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EXPOSURE TO TOBACCO SMOKE AND MARKERS OF SUBCLINICAL ATHEROSCLEROSIS IN CHILDREN AND ADOLESCENTS

The STRIP Study

by

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To my family

ABSTRACT

Katariina Kallio

Exposure to tobacco smoke and markers of subclinical atherosclerosis in children and adolescents. The STRIP study. Research Centre of Applied and Preventive Cardiovascular Medicine and the Departments of Pediatrics and Clinical Physiology and Nuclear Medicine, University of Turku, Turku, Finland; Department of Pediatrics, University of Helsinki, Helsinki, Finland. Annales Universitatis Turkuensis, Medica-Odontologica, Turku, Finland, 2009.

Background: Measurement of serum cotinine, a major metabolite of nicotine, provides a valid marker for quantifying exposure to tobacco smoke. Exposure to tobacco smoke causes vascular damage by multiple mechanisms, and it has been acknowledged as a risk factor for atherosclerosis. Multifactorial atherosclerosis begins in childhood, but the relationship between exposure to tobacco smoke and arterial changes related to early atherosclerosis have not been studied in children.

Aims: The aim of the present study was to evaluate exposure to tobacco smoke with a biomarker, serum cotinine concentration, and its associations with markers of subclinical atherosclerosis and lipid profile in school-aged children and adolescents.

Subjects and Methods: Serum cotinine concentration was measured using a gas chromatographic method annually between the ages 8 and 13 years in 538-625 children participating since infancy in a randomized, prospective atherosclerosis prevention trial STRIP (Special Turku coronary Risk factor Intervention Project). Conventional atherosclerosis risk factors were measured repeatedly. Vascular ultrasound studies were performed among 402 healthy 11-year-old children and among 494 adolescents aged 13 years.

Results: According to serum cotinine measurements, a notable number of the school aged children and adolescents were exposed to tobacco smoke, but the exposure levels were only moderate. Exposure to tobacco smoke was associated with decreased endothelial function as measured with flow-mediated dilation of the brachial artery, decreased elasticity of the aorta, and increased carotid and aortic intima-media thickness. Longitudinal exposure to tobacco smoke was also related with increased apolipoprotein B and triglyceride levels in 13-year-old adolescents, whose body mass index and nutrient intakes did not differ.

Conclusions: These findings suggest that exposure to tobacco smoke in childhood may play a significant role in the development of early atherosclerosis.

Key Words: arterial elasticity, atherosclerosis, children, cotinine, endothelial function, environmental tobacco smoke, intima-media thickness, risk factors, ultrasound

TIIVISTELMÄ

Katariina Kallio

Altistuminen ympäristön tupakansavulle ja varhaiset valtimokovettumatautiin liittyvät verisuonimuutokset lapsilla ja nuorilla. STRIP tutkimus. Sydäntutkimuskeskus, Lastentautioppi ja Kliinisen fysiologian ja isotooppilääketieteen oppiaine, Turun yliopisto, Turku; Lastentautioppi, Helsingin yliopisto, Helsinki. Annales Universitatis Turkuensis, Medica-Odontologica, Turku, 2009.

Tausta: Mittaamalla nikotiinin aineenvaihduntatuotteen kotiniinin pitoisuutta seerumissa voidaan luotettavasti selvittää tupakansavulle altistumisen määrää. Tupakansavulle altistumisen vaikutukset verenkiertoelimistöön ovat moninaiset ja passiivinen tupakointi on todettu yhdeksi valtimokovettumataudin riskitekijäksi. Valtimokovettumataudin syntyyn vaikuttaa useat tekijät ja tautiprosessi alkaa jo lapsuudessa. Lapsilla ei ole tutkittu passiivisen tupakoinnin yhteyttä valtimokovettumataudin varhaisiin verisuonimuutoksiin.

Tavoitteet: Väitöskirjatyön tavoitteena oli tutkia terveiden lasten ja nuorten altistumista tupakansavulle seerumin kotiniinipitoisuuden avulla ja altistumisen yhteyttä valtimokovettumataudin varhaisiin rakenteellisiin ja toiminnallisiin valtimomuutoksiin sekä rasva-aineenvaihduntaan.

Menetelmät: Väitöskirjatutkimus perustuu pitkittäiseen, satunnaistettuun varhaislapsuudessa alkaneeseen SepelvaltimoTaudin Riskitekijöiden InterventioProjektiin (STRIP). Seerumin kotiniinipitoisuus määritettiin kaasukromatografisella menetelmällä vuosittain 538-625 lapselta 8 ja 13 ikävuoden välillä. Totunnaiset valtimokovettumataudin riskitekijät määritettiin toistuvasti. Verisuonten ultraäänikuvaukset tehtiin 11 vuoden iässä 402 lapselle ja 13 vuoden iässä 494 nuorelle.

