to the decreased capacity of their immune system to fight exgenous micro-organisms (Berendes et al., 1957; Bedard and Krause, 2007). However, studies over the last two decades have been focused on the role of NADPH oxidase in cardiovascular physiology and pathophysiology, and ROS and NADPH oxidase have been implicated in numerous cell processes and pathogeneses associated with atherosclerosis, including endothelial dysfunction, activation of inflammatory signalling pathways, upregulation of adhesion molecules, proliferation of vascular smooth muscle cells and oxidation of lipoproteins (Griendling et al., 1994; Griendling et al., 2000b; Griendling, 2004; Selemidis et al., 2008).

The study by Guzik et al. showed an association of the activity of NADPH oxidase with clinical risk factors and reduced NO-mediated endothelial dysfunction in atherosclerosis (Guzik et al., 2000b). Azumi et al. examined the expression of p22phox in atherosclerotic and non-atherosclerotic coronaries and found that the severity of atherosclerotic lesions and the features of plaque stability correlated with p22phox over-expression in coronary arteries (Azumi et al., 1999). In addition, Sorescu et al. demonstrated that Nox4 was increased in vascular cells in early progression of atherosclerotic plaques and decreased in more advanced stages of lesions in human coronary arteries, whereas gp91phox and p22phox were greatly increased in macrophages throughout the progression of atherosclerotic plaques, thus suggesting a possible causal link between the classic NADPH oxidase and the development of lesions. In this study, the contribution of gp91phox and p22phox to lesion progression was caused by infiltrated monocytes (Sorescu et al., 2002). Therefore, although vascular NADPH oxidase isoforms play an important role in the development of atherosclerosis, ROS generation from infiltrated monocyte NADPH oxidase also contributes to atherosclerotic lesion formation (Kalinina et al., 2002; Cathcart, 2004; Zalba et al., 2005a). Monocyte-derived superoxide can lead to LDL oxidation, which alters basic cell functions, such as adhesion and proliferation, and stimulates inflammatory and thrombotic processes in the arterial wall (Dorffel et al., 2001; Cathcart, 2004).

Recently, a number of studies on transgenic mice with the deletion or over-expression of certain NADPH oxidase subunits have examined the contribution of NADPH oxidase to the risk and the development of atherosclerosis (Selemidis et al., 2008). The deletion of p47phox showed a beneficial effect on preventing hypertension in the hypertension animal models, whereas the deletion of Nox1 and Nox2 as well as targeted overexpression of Nox1 indicated the relative roles that each subunit played in the pathogenesis of hypertension. The overexpression of human Nox1 in SMCs of mice induced a thickened medial layer and a vascular hypertrophic response (Dikalova et

al., 2005). Nox1 or Nox2 deletion in the hypertension models prevented the Ang II-induced increase in ROS production. Meanwhile, aortic medial thickening was markedly attenuated in Nox2 knockout mice, and Nox1 deletion prevented the impairment of endothelium-dependent vasorelaxation induced by experimental hypertension. However, Nox2 deletion had no effect on improving impaired endothelium-dependent vasorelaxation, and Nox1 deletion had no effect on VSMC proliferation and medial hypertrophy for the transgenic mice (Wang et al., 2001; Landmesser et al., 2002; Matsuno et al., 2005; Guzik et al., 2007; Yogi et al., 2008). The deletion of either p47phox or Nox2 has also been investigated in ApoE$^{-/-}$ mice, the most widely studied animal model of atherosclerosis. No difference in the extension of atherosclerosis was found between the p47phox- or Nox2-null, NADPH oxidase-deficient ApoE$^{-/-}$ mice and straight ApoE$^{-/-}$ mice – however, lesion burden was significantly reduced in the p47 phox-deficient animals (Hsich et al., 2000; Barry-Lane et al., 2001; Guzik et al., 2007; Vendrov et al., 2007).

2.4 The p22phox of NADPH oxidase

2.4.1 The p22phox and CYBA gene

The p22phox is a component of NADPH oxidase. In phagocytes, p22phox and pg91phox (Nox 2) are essential subunits of flavocytochrome b558, and the absence of either protein results in a non-functional NADPH oxidase. It has been known that p22phox is also expressed in a variety of non-phagocytic cells, and p22phox can interact with distinct Nox proteins including Nox1 to Nox4 (Babior, 1999; Lassegue and Clempus, 2003; Quinn and Gauss, 2004). Many studies have indicated that the p22phox association is essential for the function of these Nox 2 homologues (Ambasta et al., 2004; Hanna et al., 2004; Ueno et al., 2005; Martyn et al., 2006; Bedard and Krause, 2007). Moreover, the transfection of antisense p22phox cDNA into vascular smooth muscle cells showed a decrease in the superoxide production, suggesting that p22phox subunit could play an essential role for the activity of NADPH oxidase (Ushio-Fukai et al., 1996).

The p22phox directly interacts with Nox proteins to form flavocytochrome b558, and both subunits are stable only as a heterodimer. However, each subunit of flavocytochrome b558 is endowed with specialized functions for the functionally active NADPH oxidase. The p22phox has two major functions: 1) binding to Nox proteins, leading to protein stabilization, and 2) serving as a docking site for the binding of cytosolic components (Vignais, 2002; Ambasta et al., 2004; Martyn et al., 2006). The gene encoding the p22phox (CYBA gene) is located on the long arm of chromosome 16 at 16q24. The CYBA gene is composed of 6 exons and 5 introns spanning 8.5 kb.

The predicted amino acid content of p22phox is 195 amino acids. Exon 6 encodes for approximately 35% of the 195 amino acids of the protein, while the other five exons encode for approximately 20–25 amino acids each (Dinauer et al., 1990).

The hydropathy profiles deduced from the gene sequences are compatible with at least two (possibly three or four) trans-membrane helices in p22phox. In the absence of crystallization data, there is no consensus on this matter. However, the weight of evidence favours this two trans-membrane structure with both the NH2 terminus and the COOH terminus facing the cytoplasm. The p22phox runs on Western blots with an apparent molecular mass of 22kDa, and it is not glycosylated (Bedard and Krause, 2007). The human CYBA gene promoter possesses a typical TATA and CCAC box and several potential consenses sequences for transcriptional factors, such as GAGA elements, γ-interferon and NF-κB. Several SP1 binding sites are also identified in close proximity to the ATG codon that can play a significant role in CYBA promoter activity (Moreno et al., 2003).

2.4.2 CYBA gene polymorphisms and their functional effects

In the international Haplotype Map Project (www.hapmap.org) database of polymorphisms in samples of European ancestry, there are at least 9 common SNPs (minor allele frequency >1%) in the 7.7-kb genomic segment. These polymorphisms have been the object of considerable research with the hypothesis that they might have important functional effects leading to significant inter-individual variation in enzyme activity. In the literature to date, there are at least three polymorphisms which have been widely investigated to show the association with cardiovascular disease and its associated traits with quite conflicting results, namely the C242T (rs4673), A640G (rs1049255) and -930 $^{A/G}$ polymorphism (rs9932581).

The C242T polymorphism (rs4673). The C242T polymorphism, located in exon 4, is considered the most interesting because of the structural change it causes in enzyme structure. The mutation results in an amino acid substitution (histidine to tyrosine) at position 72 of the protein (Inoue et al., 1998). Although it has been suggested that the histidine has a role in the binding of heme, the histidine of the p22phox would not be required for the electron transfer or heme binding (Biberstine-Kinkade et al., 2001; Biberstine-Kinkade et al., 2002). Moreover, the mutation is not associated with chronic granulomatous disease, which has been seen in patients with the defects in other components of the phagocytic NADPH oxidase. All in all, it is likely that the mutation might only have relatively subtle effects on the function of NADPH oxidase, which remains to be

elucidated. It is equally possible that it is simply a genetic marker of other functional variants either in the same gene or elsewhere (Soccio et al., 2005; San Jose et al., 2008).

The functional effect of the C242T polymorphism on enzyme activity has been investigated in several studies (Guzik et al., 2000a; Wyche et al., 2004; Moreno et al., 2006). Guzik et al. first provided evidence supporting the relation of the C242T polymorphism with O_2^- production. Compared with the C allele of the C242T polymorphism, the T allele was associated with reduced basal and NADH-stimulated superoxide production on the segments of human saphenous veins and mammary arteries from atherosclerotic patients (Guzik et al., 2000a). Another study by Wyche et al. showed that adults with the TT genotype had a significant reduction in the respiratory burst from neutrophils. In order to exclude a possible explanation of decreased NADPH oxidase reactivity due to reduced expression of the p22phox protein, the levels of p22phox were also assessed by Western analysis. By the same protein levels for all genotype, TT carriers had only approximately 30% O_2^- production compared to wild-type carriers (Wyche et al., 2004).

In addition, another piece of evidence for the existence of a functional effect of the C242T polymorphism on enzyme activity derives from the investigation of essential hypertension. The study results showed that hypertensive TT carriers had reduced NADPH oxidase-mediated O_2^- production when compared with CC carriers, whereas no difference was detected in the normotensive subgroup (Moreno et al., 2006). Consistently, a recent study by Arca et al. reported that the 242T allele was associated with reduced systemic oxidative stress represented as plasma levels of 8-hydroxy-2'-deoxyguanosine(8-OHdG)(Arca et al., 2008). In contrast, some studies reported that there were no effects or that the effects of the C242T polymorphism were opposite on the activity of NADPH oxidase (Shimo-Nakanishi et al., 2004; Macias-Reyes et al., 2008; Schirmer et al., 2008).

The -930 $^{A/G}$ polymorphism (rs9932581). The -930$^{A/G}$ polymorphism is located in the CYBA gene promoter, at position -930 from the ATC codon. The analysis of the promoter sequence showed that the -930 $^{A/G}$ polymorphic site lay on a potential binding site for C/EBP transcription factors (Moreno et al., 2003; San Jose et al., 2004). The functional effect of the -930$^{A/G}$ polymorphism resulted in a higher transcriptional activity of the CYBA gene in SHR (Spontaneously hypertensive rats) and in patients with essential hypertension. Moreover, the expression of the CYBA gene was influenced by the -930 $^{A/G}$ polymorphism in SHR and hypertensive subjects, whereas no effect was found in WKY (Wistar kyoto rats) and normotensive subjects (Moreno et al., 2003; San Jose et al., 2004; Wyche et al., 2004). Several findings showed a relevant role of C/EBPs in experimental hypertension (Zalba et al., 2001; Moreno et al., 2003; San

Jose et al., 2004); C/EBPs expression was nearly absent in WKY vascular SMCs, whereas it was abnormally increased in SHR SMCs. These observations suggested that the -930$^{A/G}$ polymorphism might influence p22phox gene expression depending on cell phenotype (San Jose et al., 2004). In addition, it could not be excluded that the -930$^{A/G}$ polymorphism may be in linkage disequilibrium with other genetic variants that might influence the transcriptional activity.

The A640G polymorphism (rs1049255). The A640G polymorphism is located in the 3' untranslated region of the CYBA gene. Recently, a functional study by Schirmer et al. indicated that the CYBA A640G polymorphism was associated with NADPH oxidase activity in a healthy population, and the superoxide production was significantly reduced in GG carriers when compared with AA individuals (Schirmer et al., 2008). In contrast, Macias-Reyes et al. reported that there are higher levels of G allele mRNA compared with A allele mRNA among the patients with angiographically verified coronary artery disease (Macias-Reyes et al., 2008). In addition, Wyche et al. reported that the CYBA A640G polymorphism has no effect on the activity of NADPH oxidase in a healthy population (Wyche et al., 2004).

2.4.3 CYBA gene polymorphisms in cardiovascular disease and its associated traits

Several polymorphisms of the CYBA gene have been identified, and the C242T polymorphism has been demonstrated to affect NADPH oxidase activity (Guzik et al., 2000a; Wyche et al., 2004). Therefore, the functional consequences of the C242T polymorphism could modify the cardiovascular risk and diseases by producing different amounts of O_2^-.

Endothelial dysfunction is the earliest hallmark of cardiovascular disease (Monnink et al., 2002). One study by a German research group showed that the C242T polymorphism is associated with increased endothelium-dependent vasodilating function of epicardial coronary arteries in patients (Schachinger et al., 2001). In contrast, an earlier study reported no functional effect for the C242T polymorphism on coronary vascular endothelial function in an American population (Li et al., 1999; Zafari et al., 2002). Brachial artery FMD is closely correlated with coronary endothelial function, thus reflecting not only peripheral but also coronary circulation (Anderson et al., 1995b; Monnink et al., 2002). Several studies have examined the endothelium-dependent (FMD) and independent (nitrate-induced) artery reactivity as an indicator of the functional consequences of the C242T polymorphism. No effects were observed in these studies for the C242T polymorphism on endothelium-dependent and independent vasodilatation (Zafari et al., 2002; Schneider et al., 2003; Fricker et al., 2004).

Since Inoue et al. first reported that the T allele of the C242T polymorphism might have a protective effect against CAD, a large number of studies using different ethnic populations from Europe, Asia and America had produced conflicting results (Inoue et al., 1998). Three Asian and two European case-control studies supported an association between the C242T polymorphism and the risk of CAD (Inoue et al., 1998; Lee et al., 2001; Shimokata et al., 2004; Arca et al., 2008; Macias-Reyes et al., 2008). In contrast, four studies suggested that the T allele was associated with more rapid progression of CAD (Cahilly et al., 2000; Nasti et al., 2006; He et al., 2007; Niemiec et al., 2007). Furthermore, there have also been many results showing no relationship between the C242T polymorphism and CAD (Cai et al., 1999; Gardemann et al., 1999; Li et al., 1999; Saha et al., 1999; Stanger et al., 2001; Zafari et al., 2002; Spence et al., 2003; Mata-Balaguer et al., 2004). When the analysis was concentrated on young patients, two research groups reported distinct results. The T allele became a risk factor for CAD in young Australian Caucasians (<45 years) (Cai et al., 1999), whereas it was a protective factor for CAD in Korean male patients under the age of 51 years (Lee et al., 2001). In their family-based study, Spence et al. also showed no association between the C242T polymorphism and the development of CAD (Spence et al., 2003). **(Table 3)**

There are conflicting results for the A640G polymorphism in relation to NADPH oxidase activity. Similarly, the associations of the A640G polymorphism with cardiovascular disease are contradictory between several studies. A recent study with a Spanish population suggested that GG carriers had a higher risk for coronary heart disease than AA carriers (Macias-Reyes et al., 2008). In contrast, the AA genotype of the A640G polymorphism was suggested to be independently associated with the presence and extent of CAD in a German population (Gardemann et al., 1999), whereas several other reports did not find such associations (Inoue et al., 1998; Zafari et al., 2002; Madamanchi et al., 2005; Park et al., 2005). It is possible that the polymorphism is simply a genetic marker of other functional variants either in the same gene or elsewhere.

Although the -930 $^{A/G}$ polymorphism has been associated with higher activity of NADPH oxidase in hypertension, the study by Sales et al. suggested that the -930 $^{A/G}$ polymorphism was not involved in cardiac or renal injury induced by hypertension (Sales et al., 2007). In contrast, a recent study with a Spanish population showed that the G allele of the -930 $^{A/G}$ polymorphism was associated with insulin resistance in obese subjects (Ochoa et al., 2008).

With an abundance of differing results, obtaining a sound replication has been a major concern in showing that the associations that have been identified reflect interesting biological processes. In addition to biological factors, the differences between the studies in terms of the frequency of a particular susceptibility variant, the genetic background or the environmental

exposure could affect the capacity to replicate results. However, questions of study design, implementation and interpretation can also be important (Hattersley and McCarthy, 2005).

Table 3. Association studies on the 242C/T polymorphism with coronary heart disease.

Study	Population	Design / Number of subjects	Source population	End point of study	Result to T allele
Inoue et al.1998	Japanese	Case-control (210/210)	Patients and matched controls	>75% stenosis	Positive*
Shimokata et al. 2004	Japanese	Case-control (1028/660)	Male hypercholesterolemia	>50% stenosis	Positive
Lee et al. 2001	Korean	Case-control (305/215)	Male patients	≥70% stenosis	Positive in age >50Y
Macias-Reyes et al. 2008	Spanish	Case-control (304/315)	Patients and matched controls	CHD	Positive in women
Arca et al. 2008	Italian	Cohort (213)	Patients with coronary stenosis	Recurrence of CHD	Positive
Saha et al. 1999	Singapore	Case-control (277/321)	Patients	>50% stenosis	No†
Gardemann et al. 1999	Germany	Case-control (1706/499)	Patients	>50% stenosis, AMI	No
Cai et al. 1999	Australian	Case-control (550/139)	Patients	≥50% stenosis	No
Zafari et al. 2002	American	Case-control (50/31+118)	Patients	≥50% stenosis	No
Stanger et al. 2001	Austrian	Case-control (108/45)	Patients	≥50% stenosis	No
Spence et al. 2003	British	Family-based (1023/388 Families)	At least one patient per family	≥70% stenosis	No
Li et al. 1999	American	Case-control (252/149)	Patients	Presence of atherosclerosis	No
Mata-balaguer et al. 2004	Spanish	Case-control (104/106)	Patients	AMI	No
Nasti et al.2006	Italian	Case-control (276/218)	Patients	≥70% stenosis	Negative‡
He et al. 2007	Chinese	Case-control (565/609)	Patients and matched controls	CHD	Negative
Niemiec et al. 2007	Polish	Case-control (172/169)	Patients and matched controls	≥50% stenosis	Negative
Cahilly et al. 2000	American	Cohort (313)	Patients with 30%–75% stenosis	Progression of atherosclerosis	Negative

Abbreviations: CHD, coronary heart disease; AMI, acute myocardial infarction. * Positive association with coronary heart disease; †No association with coronary heart disease; ‡ Negative association with coronary heart disease.

3 AIMS OF THE STUDY

The imbalance between the production and removal of ROS, a condition termed oxidative stress, plays a pivotal role in the pathogenesis of atherosclerosis and its associated complications. One of the major sources of ROS is NADPH oxidase. The p22phox is a component of NADPH oxidase, and it is essential for enzyme activity. The CYBA gene encodes p22phox, and several functional polymorphisms of the CYBA gene have been demonstrated to modulate the activity of NADPH oxidase. However, the studies on the association of the CYBA gene polymorphisms with cardiovascular disease and its associated traits have produced conflicting results.

The present studies used two Finnish populations comprising 2,283 young adults and 402 high-risk patients to elucidate the relationship of CYBA gene polymorphisms (rs4673, rs1049255, rs7195830 and rs9932581) with atherosclerosis at different stages of disease. The measured CRP level, brachial artery FMD and carotid artery IMT were used as surrogate markers for the detection of sub-clinical atherosclerosis in the Cardiovascular Risk in Young Finns Study. Angiographically verified coronary arterial stenosis (50% or 75%) was considered as coronary artery disease in the current studies in the Finnish Cardiovascular Study. The specific aims of the studies were:

1. To investigate whether the CYBA C242T gene polymorphism is associated with brachial artery FMD in a population of young adults, and whether common risk factors of atherosclerosis (e.g. age, sex, smoking and obesity) modify the genetic effect (I).

2. To examine the interaction effect of the CYBA -930 $^{A/G}$ polymorphism and common risk factors on carotid IMT in a population of young adults, and to elucidate the possible contribution of this common genetic variant to the individual differences in response to environmental challenge (II).

3. To analyse the role of CYBA gene polymorphisms and their haplotypes in the chronic inflammatory process represented by CRP level in a population-based sample of young adults. (III)

4. To study the association of CYBA C242T gene polymorphism with angiographically verified CAD in a high-risk Finnish Caucasian population. (IV)

4 SUBJECTS AND METHODS

4.1 Ethical aspects

4.1.1 The Cardiovascular Risk in Young Finns Study

The study was approved by the Ethics Committee of the Turku University Central Hospital and the University of Turku, Finland. Each subject gave written informed consent.

4.1.2 The Finnish Cardiovascular Study (FINCAVAS)

The study was approved by the Ethical Committee of the Hospital District of Pirkanmaa, Finland, and all subjects gave informed consent prior to the interview and measurements as stipulated in the Declaration of Helsinki.

4.2 Subjects

4.2.1 The Cardiovascular Risk in Young Finns Study (I–III)

The Cardiovascular Risk in Young Finns Study is an ongoing five-centre, prospective cohort study of atherosclerosis risk factors in Finnish children and adolescents. 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 (www.utu.fi/med/cardio/youngfinnsstudy) (Åkerblom et al., 1985; Raitakari et al., 2008). The first cross-sectional survey was conducted in 1980 with 3,596 responders. Between 1980 and 1992, these cohorts were followed up at 3-year intervals. The latest 21-year follow-up was performed in 2001, enrolling 2,283 subjects aged 24 to 39 years from the original cohort. Non-invasive ultrasound studies were first introduced to the study to measure markers of sub-clinical atherosclerosis. These included carotid artery IMT and brachial artery endothelium-dependent FMD. In addition, novel risk factors such as highly sensitive CRP (hsCRP) levels were analysed, and blood samples for genetic analyses were also added to the 21-year follow-up protocol (Raitakari et al., 2008).

Study I. A sub-population for Study I included 2,058 subjects for whom complete data on the brachial artery FMD and the genotype of the C242T polymorphism (rs4673) were available.

Study II. A total of 2,179 subjects were included in Study II. 104 subjects were excluded due to missing data and unsuccessful genotyping of the -930 $^{A/G}$ polymorphism (rs9932581).

Study III. A total of 2,059 subjects were included in Study III. 224 subjects were excluded due to a hsCRP level exceeding 10 mg/l, missing data and unsuccessful genotyping of the C242T polymorphism.