Tulokset: Kotiniinimääritysten perusteella merkittävä osa lapsista ja nuorista oli altistunut tupakansavulle mutta altistumisen taso oli kohtuullista. Tupakansavulle altistuminen oli yhteydessä huonontuneeseen olkavaltimon sisäkalvon toimintaan, huonontuneeseen aortan joustavuuteen sekä suurentuneeseen kaulavaltimon ja aortan seinämäpaksuuteen. Toistuva tupakansavulle altistuminen oli yhteydessä suurentuneeseen apolipoproteiini B ja triglyseridi pitoisuuteen 13-vuotiailla nuorilla, joiden painoindeksissä ja ravinnon saannissa ei ollut eroa.

Johtopäätökset: Tulosten perusteella vaikuttaa siltä, että altistuminen tupakansavulle lapsuudessa on yhteydessä valtimokovettumataudin syntyyn.

Avainsanat: kotiniini, lapset, riskitekijät, seinämäpaksuus, sisäkalvon toiminta, ultraääni, valtimoiden elastisuus, valtimokovettumatauti, ympäristön tupakansavu

CONTENTS

ABBREVIATIONS

aD	aortic distensibility
aDd	diastolic diameter of the aorta
aDs	systolic diameter of the aorta
aIMT	aortic intima-media thickness
ALSPAC	Avon Longitudinal Study of Parents and Children
ApoA-I	apolipoprotein A-I
ApoB	apolipoprotein B
ARIC	Atherosclerosis Risk in Communities
aSI	aortic stiffness index
AUC	area under curve
aYEM	aortic Young's elastic modulus
BMI	body mass index
BPd	diastolic blood pressure
BPs	systolic blood pressure
CARDIA	Coronary Artery Disease Risk Development in Young Adults
cD	carotid distensibility
CHD	coronary heart disease
CI	confidence interval
cIMT	carotid intima-media thickness
CRP	C-reactive protein
cSI	carotid stiffness index
CV	coefficient of variation
cYEM	carotid Young's elastic modulus
DISC	Dietary Intervention Study in Children
E%	percentage of total daily energy intake
ETS	environmental tobacco smoke
FMD	flow-mediated dilation
HDL	high-density lipoprotein
hsCRP	high-sensitivity C-reactive protein

IMT	intima-media thickness
LDL	low-density lipoprotein
Lp(a)	lipoprotein(a)
NHANES	National Health and Nutrition Examination Survey
NMD	nitrate-mediated dilation
NO	nitric oxide
PDAY	Pathological Determinants of Atherosclerosis in Youth
PWV	pulse wave velocity
SD	standard deviation
SEM	standard error of mean
STRIP	Special Turku coronary Risk factor Intervention Project
VLDL	very low-density lipoprotein

LIST OF ORIGINAL PUBLICATIONS

The present thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I Kallio K, Jokinen E, Hämäläinen M, Kaitosaari T, Volanen I, Viikari J, Rönnemaa T, Simell O: Impact of repeated lifestyle counselling in an atherosclerosis prevention trial on parental smoking and children's exposure to tobacco smoke. Acta Paediatrica 2006;95:283-290.

II Kallio K, Jokinen E, Raitakari OT, Hämäläinen M, Siltala M, Volanen I, Kaitosaari T, Viikari J, Rönnemaa T, Simell O: Tobacco smoke exposure is associated with attenuated endothelial function in 11-year-old healthy children. Circulation 2007;115:3205-3212.

III Kallio K, Jokinen E, Hämäläinen M, Saarinen M, Volanen I, Kaitosaari T, Viikari J, Rönnemaa T, Simell O, Raitakari OT: Decreased aortic elasticity in healthy 11-year-old children exposed to tobacco smoke. Pediatrics 2009;123:e267-e273

IV Kallio K, Jokinen E, Saarinen M, Hämäläinen M, Volanen I, Kaitosaari T, Rönnemaa T, Viikari J, Raitakari OT, Simell O: Arterial intima-media thickness, endothelial function and apolipoproteins in adolescents frequently exposed to tobacco smoke. Submitted.

In addition, some previously unpublished data are presented.

1. INTRODUCTION

Cigarette smoking has been identified as the most important source of premature morbidity and mortality.[1] Never-smokers live approximately 10 years longer than heavy smokers, and their extra years of life are of better quality.[2] The relationship between cigarette smoking and coronary heart disease (CHD) was first reported in 1940,[3] and at present, smoking is one of the major risk factors for CHD.[4] In the mid 1980s several studies were published in which exposure to environmental tobacco smoke (ETS) was proved dangerous also to those who were involuntarily exposed. Since then epidemiological data on the relationship between passive smoking and heart disease have been accumulating,[5-8] concluding that passive smoking increases the CHD risk of a non-smoker by a third,[5-8] or even by a half.[9] ETS, however, is a preventable cause of morbidity and mortality.[10] The mechanisms by which passive smoking increases the risk of CHD are multiple and interact with each other. Exposure to ETS increases platelet aggregation, inflammation, endothelial dysfunction, arterial stiffness, oxidation of low-density lipoprotein (LDL) cholesterol, oxidative stress, and atherosclerosis.[11,12] Some of the damage to the cardiovascular system caused by ETS has also been reported among children.[12]