4.2.2. A subpopulation of the Finnish Cardiovascular Study (IV)

The Finnish Cardiovascular Study (FINCAVAS) was designed to find exercise test markers predicting future cardiovascular events and deaths (Nieminen et al., 2006). Originally, all consecutive patients who underwent exercise stress tests at Tampere University Hospital between October 2001 and December 2007 were invited to participate in the study. By the end of 2004, 2,290 patients had been enrolled. Of these patients, 542 also underwent coronary angiography at the Department of Cardiology. For Study IV, study subjects were selected from these 542 patients. Patients were eligible for the study only if they had risk factors including smoking, body overweight, hypertension, hypercholesterolemia or diabetes mellitus. 402 patients were finally included in the analysis. 139 were excluded due to lacking blood samples or incomplete angiographic data, and one patient was excluded because he had none of the risk factors examined in the study.

4.3 Methods

4.3.1 Physical examination and questionnaires (I–IV)

4.3.1.1 The Cardiovascular Risk in Young Finns Study (I–III)

Data including age, sex, smoking habits, physical activity, use of hormonal contraceptives, pregnancy, lactation, recent infection and chronic rheumatic diseases were acquired using questionnaires. Weight and height were measured and body mass index (BMI) calculated (kg/m²). A BMI over 25 kg/m² was defined as overweight, and a BMI of over 30 kg/m² was defined as obese (De Backer et al., 2003). The category of smokers (ever-smokers) included both current and ex-smokers (former smokers). Current smokers were defined as those who were currently smoking on a daily or weekly basis. Ex-smokers included those who reported regular smoking in the past but no current smoking (Study I, II). Among non-smokers, those who had been regularly exposed to environmental tobacco smoke at home or in the workplace were classified as non-smoking passive smokers.(Study III) Pack-years were assessed for the smokers; Pack-year = (the number of cigarettes per day/20)*year of smoking (Study II). A metabolic equivalent index (MET) for physical activity was calculated from the product of intensity, frequency, duration and commuting physical

activity (Ainsworth et al., 1993). The subjects were divided into the tertiles of high, medium and low physical activity according to their MET index, and those for whom complete data was not available were classified into the non-responders category (Study III). Blood pressure (BP) in a sitting position was measured by trained research nurses after a 5-minute rest with a random zero sphygmomanometer. Korotkoff's fifth sound was used as the sign of diastolic BP and first sound as the sign of systolic BP. The mean of three measurements on three different visits was used in the analysis. The subjects were defined as hypertensive if the systolic BP was ≥140mmHg and/or diastolic BP ≥ 90mmHg, and/or if they had already been treated with (antihypertensive) drugs.

4.3.1.2 The Finnish Cardiovascular Study (IV)

Data including age, sex, smoking habits, history of hypertension, hypercholesterolemia, diabetes mellitus and medication were collected using a computer-based questionnaire form at an interview or hospital records at Tampere University Hospital. The data on angiography procedures were collected from the Hospital Information System of Tampere University Hospital. Weight and height were recorded and BMI calculated (kg/m²). Blood pressure in a sitting position was measured using a mercury sphygmomanomenter. Subjects were considered smokers if they were either current or ex-smokers. They were defined as hypertensive if the systolic BP was ≥140mmHg and/or diastolic BP ≥ 90mmHg, or if they had already been treated with antihypertensive drugs. Subjects were classified as hypercholesterolemic if their total cholesterol level was ≥6.21mmol/L or if they had already been treated with cholesterol-lowering drugs. Patients were defined as diabetic if their fasting blood glucose concentration was ≥7.1mmol/L or if they were already undergoing treatment for diabetes. Body overweight was defined as BMI≥25kg/ m² (Nieminen et al., 2006).

4.3.2 Blood collection and analyses (I–IV)

Bloods were drawn for the biochemical and genetic analyses after an overnight fast. Assays for the Cardiovascular Risk in Young Finns Study were done in duplicate in the laboratory of the Research and Development Unit of the Social Insurance Institution, Turku, Finland (Study I, II, III). Assays for Finnish Cardiovascular Study were done at the Laboratory of Tampere University Hospital (Study IV). Standard enzymatic methods were employed for the determination of serum total cholesterol, high-density lipoprotein cholesterol (HDL-C) and triglycerides. Low-density lipoprotein cholesterol (LDL-C) concentration was calculated with the Friedewald formula (Friedewald et al., 1972). Serum glucose was measured enzymatically (glucose dehydrogenase, Olympus Diagnostica GmbH, Hamburg, Germany). Serum creatinine was determined by routine laboratory methods. An estimated glomerular filtration rate (GFR) was calculated with the

Cockroft-Gault formula (Cockcroft and Gault, 1976). Serum high-sensitive CRP protein was assessed using an automated analyzer (Olympus AU 400; Tokyo, Japan) and a highly sensitive turbidimetric immunoassay kit (CRP-UL-assay; Wako Chemicals, Neuss, Germany). The lower detection limit reported for the assay was 0.06mg/L. The inter-assay coefficient of variation was 3.33% at the mean level of 1.52 mg/L and 2.65% at the mean level of 2.51 mg/L.

4.3.3 Ultrasound measurements (I, II)

The ultrasound study of brachial artery FMD and carotid IMT were performed using Sequoia 512 ultrasound mainframes (Acuson, Mountain View, California, USA) with 13.0 MHz linear array transducers (Raitakari et al., 2003; Juonala et al., 2004b). All studies were performed simultaneously between September 2001 and January 2002 in the five cities of the multi-centre study (Helsinki, Kuopio, Oulu, Tampere and Turku).

4.3.3.1 Brachial artery flow-mediated dilatation (FMD) (I)

The left brachial arterial flow was interrupted for 4.5 min by a cuff placed on the proximal forearm at a pressure of 250 mmHg. The arterial diameter was measured at a fixed distance from an anatomic marker at rest (baseline) as well as 40, 60 and 80 seconds after cuff deflation. The average of the three measurements at each time point was used to derive the maximum brachial artery FMD, which is the greatest value between 40 and 80 seconds. FMD was the increase in vessel diameter after reactive hyperemia, expressed as the percentage relative to a resting scan. The long-term variation of study was assessed via re-examining a 2.5% random sample after the initial visit. The scans were measured twice by the same reader. The 3-month between-visit coefficient of variation was 3.2% for brachial diameter and 26.0% for FMD.

4.3.3.2 Carotid artery intima media thickness (IMT) (II)

The posterior (far) wall of the left carotid artery was scanned according to a standardized protocol.(Raitakari et al., 2003) A resolution box function (zoom) was used to record an image of 25mm in width and 15mm in height. 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 on optical disks for subsequent off-line analysis. The digitally stored scans were manually analyzed by a single reader blinded to the subjects' details, with the analyses performed using ultrasonic callipers. The best-quality end-diastolic frame was selected from the five-second scan (incident with the R-wave on a continuously

recorded electrocardiogram). From this image, a minimum of four measurements of the carotid wall was taken approximately 10 mm proximal to the bifurcation to derive the mean and maximal IMT. To assess the reproducibility of IMT measurements, 60 participants were re-examined 3 months after the initial visit. The between-visit coefficient of variation for IMT measurements was 6.4%. To assess the reproducibility of IMT image analyses, 113 scans were reanalyzed by a second observer. The between-observer coefficient of variation was 5.2%.

4.3.4 Coronary angiography (IV)

Coronary angiography was performed using the standard Judkins' technique (Judkins, 1967). All the coronary angiographies were analysed by the same dedicated cardiologist. A percentage of stenosis in the ostial, proximal, middle and distal parts of the left main (LM), the left anterior descending (LAD), the left circumflex (LCX) and the right coronary artery (RCA) was recorded. The same parameters were also assessed for major branches: the diagonal branches I and II as well as the first septal branch of the LAD; the left obtuse marginal I and II as well as posterior descending branches of the LCX; the posterior descending, right ventricular and posterior left ventricular branches of the RCA; and the left intermediate marginal branch. The stenoses were classified into seven categories: 0%, <50%, 50%–74%, 75%–89%, 90%–98%, 99% and 100% (Nieminen et al., 2006). By means of coronary angiography, the patients with significant coronary arterial stenosis affecting at least one vessel were defined as CAD patients, and patients with no stenosis or stenosis under a cut-off point were defined as control subjects in the study. The analysis was first carried out by comparing subjects with ≥50% (n=250) to those with <50% (n=152) stenosis, after which the second analysis was carried out in the same study group by comparing those with ≥75% (n=209) to those with <75% (n=193) of stenosis.

4.3.5 DNA extraction, packing tag SNPs and genotyping (I–IV)

Whole venous bloods were drawn into EDTA tubes and then stored at -70°C. Genomic DNA was purified using the Quiagen QIAmp DNA Blood Mini Kit (Quiagen Inc., Hilden, Germany) or by the BioRobot M48 Workstation (Quiagen Inc., Hilden, Germany) according to the manufacturers' instructions.

Using the Seattle SNPs Genome Variation Server (http://gvs.gs.washington.edu/GVS), six tagSNPs (minor allele frequency≥5%) of the CYBA gene (including 3kb upstream and downstream of gene) were found for the coverage of a total of three linkage disequilibrium bins (r^2>0.8) in a European-descent population studied by Perlegen Sciences. Based on these data, three tagSNPs (rs4673, rs1049255, rs7195830) chosen from each bin were investigated in the Cardiovascular Risk

in Young Finns Study. In addition, the CYBA -930 $^{A/G}$ gene polymorphism (rs9932581) in the promoter region was also eligible for both the Cardiovascular Risk in Young Finns Study and the Finnish Cardiovascular Study, because it had been reported to yield a functional effect on the activity of NADPH oxidase.

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 published in the GeneBank or Celera databases, designed in conjugation with Applied Biosystems with the aid of the Assays by Design service. Polymerase chain reaction (PCR) for CYBA gene polymorphisms was performed in 384 well plates following a standard protocol for TapMan MGB probes. A 5µl final volume containing genomic DNA, 2×Universal Master Mix, 900 nM of each primer, and 200 nM of each probe was used in PCR. PCR pipetting steps were carried out with the Tecan© robot with the Freedom EVOware, ver. 1.0 programme (Tecan treding AG, Männedorf, Switzerland). After the PCR was finished, end-point fluorescence was measured and allelic discrimination performed using the ABI prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Random duplicates were used as a quality control. All Genotypes were analysed in the Centre for Laboratory Medicine, Department of Clinical Chemistry, Laboratory of Atherosclerosis Genetics at Tampere University Hospital.

4.4 Statistical analyses

The continuous variables are presented as mean ± standard deviation (SD) in all tables. Differences across study groups were examined using the χ^2-test for categorical variables, the Student's t-test and analysis of variance (ANOVA) for continuous variables. Due to skewed distributions, the values for triglycerides and hsCRP were log10-transformed, and blood glucose reciprocal-transformed before the analyses (Studies I–III). In Study I, multiple linear regression analysis was used to examine whether the gene polymorphism was an independent predictor of FMD. In Study II, a linear regression model was employed to test the relation between IMT and the interaction of the studied gene polymorphism with risk factors. To further examine the interaction effect on IMT, stratified analysis was performed with analysis of covariance (ANCOVA). In Study III, stepwise multiple linear regression analysis was used to examine whether the gene polymorphisms or carriage of haplotypes were independent predictors of hsCRP. In Study IV, the multiple logistic regression method was used to determine the independent risk factors for CAD, and the odds ratio (OR) of CAD was also calculated for the study subjects exposed to the risk factors. All statistical analyses were two-sided and performed with the statistical software SPSS (versions13.0–14.0;

SPSS, Chicago, IL). P values below 0.05 were considered statistically significant. The LDA (linkage disequilibrium analyser 1.0) freeware was used to test the linkage disequilibrium between each pair of loci (Ding et al., 2003). Haplotypes were inferred with the PHASE version 2.1.1 programme, which uses a Bayesian statistical method for reconstructing haplotypes from population genotype data and lists the most probable haplotype pairs for each individual (Stephens and Donnelly, 2003).

5 RESULTS

5.1 Characteristics of the study populations, the CYBA allele frequencies and haplotypes

The distribution of CYBA genotypes and allele frequencies in the Cardiovascular Risk in Young Finns Study (n=2283) and a subpopulation of the Finnish Cardiovascular Study (n=402) are provided in Table 4A and B. The genotype distributions in two populations were in Hardy-Weinberg's equilibrium.

Table 4. Distribution of CYBA gene polymorphisms and allele frequencies in two populations:
A. Cardiovascular Risk in Young Finns Study (n=2283).

SNP	Genotype, n (%)				Allele Frequency	
rs4673	N*	CC	CT	TT	C	T
	2237	1482 (66.2%)	669 (29.9%)	86 (3.8%)	0.81	0.19
rs9932581	N	GG	GA	AA	G	A
	2243	774 (34.5%)	1077 (48.0%)	392 (17.5%)	0.58	0.42
rs1049255	N	AA	AG	GG	A	G
	2264	656 (29.0%)	1112 (49.1%)	496 (21.9%)	0.54	0.46
rs7195830	N	GG	GA	AA	G	A
	2274	964 (42.4%)	1025(45.1%)	285 (12.5%)	0.65	0.35

*N, number for successful genotyping

B. A Subpopulation of the Finnish Cardiovascular Study (n=402).

SNP	Genotype, n (%)				Allele Frequency	
rs4673	N*	CC	CT	TT	C	T
	402	258 (64.2)	124 (30.8)	20 (5.0)	0.80	0.20
rs9932581	N	GG	GA	AA	G	A
	394	146 (37.1)	181 (45.9)	67 (17.0)	0.60	0.40

*N, number for successful genotyping

Among three selected tagSNPs, the relationship between genotypes at each pair of polymorphic sites was in linkage disequilibrium in the Young Finns Study (n=2283) **(Table 5)**. The results were comparable to a European-descent population studied by Perlegen Science. However, the -930 $^{A/G}$ polymorphism (rs9932581) was shown to segregate independently from the C242T polymorphism. Therefore, the haplotypes were estimated from three SNPs (rs4673, rs1049255, rs7195830) using the PHASE programme.

Table 5. Pair-wise LD strength among the 4 CYBA SNPs was measured in the Young Finns Population (n=2283).

SNP1	SNP2	r^2	D’	χ^2	p.value
rs7195830	rs1049255	0.4495	0.9796	2034.79	<0.0001
rs7195830	rs 4673	0.1132	0.9491	510.51	<0.0001
rs7195830	rs9932581	0.0029	0.0876	13.32	0.0003
rs1049255	rs 4673	0.0557	0.4538	250.60	<0.0001
rs1049255	rs9932581	0.0296	0.2195	133.46	<0.0001
rs4673	rs9932581	<0.0001	0.0052	0.039	0.8426

The haplotype reconstruction resulted in eight haplotypes. The frequencies of haplotypes are given in Table 6.

Table 6. Haplotypes in the Young Finns Population (n=2283).

Haplotype	242C>T (rs4673)	640A>G (rs1049255)	673A>G (rs7195830)	Frequency (%)
1	C	A	G	13.6
2	C	A	A	34.4
3	C	G	G	32.9
4	C	G	A	0.3
5	T	A	G	5.2
6	T	A	A	0.3
7	T	G	G	13.3
8	T	G	A	0.009

The characteristics of the study populations are shown in Table 7 and Table 8. The study subjects in the Cardiovascular Risk in Young Finns Study (n=2283) formed a low-risk population: they were young, with 13.8% (n=315) suffering from hypertension and 1% (n=23) from diabetes. However, many of the subjects (52.7%, n=1203) had a smoking habit, and 43.1% (n=980) were overweight (BMI≥25 kg/m²). Compared with the complete population (n=2283), the subjects in Study III had significantly lower hsCRP levels. In contrast, the study subjects in a subpopulation of the Finnish Cardiovascular Study (n=402) formed a high-risk population: 5% (n=21) had one risk factor for CAD, 32% (n=87) had two, 30% (n=120) three, 35% (n=141) four, and 8% (n=33) as many as five. Moreover, the prevalence of risk factors remained very high in the control population as well. The prevalence of hypertension, hypercholesterolemia, smoking and body overweight was 95%, 57%, 52% and 74% among the controls without 50% coronary stenosis, respectively. The risk factors examined in the study were body overweight, smoking, diabetes mellitus, hypertension and hypercholesterolemia.

Table 7. Characteristics of the populations: 2,283 subjects in the Young Finns Study; 2,058 subjects in Study I; 2,179 subjects in Study II; and 2,059 subjects in Study III. The continuous variables are presented as mean ± SD. P values were obtained by ANOVA. * P<0.05 compared with all subjects, subjects in Study I and Study II.

	All (n=2283)	Study I (n=2058)	Study II (n=2179)	Study III (n=2059)	P value
Age, (years)	32 ± 5	32 ± 5	32 ± 5	32 ± 5	0.974
Male sex, n (%)	1026 (44.9)	907 (44.1)	980 (45.0)	935 (45.4)	0.853
Body mass index, kg/m²	25 ± 4	25 ± 4	25 ± 4	25 ± 4	0.776
Smoking, n (%)	1203 (52.7)	1082 (52.6)	1151 (52.8)	1083 (52.6)	0.782
Systolic BP, mm Hg	122 ± 15	122 ± 14	122 ± 15	122 ± 15	0.923
Diastolic BP, mm Hg	73 ± 9	73 ± 9	73 ± 9	73 ± 9	0.991
Total cholesterol, mmol/l	5.16 ± 0.98	5.16 ± 0.99	5.16 ± 0.98	5.15 ± 0.97	0.980
HDL cholesterol, mmol/l	1.29 ± 0.32	1.29 ± 0.32	1.29 ± 0.32	1.29 ± 0.32	0.979
LDL cholesterol, mmol/l	3.27 ± 0.86	3.27 ± 0.86	3.28 ± 0.85	3.27 ± 0.84	0.982
Triglycerides, mmol/l	1.34 ± 0.85	1.35 ± 0.87	1.32 ± 0.79	1.31 ± 0.77	0.793
Glucose, mmol/l	5.05 ± 0.83	5.04 ± 0.84	5.04 ± 0.776	5.04 ± 0.77	0.936
C-reactive protein, mg/l	1.93 ± 3.95	1.88 ± 3.70	1.93 ± 4.00	1.36 ± 1.70*	0.008

Table 8. Characteristics of a subpopulation of the Finnish Cardiovascular Study (n=402). The values are presented as n (%) or mean ± SD.

Characteristic	All subjects (n=402)	CAD50* (n=250)	Control50 (n=208)	CAD75ǂ (n=209)	Control75 (n=193)
Male	281(70)	193(77)	88 (58)	163 (78)	118 (61)
Age, years	59 ± 11	62 ± 10	55 ± 11	63 ± 10	55 ± 11
Body mass index, kg/m²	27.3 ± 4.3	27.3 ± 4.1	27.2 ± 4.6	27.3 ± 4.0	27.3 ± 4.6
Risk factors, n (%)					
Body overweight	300 (75)	187 (75)	113 (74)	159 (76)	141 (73)
Smoker	226 (56)	147 (65)	79 (35)	122 (58)	104 (54)
Diabetic	97 (24)	72 (29)	25 (16)	62 (30)	35 (18)
Hypertension	382 (95)	293 (96)	145 (95)	201 (96)	183 (95)
Hypercholesterolemia	279 (69)	193(77)	86 (41)	161 (77)	118 (61)

* CAD cases with 50% coronary stenosis; ǂ CAD cases with 75% coronary stenosis

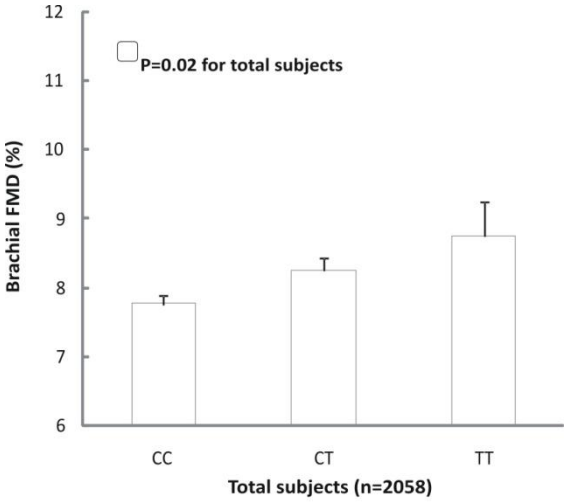
5.2 CYBA C242T gene polymorphisms and brachial artery FMD (I)

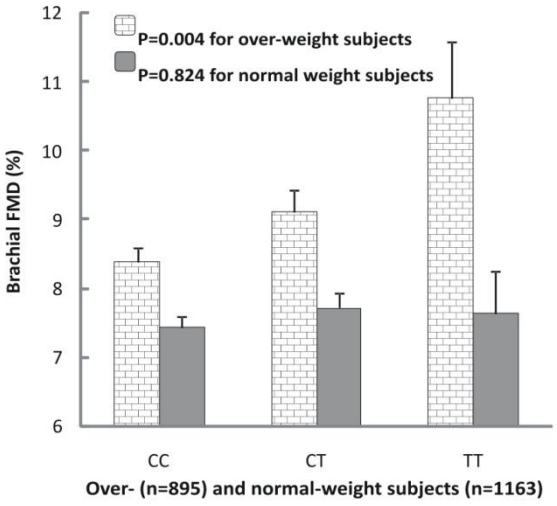
The T allele frequency of the CYBA C242T gene polymorphism was in line with hardy-Weinberg's equilibrium for all study subjects (n=2058) as well as for the subgroups stratified by smoking status and body size.

FMD, defined as the increased percentage in brachial artery diameter after reactive hyperaemia was assessed by ultrasound. In all subjects, the mean value of brachial artery FMD was significantly influenced by genotype and displayed a genotype-dependent effect **(Figure 6).** The mean values were 8.0± 4.4% for all study subjects, with 7.8± 4.4, 8.2± 4.5 and 8.7± 4.5% in subjects with the CC, CT and TT genotype of the CYBA C242T gene polymorphism, respectively. The relationship of FMD with genotype was significantly present in the overweight (n=895) and ever-smoking population (n=1080), but not among the normal-weight (n=1163) and non-smoking subjects (n=964). Moreover, no relationship was found in former smokers (n=305, β=0.075, P=0.143) **(Figure 6)**.

Multivariate linear regression analysis indicated that the CYBA C242T polymorphism was an independent determinant of FMD in the entire study population. The statistically significant relationship between the CYBA C242T polymorphism and FMD was observed in overweight subjects and ever-smokers, but not in the normal-weight subjects and non-smokers. **(Table 9)**

Figure 6. Bar graphs showing the brachial artery FMD (mean ± SEM%) of the study groups(n=2,058) stratified by the genotype of the CYBA C242T polymorphism in all, in overweight and normal-weight subjects, and in smoking and non-smoking subjects. The P values are from a linear regression analysis testing a linear relationship between the genotypes and FMD.





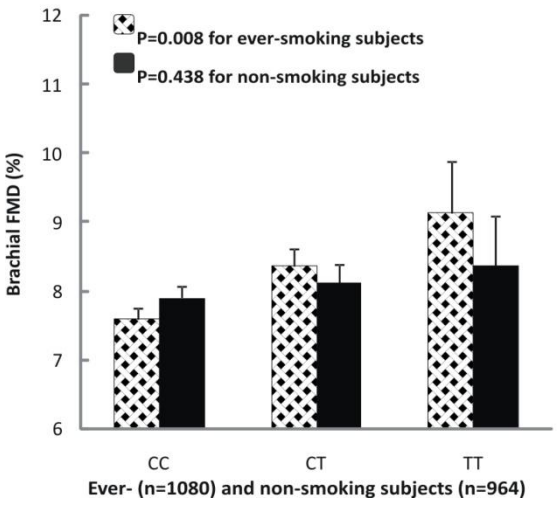


Table 9. Multiple linear regression for predicting brachial artery FMD in the entire study population as well as subgroups stratified by smoking status and body size. Statistical model: the C242T polymorphism; age; sex; BMI; smoking; systolic/diastolic BP; glucose; hsCRP; total, HDL and LDL cholesterol; and triglycerides were entered as predictors into the multiple linear regression model.

Predictors	All Subjects n=2085	BMI≥25 n=895	BMI<25 n=1163	Smokers n=1082	Non-Smokers n=962
	B±SE	B±SE	B±SE	B±SE	B±SE
C242T polymorphism	0.399± 0.170 *	0.734± 0.250**	0.071± 0.232	0.651± 0.233**	-0.034± 0.019
Age	0.009± 0.020	0.000± 0.032	0.010± 0.027	-0.007± 0.028	0.022± 0.027
Male sex	-1.522± 0.246***	-1.694± 0.378***	-1.530± 0.336***	-1.416± 0.332***	-1.620± 0.365***
Body mass index	0.132± 0.028***	0.055± 0.046	0.160± 0.073**	0.123± 0.038***	0.136± 0.041***
Smoking	0.057± 0.191	-0.218± 0.288	0.259± 0.256	--	--
Systolic BP	-0.034± 0.012**	-0.042± 0.017**	-0.028± 0.018	-0.039± 0.017**	-0.034± 0.019
Diastolic BP	0.014± 0.018	0.021± 0.026	0.012± 0.027	0.010± 0.025	0.022± 0.027
Glucose	0.762± 5.059	4.125± 7.488	-2.374± 6.893	7.601± 6.951	-6.147± 7.386
Total cholesterol	-0.511± 0.580	-0.150± 0.716	-2.310± 1.370	-0.494± 0.717	-0.314± 0.983
HDL cholesterol	1.331± 0.679*	0.765± 0.913	3.107± 1.416**	1.827± 0.869	0.662± 1.106
LDL cholesterol	0.556± 0.568	-0.001± 0.689	2.540± 1.368	0.460± 0.700	0.478± 0.967
Triglycerides	1.947± 1.097	1.377± 1.597	3.939± 1.933**	1.769± 1.471	1.894± 1.684
C-reactive protein	0.231± 0.206	0.160± 0.311	0.300± 0.275	0.383± 0.283	1.104± 0.300
Adjusted R^2	0.069	0.082	0.050	0.080	0.058
Significance (ANOVA)	<0.001	<0.001	<0.001	<0.001	<0.001

B: Regression coefficient; SE: Standard error; ANOVA: Analysis of variance; *=P<0.05, **=P<0.01, ***=P<0.001

5.3 CYBA -930A/G polymorphism and risk factor interactions in relation to carotid IMT (II)

The allele frequencies of the CYBA -930 $^{A/G}$ gene polymorphism were in line with Hardy-Weinberg's equilibrium for all study subjects (n=2179) as well as the subgroups stratified by hypertension and smoking status.

Smoking is an important risk factor for the development of atherosclerosis, and IMT is a marker of subclinical atherosclerosis. However, the vascular changes in response to smoking vary from individual to individual. Among all subjects, the mean and maximal IMT values were significantly higher among smokers (current and ex-smokers) than non-smokers in the present study. The differences were most significant in subjects with the GG genotype, borderline significant for the GA genotype and non-significant for the AA genotype. All these results remained similar after adjusting for potential risk factors including age, sex, BMI, systolic BP, diastolic BP, blood glucose, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, hsCRP and estimated glomerular filtration rate. (Table 8) Similarly, the mean and maximal IMT values were increased in subjects with high BP in comparison to those with normal BP. The differences remained significant in subjects with the GG genotype, whereas they were non-significant among all subjects as well as those with the GA or the AA genotype after adjusting for the potential risk factors mentioned above. **(Table 10)**

In the entire population, the interaction effect of the -930 $^{A/G}$ polymorphism and risk factors, such as smoking or hypertension, was examined with a linear regression model. The interaction of genotype with smoking was associated with mean and maximal IMT, and it remained significant (β=-0.168, P=0.042 and β =-0.190, P=0.022, respectively) when the model was further adjusted for the aforementioned risk factors. Similarly, there was a relationship between mean and maximal IMT and the interaction of genotypes with hypertension, and the relation remained significant after adjustment for potential risk factors (β=-0.225, P=0.012 and β =-0.204, P=0.023, respectively).

The magnitude of the interaction was further examined by performing a stratified analysis according to smoking status or hypertension. The mean and maximal IMT values were different across the genotypes, and the results remained similar after adjusting for potential risk factors among smokers and non-smokers as well as hypertensive and normotensive subjects. **(Table 11)**

Table 10. Differences in carotid artery IMT (mm) between smokers and non-smokers, as well as high BP and normal BP among all study subjects and the study groups stratified by genotype. The variables are presented as mean ± SD. P values were obtained by ANOVA, and p' values were ANCOVA-adjusted for potential risk factors including age, sex, BMI, systolic BP, diastolic BP, blood glucose, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, hsCRP and estimated glomerular filtration rate.

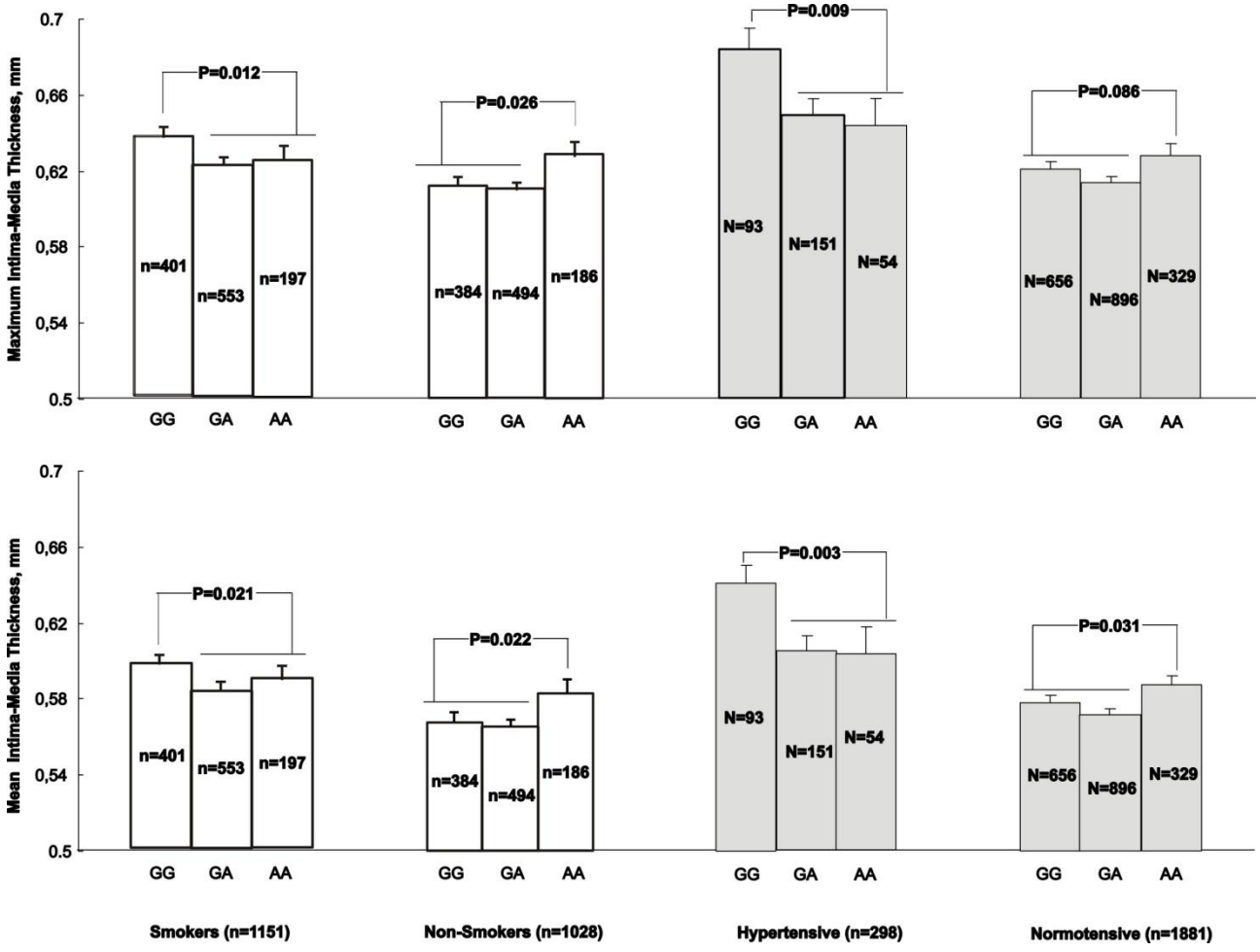
	Smoker	Non-smoker	p	p'	Hypertensive	Normotensive	p	p'
All	n=1151	n=1028			n=298	n=1881		
Mean IMT	0.59±0.09	0.57±0.09	0.002	0.005	0.62±0.10	0.58±0.09	<0.001	0.256
Max IMT	0.63±0.10	0.62±0.10	0.002	0.008	0.66±0.10	0.62±0.10	<0.001	0.264
GG	n=401	n=348			n=93	n=656		
Mean IMT	0.60±0.09	0.57±0.09	0.001	0.005	0.64±0.10	0.58±0.09	<0.001	0.002
Max IMT	0.64±0.10	0.61±0.10	0.001	0.004	0.68±0.10	0.62±0.10	<0.001	0.003
GA	n=553	n=494			n=151	n=896		
Mean IMT	0.58±0.09	0.57±0.09	0.051	0.060	0.60±0.10	0.57±0.09	<0.001	0.748
Max IMT	0.62±0.09	0.61±0.01	0.055	0.068	0.65±0.10	0.61±0.09	<0.001	0.748
AA	n=197	n=186			n=54	n=329		
Mean IMT	0.59±0.09	0.59±0.10	0.82	0.67	0.60±0.10	0.59±0.10	0.261	0.167
Max IMT	0.63±0.10	0.63±0.10	0.60	0.44	0.64±0.10	0.63±0.10	0.263	0.133

Among smokers, or among hypertensive subjects, those with the GG genotype had similarly higher mean and maximal IMT values when compared with carriers of the A allele. In contrast, the mean and maximal IMT were lower in carriers of the G allele than in subjects with the AA genotype among non-smokers or normotensive subjects. The results are shown in **Figure 7.**

Table 11. Differences in carotid artery IMT (mm) between the genotypes among smokers and non-smokers as well as subjects with high and normal BP. The variables are presented as mean ±SD. * <0.05 vs.GG, †<0.05 vs GA. P values were obtained by ANOVA, and p' values were ANCOVA-adjusted for potential risk factors including age, sex, BMI, systolic BP, diastolic BP, blood glucose, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, hsCRP and estimated glomerular filtration rate.

	GG	GA	AA	p	p'
Smoker	n=401	n=553	n=197		
Mean IMT	0.60±0.09	0.58±0.09*	0.59±0.09	0.054	0.047
Max IMT	0.64±0.10	0.62±0.09*	0.63±0.10	0.044	0.039
Non-smoker	n=348	n=494	n=186		
Mean IMT	0.57±0.09	0.57±0.09	0.59±0.10†	0.048	0.061
Max IMT	0.61±0.10	0.61±0.01	0.63±0.10*†	0.056	0.067
Hypertensive	n=93	n=151	n=54		
Mean IMT	0.64±0.10	0.60±0.10*	0.60±0.10*	0.010	0.014
Max IMT	0.68±0.10	0.65±0.10*	0.64±0.10*	0.018	0.031
Normotensive	n=656	n=896	n=329		
Mean IMT	0.58±0.09	0.57±0.09	0.59±0.10†	0.030	0.037
Max IMT	0.62±0.10	0.61±0.09	0.63±0.10†	0.060	0.069

Figure 7. Bar graphs show the maximum and mean IMT of the study groups stratified by genotype among smokers and non-smokers as well as hypertensive and normotensive subjects. Values are means±SEM. P values were obtained by ANCOVA; the effect of the -930 $^{A/G}$ polymorphism was assumed to be G recessive (GG vs. AA and GA combined) among smokers and hypertensive subjects, or G dominant (GA and GG combined vs. AA) among non-smokers and normotensive subjects; age, sex, BMI, systolic BP, diastolic BP, blood glucose, total cholesterol, HDL, LDL-cholesterol, triglycerides, hsCRP and GFR were used as covariates.



5.4 CYBA gene polymorphisms and chronic inflammation measured as C-reactive protein level (III)

The genotype frequencies for four polymorphisms of the CYBA gene were in Hardy-Weinberg's equilibrium. The linkage disequilibrium between each pair of loci was examined in the present study population (n=2059). The results (Study III) were comparable to the entire population of the Cardiovascular Risk Young Finns Study (n=2283, see Tables 5 and 6). The haplotype reconstruction resulted in eight haplotypes; three haplotypes were not used in the analyses due to low frequency (<1%, see Table 6).

The differences in hsCRP levels across the genotypes of CYBA polymorphisms are summarized in Table 12. A significant difference in hsCRP levels was found between the genotypes of the C242T polymorphism (rs4673) after adjustment for covariates.

The study also examined the effect of CYBA haplotypes on hsCRP levels. **(Table 13)** The level of hsCRP differed between carriers and non-carriers of haplotype 5, with the carriers having the lowest hsCRP level. The difference was significant after adjusting for covariates (covariates were the same as those listed in Table 12).

The multiple linear regression models were used to test the relationship between the CYBA gene polymorphisms (model 1) /or CYBA haplotype carriage (model 2) and the level of hsCRP. The genotype effect of the C242T polymorphism was assumed to be recessive (CC+CT vs TT). The stepwise multiple linear regression analysis indicated that the C242T polymorphism and CYBA haplotype 5 carriage (in separate models) were independent determinants of hsCRP levels in the population. **(Table 14)**

Table 12. The relationship between CYBA gene polymorphisms and hsCRP level in study population II (n=2059). Values of hsCRP are presented as mean±SD. P values were obtained as follows: * ANOVA; ** ANCOVA, with age, sex and BMI used as covariates; *** ANCOVA, with age, sex, BMI, smoking, physical activity, hormonal contraceptives, lactation, pregnancy, systolic/or diastolic BP, blood glucose, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, history of recent infection and chronic rheumatic disease as covariates.

SNP (n)	CRP (mg/l)	p*	p**	p***
rs4673				
CC (1373)	1.35±1.71	0.189	0.045	0.036
CT (609)	1.40±1.72			
TT (77)	1.03±1.40			
rs1049255				
AA (608)	1.37±1.74	0.315	0.667	0.505
AG (1008)	1.33±1.70			
GG (443)	1.40±1.68			
rs7195830				
AA (266)	1.33±1.52	0.598	0.439	0.699
AG (940)	1.42±1.81			
GG (853)	1.29±1.64			
rs9932581				
GG (701)	1.35±1.64	0.394	0.416	0.539
GA (995)	1.38±1.75			
AA (363)	1.31±1.68			

Table 13. hsCRP levels according to CYBA haplotype in the YoungFinns population (n=2059). hsCRP was presented as mean±SD; P values were obtained as follows: * ANOVA; ** ANCOVA, with age, sex and BMI as covariates; *** ANCOVA, with age, sex, BMI, smoking, physical activity, hormonal contraceptives, lactation, pregnancy, systolic/or diastolic BP, blood glucose, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, history of recent infection and chronic rheumatic disease as covariates. Haplotype composed of the SNPs rs4673C>T/ rs1049255A>G/ rs7195830 A>G; Haplotype carriers were presented as '+', and non-carriers as '–'

Carriage of haplotype (n)	CRP (mg L^{-1})	p*	p**	p***
h1				
CAG+ (578)	1.33±1.68	0.943	0.490	0.242
CAG- (1481)	1.36±1.71			
h2				
CAA+ (1194)	1.40±1.74	0.442	0.314	0.839
CAA- (865)	1.30±1.64			
h3				
CGG+ (1061)	1.37±1.75	0.743	0.785	0.733
CGG- (998)	1.34±1.65			
h5				
TAG+ (138)	1.10±1.56	0.057	0.014	0.021
TAG- (1921)	1.37±1.71			
h7				
TGG+ (569)	1.38±1.69	0.695	0.816	0.906
TGG- (1490)	1.35±1.71			

Table 14. Stepwise multiple linear regression analysis for predicting hsCRP

Statistical model 1		Statistical model 2	
Variables	B±SE	Variables	B±SE
C242T polymorphism	0.122 ±0.047**	Carriage of h5	0.081 ±0.036*
Age	-0.005 ±0.002**	Age	-0.005 ±0.002**
Sex	-0.08 ±0.022***	Sex	-0.08 ±0.022***
BMI	0.042 ±0.002***	BMI	0.042 ±0.002***
Physical activity	-0.027 ±0.009**	Physical activity	-0.027 ±0.009**
HDL cholesterol	0.068 ±0.034*	HDL cholesterol	0.068 ±0.034*
Triglycerides	0.333 ±0.049***	Triglycerides	0.330 ±0.049***
Hormonal contraceptives	0.263 ±0.027***	Hormonal contraceptives	0.261 ±0.027***
Pregnancy	0.438 ±0.064***	Pregnancy	0.441 ±0.064***
Lactation	0.152 ±0.061*	Lactation	0.156 ±0.061*
Rheumatics	0.317 ±0.079***	Rheumatics	0.319 ±0.079***
Recent infection	0.127 ±0.041**	Recent infection	0.128 ±0.041**
Adjusted model R^2	0.278	Adjusted R^2	0.278
Significance (ANOVA)	<0.0001	Significance (ANOVA)	<0.0001

B: Regression coefficient, SE: Standard error, ANOVA: Analysis of variance

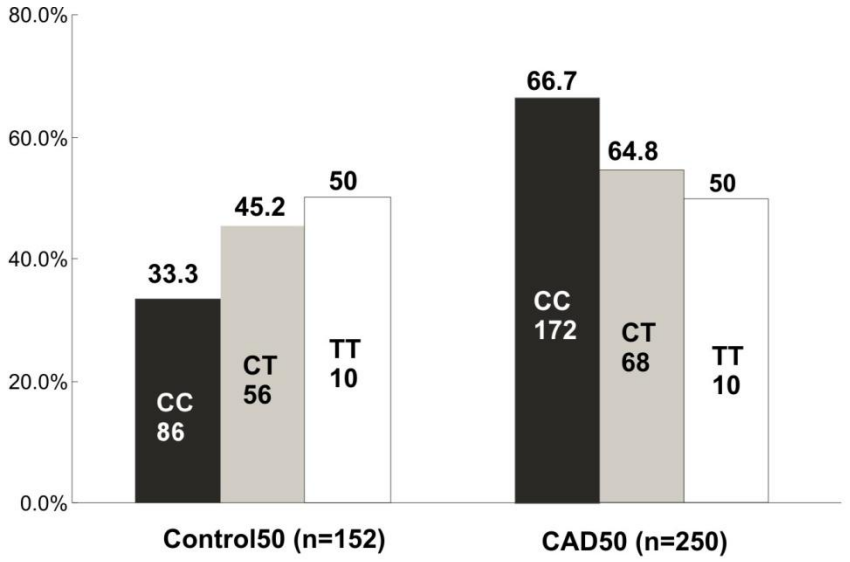
*P<0.05, **P<0.01, ***P<0.001

5.5 CYBA gene polymorphisms and coronary artery disease (IV)

The distribution of the genotypes and allele frequencies of the C242T and -930 $^{A/G}$ polymorphism in the study population are summarized in Table 4 and the characteristics of the population in Table 8. The allele frequencies of both gene polymorphisms were in line with the Hardy-Weinberg equilibrium. The distribution of the genotypes of the C242T polymorphism among CAD patients (50% or 75% stenosis) and respective control subjects are shown in **Figure 8.**

The prevalence of the T allele (TT+TC genotypes) was significantly lower among CAD patients than the control subjects (χ^2=6.141, P=0.013 for the first analysis; χ^2=5.118, P=0.024 for the second analysis). The crude OR of the genotypes of the C242T polymorphism (TT+TC vs CC) between CAD patients and control subjects was 0.591 (95% CI 0.389-0.897, P=0.013) and 0.623 (95% CI 0.414-0.940, P=0.024), respectively. The multiple logistic regression analysis indicated that the C242T polymorphism was an independent determinant of CAD in this high-risk Finnish population. **(Table 15)** In contrast to the C242T polymorphism, there was no relationship between the -930 $^{A/G}$

Figure 8. Bar graphs show a percentage of cases or controls from the total number of each category according to genotype (CC, CT and TT). The analysis was first carried out by comparing subjects with ≥50% (CAD50, n=250) to those with <50% (Control50, n=152) stenosis, and a second analysis was carried out in the same study group comparing those with ≥75% (CAD75, n=209) to those with <75% (Control74, n=193) of stenosis.



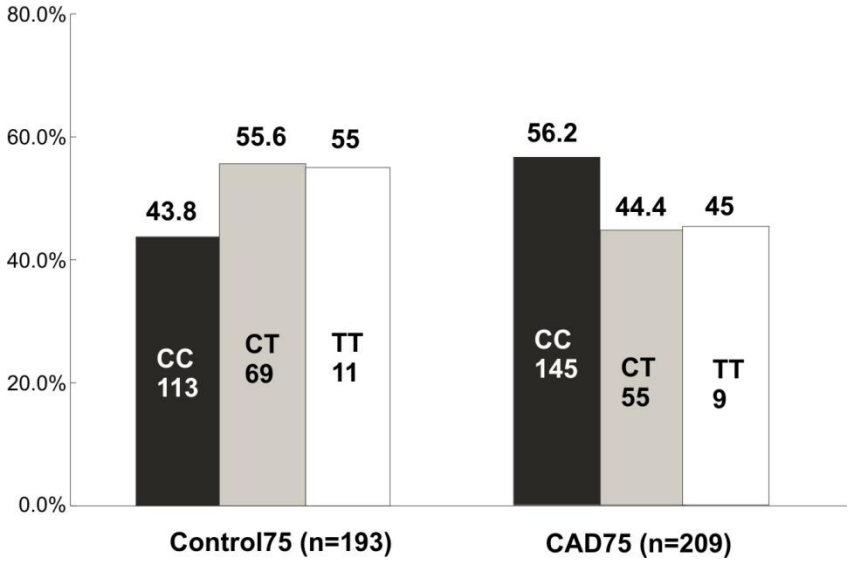


Table 15. The multiple logistic regression analysis of the relationships between explanatory variables and angiographically verified coronary artery disease

Variable	CAD (50% Stenosis)		CAD (75% Stenosis)	
	OR (95% CI)	P value	OR (95% CI)	P value
Male	3.038 (1.781 – 5.182)	< 0.001	2.623 (1.561 – 4.405)	< 0.001
Age	1.079 (1.052-1,106)	< 0.001	1.082 (1.056-1.109)	< 0.001
Overweight	0.991 (0.938 – 1.046)	0.733	0.991 (0.940 – 1.045)	0.738
Smoker	1.340 (0.798-2.252)	0.268	1.366 (0.827-2.258)	0.223
Diabetes	2.233 (1.232 – 4.048)	0.008	1.886 (1.091 – 3.260)	0.023
Hypertension	0.288 (0.089 – 0.936)	0.038	0.495 (0.160 – 1.530)	0.222
Hypercholesterolemia	2.620 (1.577-4.352)	< 0.001	2.040 (1.240-3.357)	0.005
C242T polymorphism	0.529 (0.327 - 0.856)	0.009	0.600 (0.377 - 0.954)	0.031

polymorphism and angiographically verified coronary artery disease in this study.

6 DISCUSSION

6.1 Study subjects

In the present studies (I–IV), a low and a high-risk population were used to investigate the association of CYBA gene polymorphisms with subclinical forms of atherosclerosis and its associated clinical complications.

Study subjects from the Cardiovascular Risk in Young Finns Study. The study subjects in the Cardiovascular Risk in Young Finns Study formed a low-risk population. A total of 4,320 children and adolescents including equal numbers of males and females from both rural and urban areas, and/or from both eastern and western Finland, were selected in the first cross-sectional survey in 1980. A total of 3,596 subjects, 83.2% of those invited, participated in the study in 1980. This was a high participation rate, and the reply obtained from the non-participants in a separate questionnaire revealed no systematic reason for non-participation. Therefore, the final sample in 1980 was considered to be representative of the total random sample (Åkerblom et al., 1985; Juonala et al., 2004a).

The studies (I–III) in this thesis are based on the information obtained during the 21-year follow-up study which was conducted in 2001. A total of 2,283 subjects aged 24–39 years, roughly 63.5% of the original study cohort, participated in the follow-up examination. When the baseline characteristics of the subjects in the 2001-year follow-up were compared to those who had dropped out, there were more men and younger subjects in the lost cohort. However, no significant differences were found for baseline risk factors among participants and dropouts after adjusted adjustment for age and sex. Therefore, the 2001 cohort can be considered as representative of the original study population (Juonala et al., 2004a; Raitakari et al., 2008).

Because of unsuccessful genotyping and missing data, the number of study subjects included in the final analysis of each study in this thesis (Studies I–III) is different from the original study cohort (n=2283). In Study III, subjects with a level of hsCRP more than 10 mg/l were excluded, which is why hsCRP levels were significantly lower in this population when compared with the original study cohort as well as the populations of the other two studies (Table 5). However, there were no significant differences in terms of the rest of the risk factors between the study populations even after adjustment for hsCRP levels. Therefore, the characteristics of the subjects in these studies were comparable to the original study cohort, and all results seem representative of the original study population. Moreover, the study cohort was randomly selected, and the sample size is

sufficient for statistical analyses in the cross-sectional study. All results may probably be generalized in populations consisting of young Caucasians.

Selection of cases and controls from the Finnish Cardiovascular Study. Study IV is a nested case-control study. The source population for this case-control study consisted of the patients who underwent both exercise stress tests and coronary angiographies between October 2001 and June 2005 at Tampere University Hospital. In addition, the eligibility criteria for the selection of both cases and controls included those patients who should have at least one cardiovascular risk factor such as smoking, body overweight, hypertension, hypercholesterolemia or diabetes mellitus. Therefore, the source population in Study IV was a high-risk population, in which the case-control study was nested, and from which the high-risk controls for the study were ideally drawn. However, it must be acknowledged that the sample size of Study IV remains small, and the results might be due to sampling variation and chance. Therefore, replication of the present observation with larger samples of different populations is required.

Defining cases and controls in Study IV was based on the status of coronary arteries by means of coronary angiography. Although an ideal method would completely separate the diseased and the disease-free subjects, a method of 100% sensitivity and 100% specificity is very rare in reality. Angiography can show the extent of coronary luminal stenosis, and an angiographic study is usually considered as positive when stenosis is severe enough to limit flow. Experimental and clinical studies have shown that when the level of stenosis exceeds 50%, the ability to increase blood flow in response to metabolic demand (the coronary flow reserve) is impaired (Gould et al., 1974; Scanlon et al., 1999). Therefore, 50% and/or 75% coronary arterial stenosis affecting at least one vessel was used as the cut-off point to categorize the population into cases and controls in Study IV. In addition, 50% and/or 75% stenosis are used as the cut-off point to define coronary artery disease in many clinical and epidemiological studies (Casas et al., 2004), thus allowing comparison of the present results with previous studies.

6.2 Methodological considerations

6.2.1 Candidate genes and association studies

Atherosclerosis or its associated complications are multi-factorial diseases caused by a variety of genes as well as environmental factors, and its mode of inheritance does not follow Mendelian laws. There are different methods to be used when studying complex diseases. Association studies have a greater power to detect the effects of common variants involved in complex diseases (Hirschhorn, 2005). During the past several years, technological advances, bioinformatic and statistical

foundations have been laid for whole-genome association analyses. These genome-wide studies have provided information about disease-associated genes, and they serve an important function in trying to uncover the complex genetics behind the conditions. However, these studies require large sample sizes of thousands of subjects, and they are expensive to conduct. Therefore, association studies are most often limited to candidate genes. The candidate gene selection can be based on genome-wide studies, previous candidate gene studies, gene expression studies, and knowledge of their function in the pathophysiology of the disease. In the present study, the CYBA gene was chosen as a target gene because it had previously been demonstrated that it is essential for a functional NADPH oxidase, and that its expression is up-regulated in atherosclerotic arteries (Ushio-Fukai et al., 1996; Wyche et al., 2004). However, the association of several polymorphisms in this gene with cardiovascular diseases has produced conflicting results (Soccio et al., 2005; San Jose et al., 2008). Differences in the genetic background of study populations, the study design and the statistic methods used might contribute to the disparate results.

The Finnish population has been considered as one of the best genetic isolates because of its genetically homogenous background (Peltonen et al., 1999). We compared the allele frequency of CYBA polymorphisms in unrelated Finnish CAD patients and controls in Study IV to identify markers that might differ significantly between the study groups. It is clear that a large enough population increases the power of an association study. Therefore, in Studies I–III, several CYBA gene polymorphisms were studied in a large population-based sample of young adults. The haplotype analysis which was used in Study III further increased the power of the association analyses. In addition, the gene-environment interactions were also examined with the Young Finns population. Study I showed that the association of CYBA C242T polymorphism with FMD was only seen in subpopulations such as smokers and overweight subjects, and Study II revealed a gene-environment interaction ($-930^{A/G}$ polymorphism-smoking/-hypertension) on IMT using a linear regression model or performing a stratified analysis according to known risk factors, such as smoking and hypertension. These studies indicated that a well-designed study and comprehensive analyses might produce more sound results.

6.2.2 Ultrasound measurements (I, II)

The ultrasound studies on brachial artery FMD and carotid artery IMT were performed under the auspices of the 21-year follow-up of the Cardiovascular Risk in Young Finns Study. The measurements were carried out simultaneously in five study centres, and the digitally stored scans were analysed by a single reader blinded to the study subjects. These non-invasive vascular markers

have been considered as surrogate markers for atherosclerosis and its associated complications (Celermajer et al., 1992; Corretti et al., 2002; Simon et al., 2002; Mancini et al., 2004).

Increased carotid IMT is a non-invasive marker of early arterial structural alteration. It has been found to relate to atherosclerotic risk factors and cardiovascular damages and predict clinical events such as myocardial infarction and stroke (Burke et al., 1995; O'Leary and Polak, 2002). Because this method is relatively simple and represents a safe, inexpensive, precise and reproducible measure, it has been widely performed in epidemiologic studies. In the 21-year follow-up of the Cardiovascular Risk in Young Finns Study, the reproducibility of the method was assessed by re-examining 60 subjects at three months after the initial visit. The between-visit coefficient of variation was 6.4%, and it is very similar to previous reports (Study II) (Kanters et al., 1997).

Brachial FMD responses are thought to reflect systemic vascular endothelial function. Endothelial dysfunction is an early functional marker of atherosclerosis. Impairment in brachial FMD has been shown to relate to the prevalence and extent of coronary atherosclerosis, and the predict cardiovascular events (Neunteufl et al., 1997; Gokce et al., 2002; Chan et al., 2003). Vascular responses to endothelium-dependent and independent stimuli can be measured using non-invasive high-resolution ultrasound. In Study I, we did not measure endothelium-independent nitrate-mediated vasodilatation that is often used as a control for the FMD test to ensure that the altered FMD capacity is a consequence of endothelial dysfunction and not a reflection of underlying smooth muscle function. This is a limitation of Study I, and it may only be improved in next follow-up study. In addition, the long-term variation detected in FMD measurement (CV 26%) was larger in Study I than some reported studies (Sorensen et al., 1995), but it was comparable with the long-term variation in other study groups (Lind et al., 2000; De Roos et al., 2003). The large variation is probably a result of physiological and technical issues (Lind et al., 2000). The long-term reproducibility of brachial artery diameter measurements was, however, excellent (CV 3.2%) in the Cardiovascular Risk in Young Finns Study (Juonala et al., 2004b). It therefore seems that much of the long-term variation in FMD is attributable rather to a physiological fluctuation in endothelial function than to measurement error.

6.3 CYBA C242T gene polymorphism and functional alteration in arterial wall (I)

Increased blood flow in the conduit arteries can provoke endothelial cells to release NO, leading to vasodilatation. Therefore, non-invasive measurement of brachial artery FMD reflects NO-

dependent endothelial function (Cooke et al., 1991). Impaired FMD is considered an early feature of endothelial dysfunction, representing a loss of NO bioavailability due to either reduced formation or accelerated degradation of NO (Cooke and Dzau, 1997; Harrison, 1997). Loss of NO bioavailability by increased ROS is a major mechanism underlying endothelial dysfunction, and NADPH oxidase is a major source of ROS (Behrendt and Ganz, 2002).

The C242T polymorphism of the CYBA gene that codes p22phox has been found to modulate superoxide production of NADPH oxidase. Functionally, superoxide production has been linearly associated with the C242T genotypes, being highest in CC, intermediate in CT, and lowest in TT homozygotes (Wyche et al., 2004). In Study I, the C242T polymorphism of the CYBA gene was associated with FMD in a population-based sample of young adults, suggesting a functional role of the C242T polymorphism in endothelial function. A significant linear trend was observed in brachial artery FMD responses across the genotypes, with the greatest difference observed between TT and CC homozygotes. Our results are in concert with a previous study by Schachinger et al., and they reported that the C242T polymorphism was an independent determinant of epicardial coronary endothelial vasodilator function, therefore suggesting a functional significance of this gene polymorphism as related to superoxide production in the vascular wall (Schachinger et al., 2001). Contrary to these findings, some studies have failed to find any effect of the C242 T polymorphism either on venous endothelial function in individuals or on forearm endothelium-dependent vasodilation in subjects with hypercholesterolemia (Schneider et al., 2003; Fricker et al., 2004). Differences in vascular territories, characteristics of populations and sample sizes may explain such discrepancies.

It has been recognized that NADPH oxidase constitutively produces low amounts of superoxide anion intercellularly in the physiological condition. However, enzymatic activity can be up-regulated in conditions of increased oxidative stress such as obesity, smoking, hypertension and diabetes (Lassegue and Clempus, 2003; Furukawa et al., 2004; Jaimes et al., 2004). Therefore, it is possible that the functional significance of the C242T polymorphism, as related to superoxide production, is more potent in situations in which NADPH oxidase may be highly activated.

In Study I, the sample size is large and therefore allows us to further examine the genetic effect by a stratified analysis according to risk factors related to superoxide production. Although the subjects in Study I formed a low-risk population with only 13.7% hypertension, 0.9% diabetes and 19.4% hypercholesterolaemia, 52.6% were smokers and 43.5% overweight. In smokers or overweight subjects, the linear trend in brachial artery FMD response across the genotypes remained significant, but not among the non-smokers or normal-weight subjects, suggesting that the functional effect of the C242T polymorphism on endothelial function might only be manifested in

an environment of higher oxidative stress such as adiposity or smoking. Altogether, the Study I showed an association between the CYBA C242T polymorphism and brachial artery FMD response in young Finnish adults, and body adiposity and smoking status can modify this association.

As a multi-factorial intermediate phenotype of atherosclerosis, endothelial dysfunction can be affected by both genetic and environmental factors, and the underlying genetic risk may be modified by the environment. A major limitation of Study I was that we did not directly measure the biochemical indices related to superoxide production to verify our hypothesis. Therefore, further studies are required to replicate these results in similar populations.

6.4 CYBA -930A/G polymorphism and risk factor interactions in relation to carotid IMT (II)

Carotid IMT is a marker of sub-clinical atherosclerosis which correlates with the presence of coronary atherosclerosis and represents an independent risk factor for CAD events and stroke (Mancini et al., 2004). In the Cardiovascular Risk in Young Finns Study, we examined the association between cardiovascular risk factors and carotid IMT, and IMT values were significantly higher among cigarette smokers than among non-smokers (Raitakari et al., 2003). This suggested that cigarette smoking is an important risk factor for the development of atherosclerosis. Many other studies have established a strong correlation between cigarette smoking and an accelerated progression of carotid atherosclerosis as well (Haapanen et al., 1989; Salonen and Salonen, 1991). Interestingly, in Study II, we found that the impact of cigarette smoking on carotid IMT was influenced by the CYBA -930 $^{A/G}$ gene polymorphism. Smoking-induced changes in carotid IMT were most significant in subjects with the GG genotype, borderline significant for the GA genotype and non-significant for the AA genotype, suggesting a gene-environment interaction in carotid IMT for this population.

Cigarette smoking is known to induce a large amount of oxidants, and these free radicals could arise directly from cigarette smoke or indirectly from endogenous sources (Church and Pryor, 1985; Ambrose and Barua, 2004). Recent studies have demonstrated that cigarette smoke-induced superoxide production is released from the NADPH oxidase, but not from other inducible intracellular sources, such as xanthine oxidase or endothelial NO synthase (Jaimes et al., 2004; Orosz et al., 2007). NADPH oxidase-mediated superoxide production has shown a significant correlation with IMT in subjects free of clinical atherosclerotic disease (Zalba et al., 2005b). Previously, the -930 $^{A/G}$ polymorphism has been shown to have differential functional effects in the development of NADPH-dependent oxidative stress (Moreno et al., 2003; San Jose et al., 2004). Therefore, our finding supports the hypothesis that cigarette smoking increases oxidative stress as a

potential mechanism for initiating atherosclerosis, whereas the -930 $^{A/G}$ polymorphism of the NADPH oxidase seems to modify the atherosclerotic process and cause a between-individual difference in IMT in response to cigarette smoking.

In Study II, a linear regression model was used to demonstrate the interaction effect of smoking with the genotypes on IMT. Moreover, the magnitude of the interaction effect was further examined by performing a stratified analysis according to smoking habits. The genetic effect on IMT differed in the presence and absence of cigarette smoking, indicating that the difference might be due to an interaction effect of cigarette smoking with the G allele of the -930 $^{A/G}$ polymorphism.

The human p22phox promoter possesses several potential consensus sequences for transcriptional factors. Because the -930 $^{A/G}$ polymorphic site lies on a potential binding site for C/EBPs transcription factors, it has been hypothesized that up-regulated C/EBP expression in essential hypertension might induce a greater effect on the transcriptional activity of the G than of the A allelic CYBA promoter. The expression of the CYBA gene was influenced by the -930 $^{A/G}$ polymorphism in the SHR and hypertensive subjects, whereas no effect was found in the WKY and normotensive subjects, further suggesting that the -930 $^{A/G}$ polymorphism influenced p22phox gene expression depending on cell phenotype (Zalba et al., 2001; Moreno et al., 2003; San Jose et al., 2004). In Study II, the study subjects were young and formed a low-risk study population with only 13.7% hypertension. However, we still found an interaction effect of hypertension and the -930 $^{A/G}$ polymorphism on carotid IMT, and BP-induced changes in carotid IMT were found in subjects with the GG genotype, but not for the AA genotype. These findings were in concert with previous functional studies and their hypotheses. In addition, we found a significant effect of the -930 $^{A/G}$ polymorphism in G allele carriers depending on the exposure to cigarette smoking. It has been observed that the C/EBP-binding activity is significantly increased in healthy smokers when compared with non-smokers (Didon et al., 2005). It is therefore tempting to speculate a potential involvement of C/EBP in the NADPH oxidase activation among the smokers included Study II.

The present study lacked the functional data on the effect of the -930 $^{A/G}$ polymorphism, whereas these data might be the useful intermediate phenotypes for predicting the relationship between the -930 $^{A/G}$ polymorphism and carotid IMT. Therefore, further investigations are required to elucidate the functional effect of the -930 $^{A/G}$ polymorphism on IMT variation.

6.5 CYBA gene polymorphisms and serum C-reactive protein level (III)

Of various inflammation markers, CRP has been used as an indicator of low-level arterial inflammation for predicting future cardiovascular events in apparently healthy individuals. The prognostic value of CRP as a marker of cardiovascular risk has been firmly established (Ridker et al., 1997; Järvisalo et al., 2002; Pearson et al., 2003; Bassuk et al., 2004; Zieske et al., 2005). A link between biomarkers of oxidative stress and elevated CRP level has been reported in individuals free of cardiovascular diseases (Abramson et al., 2005; Dohi et al., 2007).

In Study III, the association between CYBA C242T gene polymorphisms and chronic inflammatory process as represented by hsCRP level was further examined in young Finnish adults. Because a CRP concentration above 10 mg/l has traditionally been considered as a clinically acute infection, the subjects were excluded if their level of hsCRP was more than 10 mg/l in Study III.

In the young Finns population, the distribution and population determinants of hsCRP level have been investigated previously, including lifestyle variables and conventional risk markers (Raitakari et al., 2005). It has been found that obesity is an important lifestyle determinant of hsCRP, explaining roughly 20% of the variation in this population. In addition, traditional risk factors have all been associated with elevated hsCRP levels, and a more intense lifestyle modification would appear appropriate for those individuals with high hsCRP. Therefore, in Study III, the relationship was further examined after comprehensive adjustment for these established risk factors. The results indicated that the C242T gene polymorphism was an independent determinant of hsCRP level.

NADPH oxidase-derived ROS has been found to regulate the expression of several pro-inflammatory mediators via the activation of the transcription factor NF-κB (Li and Verma, 2002; Mogensen et al., 2003). The production which pro-inflammatory cytokines elicits, like interleukin 6, can stimulate the liver to produce CRP (Libby, 2002). The C242T polymorphism has been associated with the activity of NADPH oxidase in several studies, which makes it likely that this genetic effect might modify the inflammatory process by producing different amounts of O_2^- .

The results from Study III are also in concert with the findings of Study I that the C242T polymorphism could modulate the brachial artery FMD response in young Finnish adults. Brachial artery FMD reflects NO bioavailability, and NO functions as antioxidant via its ability to neutralize the superoxide. A decline in NO bioavailability is usually accompanied by many other alterations in endothelial function including altered anti-inflammatory properties (Celermajer, 1997; Landmesser et al., 2004). The activation of endothelial cells has been associated with a phenotype that promotes the recruitment of inflammatory cells to sites of vascular injury, and these cells generate various inflammatory molecules which provoke high oxidative stress in vasculature (Chandel et al., 2001;

Bonetti et al., 2003; Park et al., 2004). The CYBA 242T allele was related to enhancing brachial FMD in Study I and to low level of hsCRP in Study III in the young Finns population, suggesting that the CYBA 242T allele might have protective effects against the activation of endothelial cells and provocation of inflammatory process in the very early stage of atherosclerosis.

According to the common disease/common variant hypothesis, the genetic architecture of a complex disease is more likely to consist of a few causal alleles, each conferring a small specific effect on the risk in an individual (Lander, 1996; Reich and Lander, 2001). The multi-marker haplotypes can serve as an effective proxy for more putative causal alleles in a candidate gene or elsewhere, which may be highly correlated with the tagSNPs in the panel. In Study III, with the exception of -930 $^{A/G}$ polymorphism that has been shown to segregate independently from the C242T polymorphism, the relationship between genotypes at each pair of polymorphic sites was in linkage disequilibrium among three tagSNP. Therefore, the haplotypes were estimated from these three SNPs in Study III. In contrast to the C242T polymorphism, no relationship was found between three single SNPs and hsCRP level. However, a significantly decreased hsCRP level was observed in carriers of haplotype 5 which contained the rs4673T, rs1049255A and rs7195830G allele. This finding of the haplotype analyses was consistent with the results of single SNP analyses, supporting the assumption that the C242T polymorphism might be a potential causal variant in the CYBA gene and responsible for the association with hsCRP level in young individuals.

6.6 CYBA C242T gene polymorphism and coronary artery disease (IV)

For more than a decade, it has been hypothesized that cardiovascular disease (CVD) as a chain of events is initiated by a myriad of related and unrelated risk factors, progressing through numerous physiological pathways and processes to the development of end-stage heart disease (Dzau and Braunwald, 1991). Now the concept of a CVD continuum has been confirmed. Moreover, it has been recognized that the modification at any point along this chain can alter disease progression (Dzau et al., 2006a; Dzau et al., 2006b). In Study I and Study III, the C242T gene polymorphism was associated with enhancing brachial FMD and reduced hsCRP level, suggesting that the CYBA 242T allele might have a protective effect modifying the cardiovascular risk in young adults. With regard to a CVD continuum, we further elucidated the relationship of the CYBA C242T gene polymorphism with coronary artery disease (CAD) in Study IV and found that the polymorphism was consistently associated with the presence of CAD in a high-risk Finnish population.

The association of the C242T polymorphism with CAD has been widely studied with conflicting results (Inoue et al., 1998; Cai et al., 1999; Cahilly et al., 2000). Differences in the

genetic background of the study populations, the study design and the statistic methods used might contribute to the disparate results.

In Study IV, a case-control study nested in a high-risk Finnish hospital cohort was conducted with CAD patients verified by coronary angiography. The T allele frequency in this population was 0.20, which was lower than the approximately 0.33 reported for several other Caucasian population (Cai et al., 1999; Gardemann et al., 1999; Li et al., 1999; Cahilly et al., 2000), but higher than the 0.13 found in a Japanese population (Inoue et al., 1998). Because we have also examined the T allele frequency of 0.19 (Table 1) in the Cardiovascular Risk in Young Finns population (n=2283), the T allele frequency in this high-risk population was consistent with the distribution in the Finnish general population. This result indicates that the genetic background of the C242T polymorphism of the CYBA gene differs between Finnish and several other Caucasian populations.

CAD is a multi-factorial disease caused by a sum of genetic and environmental risk factors, and the C242T gene polymorphism may thus only play a minor role in the development of the disease. It was mentioned in Study I that the CYBA gene may not exert a large effect in a healthy individual, because the vascular NADPH oxidase constitutively produces low amounts of intracellular superoxide anions in physiological conditions. The previous results of Study I indeed showed that the association between the C242T polymorphism and the brachial artery FMD response only remained stronger in smokers and overweight subjects but not in their counterpart groups. Study IV was conducted with a high-risk population, and the effect of C242T polymorphism was significantly associated with CAD. The well-established acquired risk factors for CAD, such as smoking, diabetes, obesity, hypertension and hypercholesterolemia, have been found to induce the excessive production of the NADPH-dependent O_2^- (Lassegue and Clempus, 2003; Furukawa et al., 2004; Jaimes et al., 2004). Therefore, it is possible that the effect of the CYBA gene might be more potent because NADPH oxidase activity was up-regulated by these risk factors. In Study IV, the effect of the C242T gene polymorphism on CAD may be highlighted in the high-risk population in which NADPH oxidase might be highly activated.

Study IV was designed to examine the assumption that the 242T allele may have a protective effect against coronary artery stenosis in a population exposed to numerous risk factors. As necessitated by this hypothesis, the prevalence of risk factors was thus very high in the control population as well (for example, 95%, 57%, 52% and 74% for hypertension, hypercholesterolemia, smoking and body overweight, respectively). The fact that the selection of a well-characterized high-risk control group was an important part of the study design led to the finding that there were no differences in the incidence of body overweight, smoking and hypertension between the CAD

patients and controls in this study. Differences in age and sex were found between the CAD patients and controls, which may affect the association study on the C242T polymorphism with CAD. However, the association remained significant after adjusting for all the potential confounders in a multiple logistic regression analysis. We concluded that the CYBA C242T gene polymorphism is associated with the presence of CAD and the 242T allele has a protective effect against CAD, although the subjects were exposed to multiple risk factors for CAD.

The small sample size is a limitation of Study IV. Therefore, the association might be due to sampling variation and chance. To confirm the findings, further studies are required to replicate the results with larger cohorts in different populations.

6.7 Significance of the study

Atherosclerosis is a multi-factorial disease caused by environmental and genetic risk factors as well as their interactions (Hopkins and Williams, 1981; Yusuf et al., 2004; Goldstein et al., 2006). In the present study, we used a candidate gene approach to study the possible impact of CYBA gene polymorphisms and gene-environment interactions on the development of atherosclerosis and its complications.

During the course of the present study, new techniques and study methods for genetic epidemiology have developed enormously. The Human Genome Project and the HapMap project have provided a larger number of information on the sequence of the human genome and the variation thereof. Recently, the genome-wide association study, which represents a powerful new tool for genetic epidemiology, has been utilised to identify hundreds of genetic variants associated with complex human diseases and traits, also providing valuable insights into their genetic architecture. Although the GWA method has an important benefit compared to the candidate gene study, the limited information available about environmental exposure and other non-genetic risk factors in GWA studies have made it difficult to identify gene-environment interactions or modifications of the gene-disease association in the presence of environmental factors (Manolio et al., 2009; Pearson and Manolio, 2008).

Based on the findings from the present study, the polymorphisms in the CYBA gene are not only associated with atherosclerosis from a very early stage to the clinical manifestation of CVD, but, more importantly, the genetic associations vary in the presence or absence of environmental exposure, suggesting that these polymorphisms act jointly with environmental factors, showing a gene-environment interaction in the process of atherosclerosis.

NADPH oxidase is the only enzyme whose sole function is to generate ROS, and it has been considered as the most important source of ROS in vascular wall and inflammatory cells. Most established risk factors for cardiovascular disease have been association with overproduction of ROS (Gimbrone, 1999; Ross, 1999; Cai and Harrison, 2000; Bonetti et al., 2003). Moreover, ROS and NADPH oxidase have been implicated in numerous cell processes and pathogeneses associated with atherosclerosis. Genetic polymorphisms in the genes encoding the NADPH oxidase subunits, such as the CYBA gene polymorphisms, modulate ROS production, influencing the progression of cardiovascular disease. The identification of groups of individuals genetically prone or resistant to oxidative stress might not only be useful for the stratification of the individuals and developing individualized approaches to care and treat in high risk individuals, but they also allow the design and interpretation of appropriate clinical trials for new dietary or pharmacological interventions with antioxidants.

7 SUMMARY AND CONCLUSIONS

In this thesis, a candidate gene approach and association analyses were used to study the possible impact of CYBA gene polymorphisms and their gene-environment interactions on the chronic inflammatory process represented as hsCRP level, early functional and structural changes in the arterial wall as well as clinical manifestations of CAD. The studies were conducted with two Finnish populations, comprising 2,283 young adults who participated in the 21-year follow-up of the Cardiovascular Risk in Young Finns Study (Studies I–III) and 402 high-risk patients who were selected from the participants of the Finnish Cardiovascular Study (Study IV). The main findings and conclusions are as follows:

1. In a population-based sample of young Finnish adults, brachial artery FMD was significantly influenced by the genotype of the CYBA C242T polymorphism and displayed a genotype-dependent effect. The relationship between FMD and the C242T polymorphism was clearly present in the overweight and ever-smoking subjects, but not in the normal-weight and non-smoking subjects, suggesting that overweight and smoking status may modify this genetic effect. (Study I)

2. In a young Finnish population, smoking/hypertension-induced changes in carotid IMT were different across genotypes, and they were most significant among carriers of the GG genotype and non-significant for the AA genotype of CYBA -930 $^{A/G}$ polymorphism. A linear regression analysis indicated that the smoking/hypertension-genotype interactions were associated with carotid IMT. Using a stratified analysis according to smoking/hypertension status, the genetic effect on IMT differed in the presence and absence of smoking/hypertension, and the difference might be due to an interaction effect of smoking/hypertension with the G allele of the -930CYBA polymorphism among smokers or hypertensive subjects (Study II)

3. The CYBA 242T allele was associated with lower hsCRP level in a population of young Finnish adults. This result was further confirmed by haplotype analyses, and the carriage of haplotype 5 (rs4673T, rs1049255A, rs7195830A) which contained the T allele of the C242T polymorphism had the lowest level of hsCRP. Multivariate linear regression analysis indicated that the CYBA C242T polymorphism was an independent determinant of hsCRP

level in this population. These findings suggest that CYBA C242T gene polymorphism might play a role in the chronic inflammatory process represented as hsCRP level at a very early stage of atherosclerosis. (Study III)

4. In a high-risk Finnish population, the prevalence of the CYBA 242T allele was significantly lower among angiographically verified CAD patients than among control subjects. This finding suggested that the T allele of the CYBA C242T polymorphism might have a protective effect against the development of CAD despite long-term exposure of study subjects to high risk factors related to oxidative stress. (Study IV)

Based on these findings, it can be concluded that CYBA gene polymorphisms such as the C242T (rs4673) and the -930 $^{A/G}$ (rs9932581) polymorphisms are associated with atherosclerosis from a very early stage to the clinical manifestations of CVD. The CYBA 242T allele consistently shows a protective association against cardiovascular risk in different stages of the disease, whereas the association of the G allele of the -930 polymorphism might contribute to early structural changes in the arterial wall in response to smoking/hypertension challenge. Moreover, the genetic associations varied in the presence or absence of environmental exposure such as cigarette smoking, obesity and hypertension, suggesting that these gene polymorphisms act jointly with environmental factors and that gene-environment interactions may have an impact on the pathogenesis of atherosclerotic disease.

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Meng Fan

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10 ORIGINAL COMMUNICATIONS

CYBA C242T gene polymorphism and flow-mediated vasodilation in a population of young adults: the Cardiovascular Risk in Young Finns Study

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Objective **Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a major source of the superoxide anion that contributes to decreased nitric oxide bioavailability in the vasculature. The C242T polymorphism of the CYBA gene that encodes *p22phox*, a component of NADPH oxidase, has been found to modulate superoxide production. We examined the relationship of the C242T polymorphism with endothelial-dependent brachial artery flow-mediated vasodilatation (FMD) in a population-based sample of young healthy adults.**

Methods **FMD, defined as the increased percentage in brachial artery diameter after reactive hyperemia, was assessed by ultrasound and the C242T polymorphism using a 5′ nuclease assay in 2058 subjects aged 24–39 years.**

Results **The mean values of brachial artery FMD were $8.0 \pm 4.4\%$ in all study subjects ($n = 2058$), and 7.8 ± 4.4, 8.2 ± 4.5, and $8.7 \pm 4.5\%$ in subjects with the CC ($n = 1362$), CT ($n = 616$), and TT ($n = 80$) genotypes of the C242T CYBA polymorphism, respectively ($P = 0.02$ for trend). The association remained significant ($P = 0.019$) in multivariate analyses adjusted for age, sex, obesity indices, smoking habits, blood pressure, serum glucose, lipids, and C-reactive protein. The relationship between FMD and the C242T polymorphism was stronger ($P = 0.004$) in overweight subjects (body mass index $\geq 25\ kg/m^2$, $n = 895$) and ever-smokers ($P = 0.008$, $n = 1082$), whereas no relationship was found in normal-weight subjects and non-smokers ($P = 0.824$ and $P = 0.438$, respectively).**

Conclusion **The C242T polymorphism of the CYBA gene seems to be related to endothelial function in a population-based sample of young healthy adults. Overweight and smoking status may modify this genetic effect. *J Hypertens* 25:1381–1387 © 2007 Lippincott Williams & Wilkins.**

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Introduction

Endothelial dysfunction is an early event in atherosclerosis. In addition to traditional and genetic risk factors, the genetic effects modified by environmental influences may contribute to the status of endothelial function during the preclinical phase of atherosclerosis [1–3]. Impairment of flow-mediated vasodilatation (FMD) is considered an early feature of endothelial dysfunction, representing the decreased bioavailability of nitric oxide in the vasculature [4,5]. This phenotype may precede the development of clinically apparent atherosclerosis and have prognostic value for cardiovascular events [6,7]. There is evidence that excessive superoxide production may lead to reduced nitric oxide bioavailability in endothelial dysfunction [8,9].

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a multisubunit protein complex consisting of membrane-bound and cytosolic subunits, is a major source of superoxide anion in the vasculature [10,11]. The *p22phox* subunit is essential for activation of the NADPH oxidase system [12]. Several polymorphisms of the CYBA gene have been identified. Among these, the C242T polymorphism is located on chromosome 16q24, exon 4, at position 242 of the CYBA gene, and results in the amino acid substitution of tyrosine for histidine at residue 72 within one of the two haem-binding sites [13]. The functional significance of the C242T polymorphism has been related to NADPH oxidase activity with subsequent production of the superoxide anion [14,15].

FMD responses can be measured in the conduit arteries by high-resolution ultrasound [16,17]. We used this non-invasive technique to study the effect of the C242T polymorphism on nitric oxide-dependent endothelial function in a population-based sample of young healthy

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adults participating in the longitudinal Cardiovascular Risk in Young Finns Study [18,19]. We tested the hypothesis that the C242T polymorphism in the CYBA gene is related to brachial artery FMD in healthy adults. As adiposity and smoking may influence vascular NADPH oxidase activation [20–24], we further examined whether overweight and smoking status modified the genetic effect.

Methods

Subjects

The Cardiovascular Risk in Young Finns Study is an ongoing five-centre, prospective cohort study of atherosclerosis risk factors in Finnish children and adolescents. Details of the study design have been described previously [18,19]. The first cross-sectional survey was conducted in 1980 with 3596 subjects at the age of 3–18 years. The latest 21-year follow-up was performed in 2001, enrolling 2283 subjects, aged 24–39 years, from the original cohort. The present analyses included 2058 subjects for whom complete data on the brachial artery FMD and genotype of the C242T polymorphism were available. All subjects provided written informed consent in 2001, and the study was approved by the local ethics committees.

Clinical characteristics and risk factor measurements

Data including age, sex, and smoking habits were acquired using questionnaires. Weight and height were measured and body mass index (BMI) was calculated (kg/m^2) [25]. A BMI over 25 kg/m^2 was defined as overweight, and a BMI of over 30 kg/m^2 was defined as obese [26]. The overweight and obese subjects were classified as the overweight subgroup in the present study. Ever-smoking was defined as either a current or former smoking habit and non-smoking as no current or former smoking habit. Blood pressure was measured using a random zero sphygmomanometer. Blood samples were drawn for the biochemical and genetic analyses after an overnight fast. Standard enzymatic methods were used for the determination of serum lipids and glucose [25]. The low-density lipoprotein (LDL) cholesterol concentration was calculated using the Friedewald formula. Fasting plasma highly sensitive C-reactive protein (CRP) concentrations were analysed by latex turbidometric immunoassay (Wako Chemicals GmbH, Neuss, Germany). The lower detection limit reported for the assay was 0.06 mg/l, and the coefficient of variation in repeated measurements was 3.3%.

DNA isolation and genotyping of the C242T polymorphism in the CYBA gene

Whole venous blood was drawn into ethylenediamine tetraacetic acid tubes and then stored at −70°C. Genomic DNA was extracted using a commercially available kit and the BioRobot M48 Workstation (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions. Polymerase chain reaction (PCR) for the C242T transition polymorphism (rs4673) in the CYBA gene was carried out in 384-well plates following a standard protocol for TaqMan MGB probes [27]. A 5 μl final volume containing genomic DNA, 2 × Universal Master Mix, 900 nmol of each primer and 200 nmol of each probe was used in the PCR. Nucleotide sequences for the primers and allele-specific probes were deduced from sequences published in the Gene Bank and Celera databases and designed in conjugation with Applied Biosystems using validated single-nucleotide polymorphism genotyping assays. After the PCR was finished, endpoint fluorescence was measured and allelic discrimination performed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, California, USA).

Ultrasound measurement of flow-mediated vasodilatation

An ultrasound study of the brachial artery was performed using Sequoia 512 ultrasound mainframes (Acuson, Mountain View, California, USA) with 13.0 MHz linear array transducers. Details of the method have been described previously [28]. In brief, the arterial flow was interrupted for 4.5 min by a cuff placed on the left proximal forearm at a pressure of 250 mmHg. The arterial diameter was measured at rest (baseline) and 40, 60, and 80 s after cuff deflation. The average of three measurements at each timepoint was used to derive the maximum FMD (the greatest value between 40 and 80 s), and FMD was the increase in vessel diameter after reactive hyperemia, expressed as the percentage relative to a resting scan. In our laboratory, the interobserver variation of mean FMD measurements ($n = 10$) was $0.63 \pm 0.45\%$ [range 0.14–1.63%; coefficient of variation (CV) $8.6 \pm 6.4\%$], and the intra-observer variation of two consecutive ($n = 10$) FMD measurements was $0.48 \pm 0.43\%$ (range 0.07–1.34%; CV $6.2 \pm 4.4\%$) [29]. In the present study, we assessed the long-term variation by re-examining some of the study subjects (2.5% random sample) after the initial visit. These scans were measured twice by the same reader. The 3-month between-visit CV was 3.2% for the brachial diameter and 26.0% for FMD.

Statistical analysis

The continuous variables are presented as mean ± standard deviation (SD). Differences across genotype groups were examined using the χ^2 test for categorical variables, linear regression, Student's *t*-test, and one-way analysis of variance (ANOVA). Multiple linear regression analysis was used to examine whether the C242T polymorphism was an independent predictor of FMD. Because of their skewed distributions, the values for triglycerides and CRP were $\log_{10}$-transformed, and blood glucose reciprocal-transformed before the analyses. As some antihypertensive and lipid-lowering medications

Table 1 Characteristics of the study groups stratified according to C242T genotype in all subjects

	All subjects ($n=2058$)		
	CC ($n=1362$)	CT ($n=616$)	TT ($n=80$)
Age (years)	32 ± 5	32 ± 5	32 ± 5
Male sex, *n* (%)	615 (45.2)	259 (42.0)	33 (41.3)
Body mass index (kg/m^2)	25 ± 4	25 ± 4	26 ± 5*
Smoking, *n* (%)[a]	711 (52.5)	332 (54.5)	39 (49.4)
Systolic BP (mmHg)[b]	122 ± 14	122 ± 14	123 ± 15
Diastolic BP (mmHg)[b]	73 ± 9	73 ± 9	75 ± 10
Glucose (mmol/l)	5.1 ± 0.8	5.0 ± 0.9	5.0 ± 0.5
Total cholesterol (mmol/l)	5.2 ± 1.0	5.1 ± 0.9*	5.1 ± 1.0
HDL-cholesterol (mmol/l)[c]	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.3
LDL-cholesterol (mmol/l)[c]	3.3 ± 0.9	3.2 ± 0.8*	3.2 ± 0.9
Triglycerides (mmol/l)	1.4 ± 0.9	1.3 ± 0.8	1.4 ± 0.9
C-reactive protein (mg/l)	1.9 ± 3.8	1.9 ± 3.5	1.9 ± 3.4

BP, Blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein. The continuous variables were presented as mean ± SD. * $P<0.05$ compared with CC genotype from two-sample *t*-test. [a] Data missing for 14 subjects. [b] Data missing for nine subjects. [c] Data missing for two subjects.

may influence NADPH oxidase, all of the analyses were repeated after the exclusion of subjects taking these medications ($n=32$) with similar results. All statistical analyses are two-sided and were performed with the statistical software SPSS (version 13.0; SPSS Inc., Chicago, Illinois, USA). *P* values less than 0.05 were considered statistically significant.

Results

The characteristics of all subjects ($n=2058$) as well as the subgroups stratified by body size and smoking status are shown in Tables 1, 2 and 3. BMI was higher in subjects with the TT genotype when compared with the CC homozygotes among all subjects. Total cholesterol and LDL-cholesterol were lower in subjects with the CT than with the CC genotype, both among all subjects and among overweight subjects. In addition, ever-smokers with the CT genotype had a higher level of CRP.

The T allele frequency was 0.19 in the entire study population as well as the subgroups stratified by smoking status, whereas this figure was 0.21 and 0.18 among the overweight and normal-weight subjects, respectively. The allele frequencies in overweight and normal-weight subjects did not differ from the corresponding frequency for the entire population ($P=0.105$ and $P=0.336$, respectively; χ^2 tests). The T allele frequency was in line with Hardy–Weinberg's equilibrium for the entire

Table 2 Characteristics of the study groups stratified according to C242T genotype in overweight and normal-weight subjects

	Overweight subjects ($n=895$)			Normal-weight subjects ($n=1163$)		
	CC ($n=565$)	CT ($n=290$)	TT ($n=40$)	CC ($n=797$)	CT ($n=326$)	TT ($n=40$)
Age (years)	32 ± 5	32 ± 5	33 ± 4	31 ± 5	32 ± 5	31 ± 5
Male sex, *n* (%)	312 (55.2)	149 (51.4)	17 (42.5)	303 (38.0)	110 (33.7)	16 (40.0)
Body mass index (kg/m^2)	29 ± 4	29 ± 4	30 ± 5	22 ± 2	22 ± 2	22 ± 2
Smoking, *n* (%)[a]	324 (57.7)	171 (59.4)	20 (50.5)	387 (48.9)	161 (49.8)	19 (48.7)
Systolic BP (mmHg)[b]	127 ± 15	128 ± 14	129 ± 15	118 ± 13	117 ± 13	118 ± 12
Diastolic BP (mmHg)[b]	76 ± 9	76 ± 9	78 ± 10	71 ± 8	71 ± 8	71 ± 8
Glucose (mmol/l)	5.2 ± 1.0	5.2 ± 1.0	5.1 ± 0.5	4.9 ± 0.7	4.9 ± 0.8	4.9 ± 0.4
Total cholesterol (mmol/l)	5.5 ± 1.1	5.3 ± 0.9*	5.3 ± 1.1	5.0 ± 0.9	4.9 ± 0.8*	4.9 ± 0.9
HDL-cholesterol (mmol/l)[c]	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.3	1.4 ± 0.3	1.3 ± 0.3	1.4 ± 0.3
LDL-cholesterol (mmol/l)[c]	3.5 ± 0.9	3.4 ± 0.9*	3.4 ± 1.0	3.1 ± 0.8	3.0 ± 0.7	3.0 ± 0.7
Triglycerides (mmol/l)	1.7 ± 1.2	1.6 ± 0.9	1.6 ± 0.8	1.1 ± 0.6	1.1 ± 0.5	1.1 ± 1.0
C-reactive protein (mg/l)	2.6 ± 4.7	2.7 ± 4.3	2.7 ± 3.9	1.3 ± 3.0	1.3 ± 2.4	1.1 ± 2.7

BP, Blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein. The continuous variables were presented as mean ± SD. * $P<0.05$ compared with CC genotype from two-sample *t*-test. [a] Data missing for 14 subjects. [b] Data missing for nine subjects. [c] Data missing for two subjects.

Table 3 Characteristics of the study groups stratified according to C242T genotype in smoking and non-smoking subjects

	Smoking subjects ($n=1082$)			Non-smoking subjects ($n=962$)		
	CC ($n=711$)	CT ($n=332$)	TT ($n=39$)	CC ($n=643$)	CT ($n=279$)	TT ($n=40$)
Age (years)	32 ± 5	31 ± 5	33 ± 4	32 ± 5	32 ± 5	31 ± 5
Male sex, *n* (%)	361 (50.8)	156 (47.0)	19 (48.7)	248 (38.6)	101 (36.2)	14 (35.0)
Body mass index (kg/m^2)	25 ± 4	26 ± 4	26 ± 5	25 ± 4	25 ± 4	26 ± 6*
Systolic BP (mmHg)[a]	122 ± 14	123 ± 14	123 ± 13	121 ± 14	122 ± 14	124 ± 17
Diastolic BP (mmHg)[a]	73 ± 9	73 ± 9	74 ± 9	73 ± 9	74 ± 9	75 ± 10
Glucose (mmol/l)	5.1 ± 0.9	5.1 ± 1.0	5.0 ± 0.5	5.0 ± 0.8	5.0 ± 0.8	5.0 ± 0.5
Total cholesterol (mmol/l)	5.2 ± 1.0	5.1 ± 0.9	5.0 ± 1.0	5.2 ± 1.0	5.0 ± 0.9*	5.1 ± 0.8
HDL-cholesterol (mmol/l)[b]	1.3 ± 0.3	1.3 ± 0.3	1.2 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.3
LDL-cholesterol (mmol/l)[b]	3.3 ± 0.9	3.2 ± 0.8	3.2 ± 0.9	3.3 ± 0.9	3.2 ± 0.8	3.2 ± 0.8
Triglycerides (mmol/l)	1.4 ± 1.0	1.4 ± 0.8	1.4 ± 1.1	1.3 ± 0.9	1.2 ± 0.7	1.4 ± 0.7
C-reactive protein (mg/l)	1.8 ± 4.0	2.0 ± 3.1*	1.5 ± 3.1†	1.9 ± 3.6	1.9 ± 4.0	2.2 ± 3.6

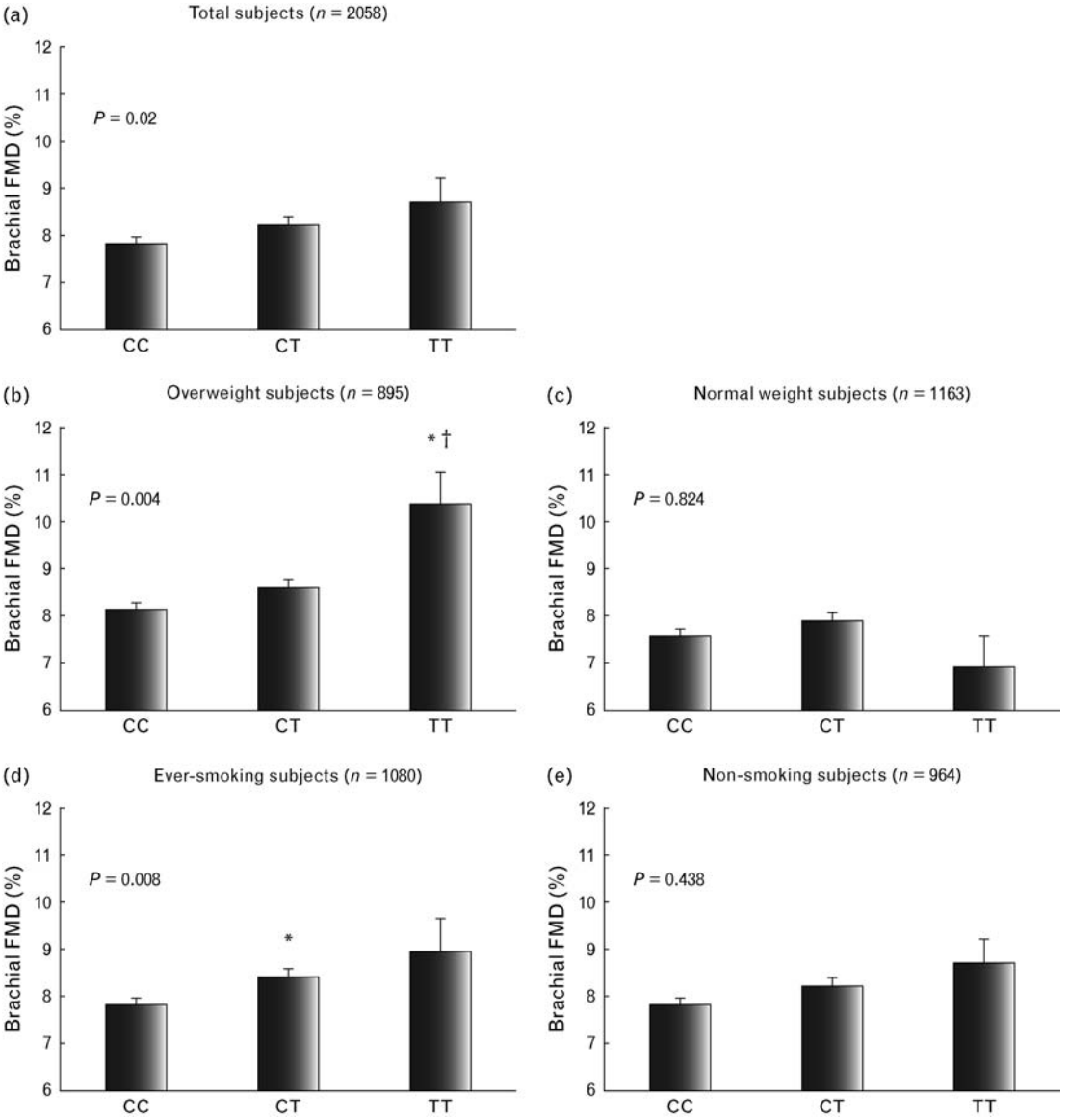
BP, Blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein. The continuous variables were presented as mean ± SD. * $P<0.05$ compared with CC genotype; † $P<0.05$ compared with CT genotype from two-sample *t*-test. [a] Data missing for nine subjects. [b] Data missing for two subjects.

population and the subgroups divided by body size and smoking status.

In all subjects, the mean value of brachial artery FMD was $8.0 \pm 4.4\%$. FMD was significantly influenced by genotype, and displayed a genotype-dependent effect ($P = 0.02$, linear regression, Fig. 1a). The relationship was clearly present in the overweight and ever-smoking subjects, but not in the normal-weight and non-smoking subjects. In the overweight subjects and ever-smokers, FMD increased significantly across the genotype groups ($P = 0.004$ and $P = 0.008$, respectively, linear regression). Using ANOVA, we found significant differences in FMD between the genotypes in the overweight ($P = 0.005$) and ever-smoking ($P = 0.028$, Fig. 1b,d) subgroups, respectively. In contrast, no significant differences in FMD values were detected across the genotype groups in the normal-weight subgroup ($P = 0.383$) and non-smokers ($P = 0.640$, Fig. 1c,e), respectively.

Multivariate linear regression analysis (statistical model in Table 4) indicated that the C242T polymorphism was

Fig. 1



Bar graphs show the brachial artery flow-mediated dilatation (FMD; mean ± SEM%) of the study groups stratified by genotype in all ($n = 2058$, panel a), overweight ($n = 895$, panel b), normal-weight ($n = 1163$, panel c), smoking ($n = 1082$, panel d) and non-smoking subjects ($n = 962$, panel e). The P values are from linear regression analysis, testing a linear relationship between the genotypes and FMD. $^{*}P < 0.05$, TT versus CC genotype; $^{\dagger}P < 0.05$, TT versus CT genotype from two-sample t-test. n is the number of observations.

Table 4 Multiple linear regression analysis for predicting brachial artery flow-mediated dilatation[a]

	All subjects		Overweight subjects		Normal-weight subjects		Smoking subjects		Non-smoking subjects	
Predictors	β	P	β	P	β	P	β	P	β	P
C242T polymorphism	0.050	0.019	0.096	0.003	0.009	0.760	0.077	0.009	0.022	0.481
Age	0.011	0.649	0.002	0.961	0.011	0.726	−0.019	0.555	0.043	0.204
Male sex	−0.174	< 0.001	−0.196	< 0.001	−0.169	< 0.001	−0.158	< 0.001	−0.190	< 0.001
Body mass index	0.129	< 0.001	0.044	0.229	0.068	0.031	0.135	< 0.001	0.116	0.004
Smoking	0.024	0.278	0.004	0.906	0.037	0.2	–	–	–	–
Systolic BP	−0.110	0.007	−0.136	0.015	−0.082	0.126	−0.148	0.006	−0.071	0.250
Diastolic BP	0.028	0.449	0.044	0.400	0.022	0.660	0.060	0.223	−0.001	0.989
Glucose	0.003	0.891	0.020	0.571	−0.011	0.724	0.029	0.380	−0.022	0.522
Total cholesterol	−0.114	0.382	−0.036	0.831	−0.474	0.092	−0.118	0.479	−0.067	0.752
HDL-cholesterol	0.097	0.050	0.056	0.400	0.214	0.028	0.124	0.053	0.530	0.501
LDL-cholesterol	0.108	0.332	0.001	0.993	0.455	0.064	0.095	0.509	0.093	0.605
Triglycerides	0.094	0.079	0.069	0.390	0.165	0.042	0.077	0.284	0.099	0.225
C-reactive protein	0.028	0.261	0.017	0.483	0.034	0.271	0.038	0.258	0.017	0.648
Adjusted R^2	0.069		0.082		0.050		0.080		0.058	
Significance (ANOVA)		< 0.001		< 0.001		< 0.001		< 0.001		< 0.001

ANOVA, Analysis of variance; BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein. [a] Statistical model: C242T polymorphism, age, sex, body mass index, waist circumference, smoking, systolic/diastolic blood pressure, glucose, C-reactive protein, total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides were entered as predictors into the multiple linear regression model.

an independent determinant of FMD in all subjects ($\beta = 0.050$, $P = 0.019$). The multivariate relationship remained significant in the overweight ($\beta = 0.096$, $P = 0.003$) and ever-smoking subjects ($\beta = 0.077$, $P = 0.009$, Table 4), but not in the normal-weight subjects ($\beta = 0.009$, $P = 0.760$) and non-smokers ($\beta = 0.022$, $P = 0.481$, Table 4). Because there is evidence that the effects of cigarette smoking on vascular physiology may be reversible after smoking cessation [30,31], we re-examined the association between FMD and the C242T polymorphism in former ($n = 305$) and current smokers ($n = 705$), respectively. The relationship remained in current smokers after adjustment for age, sex, obesity indices, blood pressure, serum glucose, lipids and CRP ($\beta = 0.079$, $P = 0.031$), whereas no relationship was found in former smokers ($\beta = 0.075$, $P = 0.143$).

Discussion

We found that the C242T polymorphism of the CYBA gene was related to the brachial artery FMD response in young healthy Finnish adults, and that body adiposity and smoking status may modify this association. Our results thus suggest a functional role of the C242T polymorphism in endothelial function.

The relationship between the C242T polymorphism of the CYBA gene and endothelial function has previously been investigated by Schachinger *et al.* [32]. The authors found that the C242T polymorphism was an independent determinant of epicardial coronary endothelial vasodilator function, therefore suggesting a functional significance of this gene polymorphism as related to superoxide production in the vascular wall. Our results with a large population-based sample of healthy adults confirm these observations. Contrary to these findings, however, some recent studies have failed to find any effect of the C242T polymorphism either on venous endothelial function in healthy individuals [33] or on forearm endothelium-dependent vasodilation in subjects with hypercholesterolemia [34]. Differences in vascular territories and sample sizes may explain such discrepancies.

Increased blood flow in the conduit arteries provokes endothelial cells to release nitric oxide, leading to vasodilatation. Therefore, FMD reflects nitric oxide-dependent endothelial function [35]. Impaired endothelial function is a marker of increased atherosclerotic risk [1]. In addition to reduced nitric oxide generation, accumulating evidence suggests that increased oxidative stress accounts for a significant proportion of endothelial dysfunction [9,36]. Increased superoxide production leads to the inactivation of nitric oxide, thus contributing to endothelial dysfunction and predisposing to the development of structural atherosclerotic vascular changes [8,9]. Membrane-associated NADPH oxidase is the primary physiological producer of reactive oxygen species, including superoxide, in the vasculature. The association of increased NADPH oxidase activity with endothelial dysfunction has been demonstrated in patients with atherosclerosis [10,36,37]. *p22phox* is a critical component of the NADPH oxidase system [12]. The C242T polymorphism of the CYBA gene that codes *p22phox* has been found to modulate superoxide production. Functionally, superoxide production has been linearly associated with the C242T genotypes, being highest in CC, intermediate in CT, and lowest in CC homozygotes [15]. Accordingly, we found a significant linear trend in brachial artery FMD responses across the genotypes, with the greatest difference observed between TT and CC homozygotes.

Interestingly, the relationship between the C242T polymorphism and FMD was modified by increased body weight and smoking habits. It is known that vascular

NADPH oxidase constitutively produces low amounts of superoxide anion intercellularly in the physiological condition [11]. Enzymatic activity can, however, be upregulated in conditions of increased oxidative stress, such as obesity and smoking [20,22,23]. Therefore, it is possible that the functional significance of the C242T polymorphism, as related to superoxide production, is more potent in situations in which NADPH oxidase may be highly activated. Our results suggest that the functional effect of the C242T polymorphism on endothelial function might be manifested only in an environment of higher oxidative stress, such as adiposity and current smoking. Future studies need to address this hypothesis in other specific high-risk populations among which oxidative stress is present and superoxide production is biochemically verified.

Brachial artery FMD is closely correlated with coronary endothelial function. Therefore, altered brachial artery FMD reactivity reflects not only the peripheral but also the coronary circulation [38]. As impaired brachial artery FMD is related to the prevalence and extent of coronary atherosclerosis and predicts cardiovascular events [39–41], TT allele carriers may be protected against not only the early pathogenesis of coronary atherosclerosis but also the later presence of coronary artery disease.

In the Young Finns population, there was an unexpected curvilinear relationship between FMD and BMI [25]. This observation has previously been discussed in detail, but it seems that an increase in body size within the non-obese range of this population of healthy young adults is associated with physiological changes that lead to enhanced brachial artery FMD responses, and counteract the opposing influences of larger vessel size and increased oxidative stress associated with greater body size [25].

Our study has some limitations. We did not measure endothelium-independent nitrate-mediated vasodilatation that is often used as a control for the FMD test to ensure that the altered FMD capacity is a consequence of endothelial dysfunction and is not a reflection of underlying smooth muscle function. Impaired and preserved responses to the smooth-muscle dependent dilator have, however, both been observed early in the process of atherogenesis [16,42]. In the present study, the long-term variation detected in FMD measurements (CV 26%) was relatively larger. The large variation is probably a result of physiological and technical issues [17]. The long-term reproducibility of brachial artery diameter measurements was, however, excellent (CV 3.2%). It therefore seems that much of the long-term variation in FMD is attributable rather to a physiological fluctuation in endothelial function than to measurement error. Furthermore, there were slight but statistically significant differences between the genotype groups in their levels of sensitive CRP and LDL-cholesterol, as well as their mean values of BMI. When we adjusted our results by these confounders using multiple linear regression analysis, however, the association between the C242T polymorphism and FMD remained significant. One additional limitation of our study was that we did not directly measure the biochemical indices related to superoxide production to verify our hypothesis.

In summary, the present study suggests a functional role of the C242T polymorphism of the CYBA gene in the modulation of brachial artery FMD in young healthy Finnish adults.

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The Association Between Cigarette Smoking and Carotid Intima–Media Thickness Is Influenced by the –930$^{A/G}$ *CYBA* Gene Polymorphism: The Cardiovascular Risk in Young Finns Study

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BACKGROUND

Smoking-induced damage to the cardiovascular system has been shown in many studies; however, the degree of damage varies from individual to individual. We hypothesized that the –930$^{A/G}$ *CYBA* gene polymorphism in the NADPH oxidase influences the association between cigarette smoking and carotid intima–media thickness (IMT) in young healthy adults.

METHODS

Cross-sectional data obtained in 2001 for the Cardiovascular Risk in Young Finns Study were used. IMT was measured with ultrasound. The genotyping was performed using a 5′-nuclease assay. A linear regression model was used to test whether the interaction between smoking and the genotypes was associated with IMT. The magnitude of the interaction effect was further examined by performing a stratified analysis according to smoking habits.

RESULTS

In the entire population, the mean and maxima IMT were higher in smokers than nonsmokers ($P = 0.005$ and 0.008, respectively). The differences were most significant in subjects with the GG genotype, borderline significant for the GA genotype, and nonsignificant for the AA genotype. The interaction of genotypes with smoking was associated with mean and maximal IMT ($P = 0.042$ and 0.022). Among smokers, subjects with the GG genotype had a higher mean and maximal IMT compared with carriers of the A allele ($P = 0.021$ and 0.012). In contrast, the mean and maximal IMT were lower for G allele carriers than subjects with the AA genotype among nonsmokers ($P = 0.022$ and 0.026). All results had been adjusted for potential risk factors related to IMT.

CONCLUSION

The –930$^{A/G}$ polymorphism modifies the association between cigarette smoking and IMT in young healthy adults.

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Smoking-induced damage to the cardiovascular system, such as increased atherosclerosis, has been shown in many clinical and epidemiological studies; however, the degree of damage varies from individual to individual.[1–4] In addition to the interaction of smoking with diet or other lifestyle factors, this between-individual difference in the response to smoking may be partly due to genetic variation. Although the exact component of cigarette smoke responsible for vasculopathy and the mechanism of smoking-related arterial damage are not fully elucidated, accumulating evidence suggests that cigarette smoke contains large amounts of free radicals, thus implicating increased oxidative stress as a potential mechanism.[5,6]

The NADPH oxidase is a major source of oxidants in the vasculature, and it consists of several membrane-bound and cytosolic proteins.[7,8] The p22phox encoded by the *CYBA* gene (cytochrome-β-245, α-polypeptide) is one of the components of the NADPH oxidase (**Figure 1**).[9] The transfection of antisense *CYBA* complementary DNA into vascular smooth muscle cells has been found to lead to a decrease in the superoxide production, suggesting that the p22phox is essential for the activity of the NADPH oxidase.[10] Several allelic variants have been identified in the *CYBA* gene.[11] Among these, the –930$^{A/G}$ *CYBA* polymorphism (rs9932581) is located at position –930 from the ATG codon in the *CYBA* gene promoter region. Linkage disequilibrium (LD) analysis has suggested that the –930$^{A/G}$ *CYBA* polymorphism is poorly associated with other

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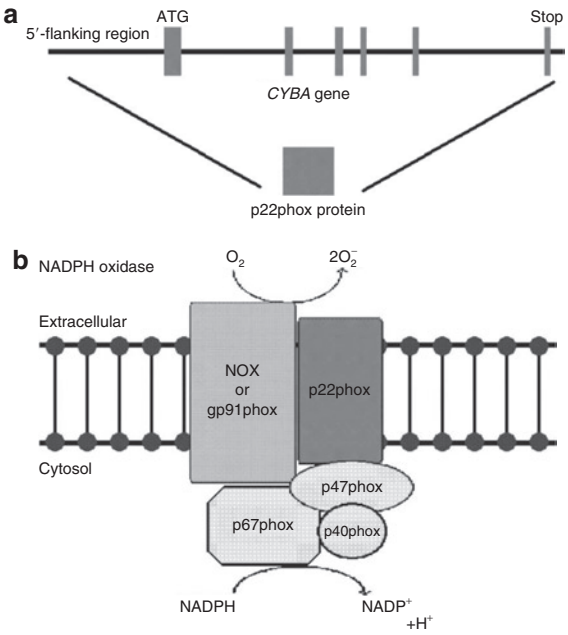

Figure 1 | Figures show a schematic of the *CYBA* gene and NADPH oxidase. (**a**) Organization of the *CYBA* gene. Boxes denote exons, and the line denotes the intron and 5′-flanking region. The *CYBA* gene encodes the p22phox protein. (**b**) Schematic figure of NADPH oxidase. The NADPH oxidase is composed of cytosolic moiety and membrane-associated proteins. The p22phox protein is one subunit of two integral membrane proteins of the NADPH oxidase.

functional *CYBA* polymorphisms, which were in a low or moderate linkage disequilibrium.[12] Although the association of this polymorphism with cardiovascular diseases is controversial, the presence of the –930$^{A/G}$ *CYBA* polymorphism has been related to higher transcriptional activity, mRNA and protein expression of the p22phox as well as the NADPH oxidase activity.[13–17] Previous studies have shown that the expression of the p22phox in human atherosclerotic coronary arteries is more intense than in nonatherosclerotic arteries, indicating possible participation of upregulated p22phox in the pathogenesis of atherosclerosis.[18] Therefore, it is likely that the genetic effect of the –930$^{A/G}$ *CYBA* polymorphism may cause a between-individual difference in susceptibility to atherogenesis because of its relation with the p22phox.

Carotid intima–media thickness (IMT) is a marker of subclinical atherosclerosis.[19] Cigarette smoking is an important risk factor for the development of atherosclerosis.[20] Many studies on smoking and IMT have established a strong correlation between cigarette smoking and an accelerated progression of carotid atherosclerosis.[21,22] Furthermore, we have previously shown that IMT values are higher among cigarette smokers than among nonsmokers in the Cardiovascular Risk in Young Finns Study.[23] In the present study, we further investigated the association of the –930$^{A/G}$ *CYBA* polymorphism with IMT in response to cigarette smoking in the young Finns population. We hypothesized that the effect of the –930$^{A/G}$ *CYBA* polymorphism of the NADPH oxidase modifies the atherosclerotic process and causes a between-individual difference in IMT in response to cigarette smoking.

METHODS

Subjects. The Cardiovascular Risk in Young Finns Study is an ongoing five-center, prospective cohort study regarding the cardiovascular risk in children and adolescents in Finland. The first cross-sectional survey was conducted in 1980 with 3,596 subjects aged 3–18 years. The latest 21-year follow-up was performed in 2001, enrolling 2,283 subjects aged 24–39 years from the original cohort. Thirty-six percent of the baseline cohort dropped out of the follow-up; the main reasons for nonparticipation have been analyzed previously.[23] In 2001, IMT was measured successfully for 2,264 subjects. In the present study, a total of 2,179 subjects were finally included in the analysis—85 were excluded due to missing data (n = 45) and unsuccessful genotyping (n = 40). All subjects provided a written informed consent in 2001, and the study was approved by the local ethics committees.

Clinical characteristics and risk factor measurements. Data including age, sex, and smoking habits were acquired using questionnaires. The category of smokers included both current and ex-smokers. Current smokers were defined as those who were currently smoking on a daily or weekly basis. Ex-smokers included those who reported regular smoking in the past but no current smoking. Pack-years were assessed for the smokers. Weight and height were measured and body mass index was calculated (kg/m^2).[24] Blood pressure (BP) in a sitting position was measured by trained research nurses after 5-min rest with a random zero sphygmomanometer. Korotkoff's fifth sound was used as the sign of diastolic BP and first sound as the sign of systolic BP. The mean of three measurements on three different visits was used in the analysis. The subjects were defined as hypertensive if the systolic BP was ≥140 mm Hg and/or diastolic BP ≥90 mm Hg and/or if they had already been treated with drugs. Bloods were drawn for the biochemical and genetic analyses after an overnight fast. Standard enzymatic methods were employed for the determination of serum lipids and glucose.[24] Low-density lipoprotein cholesterol concentration was calculated with the Friedewald formula. The estimated glomerular filtration rate was calculated with the Cockroft–Gault formula.[25] Patients were defined as diabetic if their fasting blood glucose concentration was ≥126 mg/dl, and/or if they had already been diagnosed as diabetic. Fasting plasma high-sensitive C-reactive protein concentrations were analyzed by means of latex turbidometric immunoassay (Wako Chemicals, Neuss, Germany).

DNA isolation and genotyping of the –930$^{A/G}$ CYBA gene polymorphism. Whole venous bloods were drawn into EDTA tubes and then stored at –70 °C. Genomic DNA was extracted using a commercially available kit and the BioRobot M48 Workstation (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genotyping of the –930$^{A/G}$ *CYBA* gene polymorphism (rs9932581) was performed for both PCR and allelic discrimination[26] using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Nucleotide sequences for the primers and allele-specific probes

were deduced from sequences published in the Gene Bank and Celera databases. The primer and probe design was carried out in conjugation with Applied Biosystems with the aid of the Assays by Design service. The reproducibility of polymorphism genotyping was 100%, as assessed by 94 blind duplicates.

Carotid artery measurements. Carotid ultrasound studies were performed using Sequoia 512 ultrasound mainframes (Acuson, Mountain View, CA) with 13.0 MHz linear array transducers. Details of the method have been described previously.[23] In brief, the posterior wall of the left carotid artery was scanned according to a standardized protocol. A 5-s moving scan was recorded and stored in digital format on optical disks for subsequent off-line analysis. The digitally stored scans were manually analyzed by a single reader blinded to the subjects' details, with the analyses performed using ultrasonic calipers. A minimum of four measurements of the carotid wall was taken to derive the mean and maximal IMT. To assess the reproducibility of IMT measurements, 60 participants were re-examined 3 months after the initial visit. The between-visit coefficient of variation for IMT measurements was 6.4%. To assess the reproducibility of IMT image analyses, 113 scans were reanalyzed by a second observer. The between-observer coefficient of variation was 5.2%.

Statistical analysis. The continuous variables are presented as mean ± s.d. Differences across study groups were examined with the χ^2-test for categorical variables, Student's *t*-test, and analysis of variance for continuous variables. A linear regression model was used to test the relation between IMT and the interaction of the −930$^{A/G}$ *CYBA* polymorphism with smoking habits. For constructing the interaction variable (smoking × genotype), smoking and genotype were coded as follows: smoking, 1 for nonsmokers, 2 for smokers; genotype, 1 for GG, 2 for GA, 3 for AA. To select variables (potential risk factors) for the model, we first used the univariate linear regression method to filter out variables that associate with IMT individually. The variables we chose for the model included age, sex, body mass index, systolic BP, diastolic BP, blood glucose, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, C-reactive protein, and estimated glomerular filtration rate. To further examine the interaction effect on IMT, stratified analysis according to smoking habits or genotype was performed using analysis of variance and covariance adjusted for the aforementioned risk factors. As some antihypertensive medications may influence the NADPH oxidase, all analyses were repeated after the exclusion of subjects taking these medications (n = 29), with similar results (data not shown). Due to skewed distributions, the values for triglycerides and C-reactive protein were $\log_{10}$-transformed and those for blood glucose reciprocal-transformed before the analyses. All statistical analyses were two-sided and performed with the statistical software SPSS (version 14.0; SPSS, Chicago, IL). *P* values below 0.05 were considered statistically significant. In addition, power calculations were made with the freeware Power*G3. *Post hoc* power calculations for smokers revealed that we had 72 and 74% power to detect a difference of 0.013 and 0.05 mm in mean and maximal IMT, respectively, between subjects with the GG genotype and carriers of the A allele at a 5% significance level. For nonsmokers, we had 74 and 76% power at a 5% significance level to detect a difference of 0.019 and 0.087 mm in mean and maximal IMT, respectively, between subjects with the AA genotype and carriers of the G allele.

Table 1 | Characteristics of smokers and nonsmokers

	Smoker (n = 1,151)	Nonsmoker (n = 1,028)	*P*	*P′*
Age, years	32 ± 5	32 ± 5	0.45	0.44
Male sex, *n* (%)	585 (50.8)	395 (38.4)	<0.001	<0.001
Body mass index, kg/m^2	25.3 ± 4.3	24.7 ± 4.4	0.001	0.010
Systolic BP, mm Hg	123 ± 15	122 ± 14	0.11	0.27
Diastolic BP, mm Hg	73 ± 9	73 ± 9	0.85	0.25
Glucose, mg/dl	90.9 ± 13.7	90.6 ± 14.3	0.58	0.55
Total cholesterol, mg/dl	201.6 ± 38.8	200.9 ± 37.5	0.65	0.95
HDL cholesterol, mg/dl	49.3 ± 12.3	51.6 ± 12.3	<0.001	0.026
LDL cholesterol, mg/dl	128.4 ± 33.7	127.0 ± 32.2	0.32	0.80
Triglycerides, mg/dl	121.5 ± 70.8	113.5 ± 69.0	0.008	0.029
C-reactive protein, mg/l	1.90 ± 3.84	1.96 ± 4.18	0.71	0.11
GFR, ml/min	133 ± 33	127 ± 30	<0.001	0.006

The continuous variables were presented as mean ± s.d. *P* values were obtained by the χ^2-test for categorical variables or the Student's *t*-test for continuous variables. *P′* values are *P* values adjusted for age and/or sex.
BP, blood pressure; GFR, glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

RESULTS

The study subjects formed a low-risk population: they were young, with 13.7% (n = 298) suffering from hypertension and 0.9% from diabetes (n = 20). However, many of the subjects (52.8%) had a smoking habit, and the pack-years in the entire population (n = 2,179) were 2.8 ± 5.5 (mean ± s.d.). Among smokers, the pack-years were significantly higher for current smokers than ex-smokers (6.5 ± 7.2 vs. 2.9 ± 4.8), and the result remained significant after adjusting for age and sex (P < 0.001). However, the pack-years did not correlate with carotid IMT after controlling for age and sex among the smokers (r = 0.019, P = 0.52 for maximal IMT; r = 0.016, P = 0.60 for mean IMT). Similarly, no correlation was found among current smokers or among ex-smokers (data not shown).

The characteristics of smokers (n = 1,151) and nonsmokers (n = 1,028) are shown in **Table 1**. In the entire population, the prevalence of smoking was higher among male than female subjects. Differences were found in body mass index, estimated glomerular filtration rate, and the levels of triglycerides and high-density lipoprotein cholesterol between the smokers and nonsmokers, and the results remained after adjusting for age and/or sex.

The G allele frequency was 0.58 in this population, and the allele frequencies were in line with the Hardy–Weinberg equilibrium (χ^2 = 0.27, P = 0.33). The allele frequencies among

smokers and nonsmokers did not differ from the corresponding frequency for the entire population (data not shown).

The characteristics of the subgroups stratified by genotype among smokers and nonsmokers are shown in Table 2. Both systolic and diastolic BP values differed significantly across genotypes among nonsmokers. Among smokers, there was no difference in pack-years across genotypes (data not shown).

Among all subjects, the mean and maximal IMT values were significantly higher among smokers, current smokers, or ex-smokers in comparison to nonsmokers (Table 3), while no statistical difference was detected between current and ex-smokers. The differences were most significant in subjects with the GG genotype, borderline significant for the GA genotype, and nonsignificant for the AA genotype (Table 3). All these results remained similar after adjusting for potential risk factors including age, sex, body mass index, systolic BP, diastolic BP, blood glucose, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, C-reactive protein, and estimated glomerular filtration rate (Table 3).

In the entire population, the interaction effect of the –930$^{A/G}$ *CYBA* polymorphism and smoking on carotid IMT was examined with a linear regression model. The interaction-term (genotype × smoking) was significantly associated with mean and

Table 2 | Characteristics of the study groups stratified by genotype among smokers and nonsmokers

	Smoker				Nonsmoker			
	GG (n = 401)	GA (n = 553)	AA (n = 197)	P	GG (n = 348)	GA (n = 494)	AA (n = 186)	P
Age, years	32 ± 5	32 ± 5	32 ± 5	0.90	32 ± 5	32 ± 5	32 ± 5	0.57
Male sex, n (%)	200 (49.9)	284 (51.4)	101 (51.3)	0.90	128 (36.8)	194 (39.3)	73 (39.2)	0.74
Body mass index, kg/m^2	25.5 ± 4.3	25.2 ± 4.3	25.3 ± 4.5	0.64	24.8 ± 4.4	24.7 ± 4.4	24.9 ± 4.3	0.83
Systolic BP, mm Hg	123 ± 16	122 ± 14	122 ± 14	0.57	120 ± 14	123 ± 15	121 ± 15	0.038
Diastolic BP, mm Hg	74 ± 9	73 ± 9	73 ± 9	0.32	72 ± 9	74 ± 9	74 ± 9	0.028
Glucose, mg/dl	91.4 ± 17.0	90.9 ± 12.6	90.1 ± 8.3	0.54	91.5 ± 17.5	90.2 ± 13.1	90.1 ± 9.8	0.36
Total cholesterol, mg/dl	203.8 ± 41.5	201.0 ± 37.5	199.0 ± 36.4	0.33	202.5 ± 39.9	198.5 ± 35.3	204.1 ± 38.4	0.14
HDL cholesterol, mg/dl	49.4 ± 12.1	49.4 ± 12.7	49.1 ± 12.0	0.97	51.7 ± 12.6	51.3 ± 12.1	52.1 ± 12.3	0.71
LDL cholesterol, mg/dl	130.0 ± 35.5	127.8 ± 32.5	127.1 ± 33.4	0.51	129.3 ± 34.0	124.5 ± 30.5	129.4 ± 32.6	0.056
Triglycerides, mg/dl	124.6 ± 79.2	121.1 ± 65.5	116.4 ± 67.0	0.41	110.1 ± 72.1	115.6 ± 69.7	114.5 ± 61.1	0.50
C-reactive protein, mg/l	1.98 ± 4.54	1.91 ± 3.58	1.68 ± 2.93	0.67	2.03 ± 4.21	1.93 ± 4.43	1.92 ± 3.36	0.93
GFR, ml/min	133 ± 33	133 ± 33	136 ± 34	0.50	127 ± 30	127 ± 31	128 ± 29	0.88

The continuous variables were presented as mean ± s.d. P values were obtained by means of the χ^2 test for categorical variables or one-way analysis of variance for continuous variables.
BP, blood pressure; GFR, glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Table 3 | The differences in carotid artery IMT between smokers and nonsmokers, as well as among current smokers, ex-smokers, and nonsmokers in all subjects and the study groups stratified by genotype

	Smoker	Nonsmoker	P	P'	Current-smoker	Ex-smoker	Nonsmoker[a]	P	P'
All	n = 1,151	n = 1,028			n = 758	n = 393	n = 1,028		
Mean IMT	0.59 ± 0.09	0.57 ± 0.09	0.002	0.005	0.58 ± 0.09	0.59 ± 0.09	0.57 ± 0.09	0.002	0.018
Max IMT	0.63 ± 0.10	0.62 ± 0.10	0.002	0.008	0.63 ± 0.10	0.63 ± 0.10	0.62 ± 0.10	0.004	0.028
GG	n = 401	n = 348			n = 266	n = 135	n = 348		
Mean IMT	0.60 ± 0.09	0.57 ± 0.09	0.001	0.005	0.59 ± 0.09	0.61 ± 0.10	0.57 ± 0.09	0.001	0.013
Max IMT	0.64 ± 0.10	0.61 ± 0.10	0.001	0.004	0.63 ± 0.10	0.65 ± 0.10	0.61 ± 0.10	0.001	0.013
GA	n = 553	n = 494			n = 359	n = 194	n = 494		
Mean IMT	0.58 ± 0.09	0.57 ± 0.09	0.051	0.060	0.58 ± 0.09	0.58 ± 0.09	0.57 ± 0.09	0.12	0.19
Max IMT	0.62 ± 0.09	0.61 ± 0.01	0.055	0.068	0.62 ± 0.10	0.63 ± 0.10	0.61 ± 0.10	0.14	0.17
AA	n = 197	n = 186			n = 133	n = 64	n = 186		
Mean IMT	0.59 ± 0.09	0.59 ± 0.10	0.82	0.67	0.59 ± 0.10	0.59 ± 0.08	0.59 ± 0.10	0.94	0.62
Max IMT	0.63 ± 0.10	0.63 ± 0.10	0.60	0.44	0.63 ± 0.10	0.63 ± 0.09	0.63 ± 0.11	0.87	0.73

The variables are presented as mean ± s.d. P values were obtained by one-way analysis of variance. P' values were obtained by one-way analysis of covariance adjusted for age, sex, body mass index, systolic BP, diastolic BP, blood glucose, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, C-reactive protein, and GFR.
BP, blood pressure; GFR, glomerular filtration rate; HDL, high-density lipoprotein; IMT, intima–media thickness; LDL, low-density lipoprotein.
[a]Referent group.

maximal IMT ($\beta = -0.196$, $P = 0.026$ and $\beta = -0.218$, $P = 0.013$, respectively), and it remained significant ($\beta = -0.168$, $P = 0.042$ and $\beta = -0.190$, $P = 0.022$) when the model was further adjusted for the aforementioned risk factors. The magnitude of the interaction was further examined by performing a stratified analysis according to smoking habits. The mean and maximal IMT values were different between the genotypes both among smokers ($P = 0.054$ and $P = 0.044$, respectively) and among nonsmokers ($P = 0.048$ and $P = 0.056$, respectively, one-way analysis of variance), and the results remained significant after adjustment for the aforementioned risk factors in smokers ($P = 0.047$ and $P = 0.039$) and nonsmokers ($P = 0.023$ and $P = 0.024$, one-way analysis of covariance). Among smokers, subjects with the GG genotype had higher mean and maximal IMT values when compared with carriers of the A allele (GG vs. GA + AA, $P = 0.024$ and $P = 0.014$, respectively, one-way analysis of variance). In contrast, the mean and maximal IMT values were lower in carriers of the G allele than in subjects with the AA genotype among nonsmokers (GG + GA vs. AA, $P = 0.015$ and $P = 0.018$, respectively). The results remained significant after adjusting for the aforementioned risk factors among smokers (**Figure 2** left-hand panel, $P = 0.012$ for maximal IMT and $P = 0.021$ for mean IMT, one-way analysis of covariance), as well as among nonsmokers (**Figure 2** right-hand panel, $P = 0.026$ for maximal IMT and $P = 0.022$ for mean IMT).

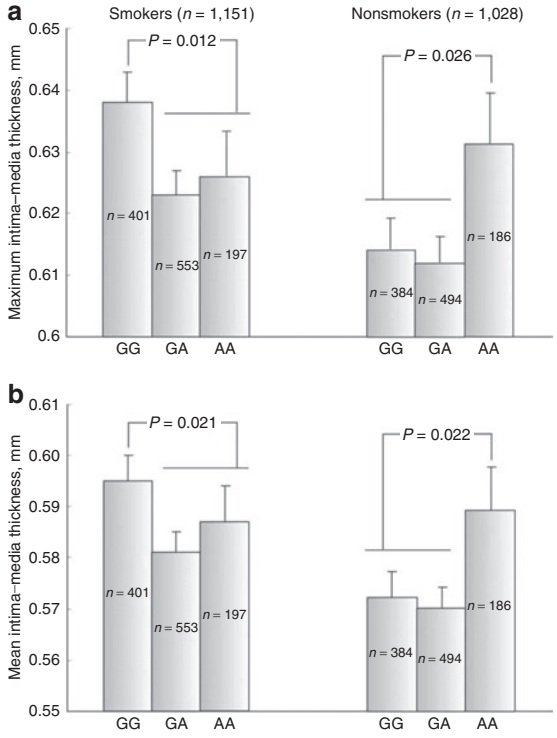

Figure 2 | Bar graphs show (**a**) the maximum and (**b**) mean IMT of the study groups stratified by genotype among smokers (left-hand panel) and nonsmokers (right-hand panel). Values are means ± s.e.m. *P* values were calculated by one-way analysis of covariance; the effect of the –930$^{A/G}$ polymorphism was assumed to be G dominant (AA vs. GA and GG) among nonsmokers or G recessive (AA and GA combined vs. GG) among smokers; and age, sex, body mass index, systolic blood pressure, diastolic blood pressure, blood glucose, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, C-reactive protein, and glomerular filtration rate were used as covariates.

DISCUSSION

In the present study, the association of cigarette smoking with IMT was influenced by the –930$^{A/G}$ *CYBA* polymorphism of the NADPH oxidase. Our results showed that the effect of cigarette smoking impacted on IMT in G allele carries, but no effect was detected in subjects with the AA genotype, suggesting that the –930$^{A/G}$ *CYBA* polymorphism might play an important role in modifying IMT in response to cigarette smoking.

Cigarette smoking is known to induce a large amount of oxidants, and these free radicals could arise directly from cigarette smoke or indirectly from endogenous sources.[27,28] In the setting of cardiovascular disease, a potentially more important source of reactive oxygen species is considered to derive from the substances in cigarette smoke that may stimulate intracellular reactive oxygen species production in the vasculature.[29–31] Recent studies demonstrated that cigarette smoke-induced superoxide production was released from the NADPH oxidase, but not from other inducible intracellular sources, such as xanthine oxidase or endothelial nitric oxide synthase.[30,31] NADPH oxidase-mediated superoxide production has shown a significant correlation with IMT in subjects free of clinical atherosclerotic disease.[32] In the present study, IMT was significantly increased among smokers in comparison to nonsmokers. However, increased IMT in response to cigarette smoking varied between the genotypes of the –930$^{A/G}$ *CYBA* polymorphism of the NADPH oxidase. Previously, the –930$^{A/G}$ *CYBA* polymorphism had shown differential functional effects in the development of NADPH oxidase-dependent oxidative stress.[17] Therefore, our finding supports the hypothesis that cigarette smoking increases oxidative stress as a potential mechanism for initiating atherosclerosis, whereas the –930$^{A/G}$ *CYBA* polymorphism of the NADPH oxidase seems to modify the atherosclerotic process and cause a between-individual difference in IMT in response to cigarette smoking. One additional finding of the present study was that the increased IMT value persisted in ex-smokers when compared with nonsmokers, and the –930$^{A/G}$ *CYBA* polymorphism similarly impacted on the association between ex-smoking and IMT. Although some studies have shown that smoking cessation could revert the changes induced by cigarette smoking,[3,33,34] others have suggested that the reversible changes are time-dependent and that the smoking-related responses gradually subside after smoking cessation.[3,34] The persisting effect of smoking after smoking cessation may keep ex-smokers in a condition of oxidative stress.[33,35] Therefore, the effect of the –930$^{A/G}$ *CYBA* polymorphism on IMT in the present study was highlighted among ex-smokers.

In the present study, the genetic effect on IMT differed in the presence and absence of cigarette smoking. The difference might be due to an interaction effect of cigarette smoking with the G allele of the –930$^{A/G}$ *CYBA* polymorphism among smokers. The functional effect of the –930$^{A/G}$ *CYBA* polymorphism

has been studied in the phagocytic cells of healthy and hypertensive subjects.[16,17,36] The studies have consistently reported that the effect of the –930$^{A/G}$ *CYBA* gene polymorphism on *CYBA* gene expression and subsequent activity depends on the hypertensive phenotype.[16,17] In contrast, the effect of the –930$^{A/G}$ polymorphism did not affect neutrophil NADPH oxidase activity in healthy adults.[36] The human p22phox promoter possesses several potential consensus sequences for transcriptional factors.[16] Because the –930$^{A/G}$ polymorphic site lies on a potential binding site for CCAAT/enhancer binding proteins (C/EBPs) transcription factors, it has been suggested that upregulated C/EBP expression in essential hypertension might induce a greater effect on the transcriptional activity of the G than of the A allelic *CYBA* promoter.[16,17] In the present study, the study subjects were young and formed a low-risk study population. Only 13.7% had hypertension, and no difference in BP values was observed between the smokers and nonsmokers. However, we found a significant effect of the –930$^{A/G}$ *CYBA* polymorphism in G allele carriers depending on the exposure to cigarette smoking. It has been observed that the C/EBP-binding activity is significantly increased in healthy smokers when compared with nonsmokers.[37] It is therefore tempting to speculate a potential involvement of C/EBP in the NADPH oxidase activation among the smokers included the present study. Although we could not provide direct evidence of this, our findings that IMT in response to cigarette smoking was increased in G allele carriers while no change was found in subjects with the AA genotype were in concordance with this hypothesis.

In the present study, IMT was higher in subjects with the AA genotype than in G allele carriers among nonsmokers. This finding was not in concert with previous observations showing that there was no effect of the –930$^{A/G}$ polymorphism on the NADPH oxidase activity in the healthy subjects with no cardiovascular risk factors and atherosclerotic burden.[16,17,36] We have no plausible explanation for this, but the differences in methodology between the present and previous studies might be behind this apparent discrepancy. All previous studies were conducted *in vitro* with the phagocytic cells obtained from healthy subjects for the study of the functional effect of the –930$^{A/G}$ *CYBA* polymorphism. However, the values of IMT across the genotypes among the nonsmokers in our study might reflect physiological adaptations to various factors including the functional effect of the –930$^{A/G}$ *CYBA* polymorphism in vascular tissues. The present study lacked the functional data on the effect of the –930$^{A/G}$ *CYBA* polymorphism, whereas these functional data might be the useful intermediate phenotypes for predicting the relationship between the –930$^{A/G}$ *CYBA* polymorphism and IMT. This is a limitation of the present study, and further investigations are required to elucidate the functional effect of the –930$^{A/G}$ polymorphism on IMT variation.

Another limitation of the study is that we lost 36% of the baseline cohort in the follow-up phase. However, we have shown that the baseline risk factors were similar among participants and dropouts in the 21-year follow-up, and the present study cohort therefore seems to be representative of the original study population.[24] Furthermore, more females were represented among nonsmokers, but this is an unlikely source of bias because we adjusted our analyses by sex. Our study was based on a larger sample of young adults. However, the genetic effect was separately examined in the smoking and nonsmoking subgroups because of an interaction effect with cigarette smoking. Power calculations showed that we had ~75% power to detect the effect of the –930$^{A/G}$ polymorphism on IMT in the subgroups. Therefore, our results need to be interpreted with caution and duplicated further with other similar populations.

The genetic effect of the –930$^{A/G}$ *CYBA* polymorphism is associated with IMT in response to cigarette smoking among young healthy Finnish adults.

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Genetics

The *p22phox* C242T gene polymorphism is associated with a reduced risk of angiographically verified coronary artery disease in a high-risk Finnish Caucasian population. The Finnish Cardiovascular Study

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Background Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a major source of the superoxide anion, which may play an important role in the development of atherosclerosis and coronary artery disease (CAD). The *p22phox*, a component of the NADPH oxidase, is essential for the activation of this enzyme, and intensive expression of the *p22phox* has been reported in human atherosclerotic arteries. However, studies on the association of the C242T polymorphism in the *p22phox* gene with CAD have produced conflicting results, and the relation of this polymorphism with CAD is not well known in a population with acquired risk factors enhancing the NADPH-dependent superoxide production.

Methods As part of the Finnish Cardiovascular Study, a case-control study was conducted with 402 high-risk Finnish Caucasian patients undergoing coronary angiography. Genotyping was performed using the 5′ nuclease TaqMan assay.

Results The prevalence of the T allele (TT + TC genotypes) was significantly lower among angiographically verified CAD patients (n = 250) than among control subjects (n = 152, $P = .013$). In contrast to subjects with the CC genotype, the T allele was found protective against CAD (odds ratio = 0.531, 95% CI 0.331-0.852, $P = .009$), and the results remained significant after adjustment for other significant coronary risk factors.

Conclusions The T allele in the C242Tpolymorphism of the *p22phox* gene had a protective effect against the development of CAD despite the exposure of study subjects to risk factors related to excessive NADPH-dependent superoxide production. (Am Heart J 2006;152:538-42.)

Increased reactive oxygen species (ROS) may play a role in the development of coronary artery disease (CAD). In addition to altering vascular function, the excessive production of ROS causes tissue injury by direct cytotoxic effects on cellular membranes and biologic macromolecules, such as proteins, lipids, and DNA.[1] Among ROS, the superoxide anion is of critical importance because many other reactive species can be derived from it.[2]

The membrane-associated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase has been implicated as a major enzymatic source of the superoxide anion in the vasculature.[3,4] The *p22phox* is a component of vascular and phagocytic NADPH oxidases. Transfection of antisense *p22phox* complementary DNA into vascular smooth muscle cells has been found to lead to a decrease in the superoxide production.[5] Thus, *p22phox* could be essential for the activation of the NADPH oxidase in vascular tissue. In addition, the expression of the *p22phox* has been found more intense in human atherosclerotic coronary arteries than non-atherosclerotic arteries, indicating that the *p22phox* might participate in the pathophysiology and pathogenesis of atherosclerotic CAD.[6]

Several genetic variations in the *p22phox* gene have been identified, and the C242T polymorphism has been

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demonstrated to affect NADPH oxidase activity.[7,8] Therefore, the functional consequences of the C242T polymorphisms in the *p22phox* gene could modify the risk of CAD by producing varying amounts of the superoxide anion. The C242T polymorphism is located in exon 4 of the *p22phox* gene and results in the amino acid substitution of histidine by tyrosine at position 72 within one of the two haem-binding sites.[9] Since Inoue et al[9] first suggested that the T allele of the C242T polymorphism might have a protective effect against CAD, several studies on the association of this polymorphism with CAD have produced conflicting results.[9-13]

Coronary artery disease is a multifactorial disease caused by a sum of genetic and environmental factors. Well-established acquired risk factors for CAD, such as smoking, diabetes mellitus, obesity, hypertension, and hypercholesterolemia, have been found to induce the excessive production of the NADPH-dependent superoxide anion.[14-18] In the present study, we hypothesized that the T allele of *p22phox* might have a protective effect against CAD after exposure of the subjects to risk factors associated with excessive production of the NADPH-dependent superoxide anion. A case-control study nested within a high-risk Finnish Caucasian population was conducted with CAD patients verified by coronary angiography.

Methods

Subjects

The original population consisted of 2290 Finnish Caucasian patients who participated in the Finnish Cardiovascular Study, an ongoing follow-up study focusing on the genetic background of exercise test responses and coronary heart disease at Tampere University Hospital, Finland, between October 2001 and December 2004. Of these patients, 542 also underwent coronary angiography at the Department of Cardiology before June 2005. The present study subjects were recruited from among these 542 patients. Patients were eligible for the study only if they had risk factors including smoking, body overweight, hypertension, hypercholesterolemia, or diabetes mellitus. The local ethical committee approved the study protocol, and a written consent after explanation of the aims and details of the study was obtained from each participant. Four hundred two patients were finally included in the analysis: 139 were excluded due to lacking blood samples or incomplete angiographic data and 1 patient was excluded because he had none of the risk factors examined in this study.

By means of coronary angiography, the patients with significant coronary arterial stenosis (≥50%) affecting at least one vessel were defined as CAD patients, and the patients with no stenosis or with stenosis <50% were defined as control subjects.

Data including age, sex, smoking habits, history of hypertension, hypercholesterolemia, diabetes mellitus, and medications were collected using a computer-based questionnaire. Weight and height were recorded and body mass index (BMI) was calculated (kg/m^2). Blood pressure in a sitting position was measured using a mercury sphygmomanometer.[19] Blood was drawn for the biochemical and genetic analyses after an overnight fast. Standard enzymatic methods were used for total cholesterol determination.[20,21] Blood glucose was measured with the glucose oxidase method.[22]

Table I. Characteristics of the entire study population

Characteristic	Study subjects
Male	281 (70)
Age (y)	59 ± 11
BMI (kg/m^2)	27.3 ± 4.3
Risk factors	402 (100)
Body overweight	300 (75)
Smoker	226 (56)
Diabetic	97 (24)
Hypertension	382 (95)
Hypercholesterolemia	279 (69)
p22phox C242T polymorphism	
CC	258 (64)
CT	124 (31)
TT	20 (5)
T allele frequency	0.20

Values are presented as n (%) or mean ± SD.

The subjects were considered smokers if they were either current or former smokers. They were defined as hypertensive if the systolic blood pressure was ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg[19] or if they had already been treated with antihypertensive drugs. Subjects were classified to have hypercholesterolemia if their total cholesterol level was ≥6.21 mmol/L[23] or if they had already been treated with cholesterol-lowering drugs. Patients were defined as diabetic if their fasting blood glucose concentration was ≥7.0 mmol/L[24] or if they were already undergoing treatment for diabetes. Body overweight was defined as BMI ≥25 kg/m^2.

DNA isolation and genotyping of the C242T *p22phox* gene polymorphism

Whole venous blood was drawn into EDTA tubes and then stored at −70°C. Genomic DNA was extracted using a commercially available kit and BioRobot M48 Workstation (Qiagen Inc, Hilden, Germany) according to the manufacturer's instructions. Polymerase chain reaction (PCR) for the C242T transition polymorphism in the *p22phox* gene was carried out in 384 well plates following a standard protocol for TaqMan MGB probes. A 5-μL final volume containing genomic DNA, 2 × Universal Master Mix, 900 nmol/L of each primer, and 200 nmol/L of each probe was used in PCR. Nucleotide sequences for the primers and allele-specific probes were deduced from published sequences in the GenBank database. The primers and probes were designed in conjugation with Applied Biosystems using the Validated SNP Genotyping Assays. After the PCR was finished, allelic discrimination was performed using the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA).

Statistical analysis

The continuous variables are presented as mean ± SD. Differences between the CAD patients and control subjects were examined with the χ^2 test for categorical variables or the Student *t* test for continuous variables. The odds ratio (OR) of

Table II. Characteristics of CAD patients and control subjects

	CAD (n = 250)	Control (n = 152)	*P*	OR (95% CI)
Male	193 (77)	88 (58)	<.001	2.463 (1.591-3.812)
Age (y)	62 ± 10	55 ± 11	<.001	1.071 (1.048-1.094)
BMI (kg/m^2)	27.3 ± 4.1	27.2 ± 4.6	.829	1.005 (0.959-1.054)
Body overweight	187 (62)	63 (62)	.919	0.976 (0.615-1.550)
Smoker	147 (59)	79 (52)	.181	1.319 (0.879-1.979)
Diabetic	72 (29)	25 (16)	.005	2.055 (1.235-3.418)
Hypertension	237 (95)	145 (95)	.790	0.880 (0.343-2.257)
Hypercholesterolemia	193 (77)	86 (57)	<.001	2.599 (1.680-4.019)
p22phox C242T polymorphism				
CC	172 (69)	86 (57)		
TT + TC	78 (31)	66 (43)	.013	0.591 (0.389-0.897)
T allele frequency	0.18	0.25	.009	0.632 (0.447-0.894)

P values were obtained by χ^2 test for categorical variables or Student *t* test for continuous variables. Values are presented as n (%) and mean ± SD. Odds ratios and 95% CI were calculated with logistic regression analysis. Odds ratio for genotypes was calculated with TT + TC versus CC.

Table III. Characteristics of the study groups stratified by genotype in the entire study population, among CAD patients, and among control subjects

	Total (N = 402)		CAD (n = 250)		Control (n = 152)	
C242T genotypes	CC (n = 258)	TT + TC (n = 144)	CC (n = 172)	TT + TC (n = 78)	CC (n = 86)	TT + TC (n = 66)
Male	177 (69)	104 (72)	130 (76)	63 (81)	47 (55)	41 (62)
Age (y)	60 ± 11	58 ± 11	63 ± 9	61 ± 11	54 ± 12	55 ± 10
BMI (kg/m^2)	27.2 ± 4.3	27.5 ± 4.3	27.2 ± 4.2	27.6 ± 4.0	27.2 ± 4.5	27.3 ± 4.7
Body overweight	193 (75)	107 (74)	127 (74)	60 (77)	66 (77)	47 (71)
Smoker	151 (59)	75 (52)	104 (61)	43 (55)	47 (55)	32 (49)
Diabetic	58 (23)	39 (27)	47 (27)	25 (32)	11 (13)	14 (21)
Hypertension	246 (95)	136 (94)	164 (95)	73 (94)	82 (95)	63 (96)
Hypercholesterolemia	177 (69)	102 (71)	130 (76)	63 (81)	47 (55)	39 (59)

Values are presented as n (%) and mean ± SD. There were no differences between the study groups when stratified by genotype in the entire study population, among CAD patients, and among control subjects.

CAD and its 95% CI for the study subjects exposed to specific risk factors were calculated with logistic regression analysis. Furthermore, the multiple logistic regression method was used to determine the independent risk factors for CAD in this population. Only the risk factors that were significantly associated with CAD in univariate analysis were included in the multiple logistic regression analysis. All statistical analyses were 2-sided and performed with the statistical software SPSS (version 13.0, SPSS Inc, Chicago, IL). A P value <.05 was considered statistically significant.

Results

The characteristics of the study subjects are given in Table I. The risk factors examined in the study were body overweight, smoking, diabetes mellitus, hypertension, and hypercholesterolemia. The study subjects formed a high-risk population: 5% (n = 21) had only one risk factor for CAD, whereas 32% (n = 87) had two, 30% (n = 120) three, 35% (n = 141) four, and 8% (n = 33) as many as five.

A total of 402 patients were angiographically defined as either CAD patients (n = 250) or control subjects (n = 152). The CAD patients were older than the control subjects. Male sex, diabetes, and hypercholesterolemia were significantly pronounced among the CAD patients. There were no differences in the incidence of body overweight, smoking, or hypertension between the CAD patients and control subjects (Table II). Furthermore, no differences were found between the study groups in their clinical characteristics when stratified by genotype in the entire study population, among CAD patients, or among control subjects (Table III).

The T allele frequency of the C242T polymorphism of the *p22phox* gene in this population was 0.20 (Table I), and the allele frequencies were in line with the Hardy-Weinberg equilibrium. The distribution of the genotypes and T allele frequency of the *p22phox* gene in the CAD patients and control subjects are shown in Table II. The T allele frequency was 0.18 for the CAD patients and 0.25 for control subjects (χ^2 = 6.796, P = .009), and the prevalence of the T allele

Table IV. The multiple logistic regression analysis of the relationships between explanatory variables and angiographically verified CAD

Variable	OR (95% CI)	*P*
Male	3.030 (1.838-4.994)	<.001
Age	1.072 (1.047-1.097)	<.001
Diabetes	2.028 (1.151-3.573)	.014
Hypercholesterolemia	2.482 (1.520-4.053)	<.001
p22phox C242T polymorphism	0.531 (0.331-0.852)	.009

Statistics: BMI, smoker, and hypertension were excluded from the multiple logistic regression analysis because there were no differences in the distribution of these variables between CAD patients and control subjects in this study (see Table II).

(TT + TC genotypes) was significantly lower among CAD patients than the control subjects (χ^2 = 6.141, *P* = .013). The crude OR of the genotypes of the *p22phox* gene (TT + TC genotypes vs the CC genotype) between CAD patients and control subjects was 0.591 (95% CI 0.389-0.897, *P* = .013).

The multiple logistic regression analysis was further used to assess the association between the C242T polymorphism and CAD with other risk factors under control. Because there were no statistically significant differences in the incidence of hypertension, body overweight, or smoking between CAD patients and control subjects, these variables were excluded from the multiple logistic regression analysis. The *p22phox* C242T polymorphism remained a significant explanatory variable in the model (see Table IV). The adjusted OR of the C242T polymorphism between CAD patients and control subjects was 0.531 (95% CI 0.331-0.852, *P* = .009). Therefore, the presence of the T allele might be an independent protective factor against the development of CAD in a high-risk population. In addition, 4 other variables were found to be independent risk factors for CAD in this population: male sex (OR 3.030, 95% CI 1.838-4.994, *P* < .001), age (OR 1.072, 95% CI 1.047-1.097, *P* < .001), diabetes (OR 2.028, 95% CI 1.151-3.573, *P* = .014), and hypercholesterolemia (OR 2.482, 95% CI 1.520-4.053, *P* < .001) (Table IV).

Discussion

The present study on the association between the C242T polymorphism of the *p22phox* gene and CAD was conducted with high-risk Finnish Caucasian patients undergoing angiography. The results show that the prevalence of the T allele (TT + CT genotypes) is significantly lower among angiographically verified CAD patients (n = 250) than among control subjects (n = 152) after exposure of the entire study population to coronary risk factors related to the excessive production of the NADPH-dependent superoxide anion. This finding indicates that the T allele of the C242T polymorphism has a protective effect against CAD in the high-risk Finnish population.

In previous reports, the association of the *p22phox* C242T polymorphism with CAD has been contradictory: Inoue et al[9] first reported that the risk of CAD was low in Japanese subjects carrying the T allele. In contrast, an Australian study found an increased risk of CAD in young Australian Caucasian carriers of the T allele,[11] and an American study suggested that the T allele was associated with more rapid progression of CAD.[13] Furthermore, there have also been studies reporting no linkage between the C242T polymorphism and CAD.[10,12,25] Differences in the genetic background of the study populations, the study design, and the statistic methods used might contribute to these disparate results.

We chose a high-risk hospital cohort to examine the protective effect of the T allele in the C242T polymorphism of the *p22phox* gene against the development of CAD. The T allele frequency in this high-risk population was 0.20, which was lower than the approximately 0.33 reported for several other Caucasian populations,[10-12] and higher than the 0.13 found in a Japanese population.[9] Because we have also demonstrated a T allele frequency of 0.19 in 1440 young healthy Finnish Caucasians (unpublished), the T allele frequency in the present high-risk population was consistent with the distribution of the Finnish general population. This result indicates that the genetic background of the C242T polymorphism of the *p22phox* gene differs between Finnish and several other Caucasian populations.

Coronary artery disease is a multifactorial disease, and genetic polymorphisms may only play a minor role in the development of the disease. The possibility is that the effect of the *p22phox* C242T polymorphism on CAD is highlighted in certain conditions. It has been known that the vascular NADPH oxidase constitutively produces low amounts of intracellular superoxide anions in physiological condition.[3,26] The *p22phox* gene may thus not exert a large effect in a healthy population. In contrast, the effect of the *p22phox* gene may be more potent when NADPH oxidase activity is up-regulated by the risk factors related to excessive NADPH-dependent superoxide production, such as smoking, diabetes mellitus, obesity, hypertension, and hypercholesterolemia.[14-18] As expected, in the present case-control study nested within a well-defined high risk population, we found that the prevalence of the T allele was significantly lower in angiographically verified CAD patients than control subjects (*P* = .013), and the association of the C242T polymorphism with CAD was statistically significant even after adjustment for several other risk factors (OR = 0.531, 95% CI 0.331-0.852, *P* = .009). Our results are in concert with the result of Inoue et al[9] in a Japanese case-control study. In contrast to the healthy controls in the Japanese study, however, the present controls were high-risk subjects with no angiographically defined CAD. Our finding further indicates that the T allele yields a protective effect

against CAD even after exposure of the subjects to the high-risk factors for CAD.

In conclusion, the T allele frequency in the C242T polymorphism of the *p22phox* gene was lower in a Finnish Caucasian population than in several other Caucasian populations. The C242T polymorphism of the *p22phox* gene was associated with the presence of CAD, and the T allele has a protective effect against CAD although the subjects were exposed to multiple risk factors for CAD. It must be acknowledged, however, that because our findings were based on relatively few Finnish Caucasians with only 152 control subjects, the association might be due to sampling variation and chance. To confirm the findings, further study is required to replicate our observations with larger cohorts of different populations.

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