A biomarker is desirable in quantifying systemic exposure of non-smokers to ETS. At present, cotinine, a major metabolite of nicotine, appears to be the most specific and sensitive biomarker for exposure to nicotine from ETS, and the measurement of cotinine concentration in biological fluids has been used most widely by scientists to evaluate ETS exposure.[13] The half-life of cotinine in blood is about 20 hours, thus reflecting the exposure to nicotine during the past 2-3 days.[14]

Children and adolescents are particularly vulnerable to the health effects of passive smoking.[10] The prevalence and levels of exposure to ETS among children and adolescents has been widely studied, but there are no reports on tobacco smoke exposure in children measured with biomarkers from Finland. It was estimated in the mid 1990s that in 7% of all the households in Finland children were exposed to ETS.[15] At that time about 950000 children under 15 years lived in Finland leading to estimations that 60000-70000 were regularly exposed to ETS.[16] Subsequently, legislation has been tightened to ban smoking in public places, but there is no data whether this has diminished the overall exposure of children or exposure in their homes.

Atherosclerosis is a chronic disease developing as a result of a life-long process. However, it is now clear that the atherosclerotic process begins in childhood,[17] decades before clinical symptoms of the disease appear. This long asymptomatic phase provides an opportunity for early detection of arterial changes. Noninvasive ultrasonic methods have increasingly gained acceptance in the determination of early vascular changes related to atherosclerosis (e.g. decreased arterial reactivity, loss of arterial elasticity, and thickening of the arterial wall), especially in young subjects and healthy populations. These methods may be useful in identifying those apparently healthy individuals at risk of vascular disease later in life.[18]

This thesis is based on the ongoing, prospective, randomized Special Turku coronary Risk factor Intervention Project for children (STRIP) study, which is aimed at decreasing the exposure of children to known environmental cardiovascular risk factors. The main objectives of the present study were to examine the levels and determinants of exposure to tobacco smoke among children and adolescents using a biomarker, serum cotinine concentration, and the associations between exposure to tobacco smoke, cardiovascular risk factors, and markers of preclinical atherosclerosis such as brachial endothelial function, arterial elasticity, and carotid and aortic intima-media thickness.

2. REVIEW OF THE LITERATURE

2.1. Atherosclerosis

2.1.1. Beginning at an early age

Atherosclerosis is a chronic disease developing as a result of a life-long process. The clinical manifestations of the atherosclerosis such as myocardial infarction, stroke, claudication, and sudden cardiac death usually occur in middle age or later. However, it is now clear that atherosclerotic process begins in childhood.[17,19,20] The first evidence of the arterial changes related to early atherosclerosis came from the autopsies of young asymptomatic soldiers killed in the Korean and Vietnam wars.[21,22] More recently, results from the Pathological Determinants of Atherosclerosis in Youth (PDAY)[23] and the Bogalusa Heart Study[24] have demonstrated that subclinical atherosclerosis is present already in the arteries of young children. Furthermore, fatty streaks have been found even in fetal arteries as a consequence of maternal hypercholesterolemia during pregnancy.[25]

The atherosclerotic process is accelerated in the presence of risk factors. Indeed, several large prospective studies in children and young adults have highlighted the importance of childhood risk factors and lifestyle in atherogenesis, for example, the Muscatine study,[26] the Bogalusa Heart Study,[27] the Cardiovascular Risk in Young Finns Study,[28] the Coronary Artery Disease Risk Development in Young Adults (CARDIA) study,[29] the Dietary Intervention Study in Children (DISC),[30] the Avon Longitudinal Study of Parents and Children (ALSPAC) study,[31] and the STRIP study.[32,33] Moreover, the extent of atherosclerotic lesions in children and adolescents increases with the number of atherosclerotic vascular disease risk factors in childhood.[24]

2.1.2. Development of atherosclerosis

The arterial wall consists of the intima, media, and adventitia, which are histologically distinct layers. The intima, formed of endothelium and of smooth muscle cells, is the innermost layer of the arterial wall and is the part most affected in the atherosclerotic process. Although atherosclerosis is considered a systemic disorder, which affects the entire arterial tree, it usually develops at certain predilection sites of large elastic and medium-sized muscular arteries.[34] The most lesion-prone regions include the coronary arteries, the terminal abdominal aorta and its major branches, and the major branches of the aortic arch.[34] The first atherosclerotic lesions typically appear in the aorta.[17]

Atherosclerosis is considered as a chronic inflammatory disease.[20] A widely accepted hypothesis, introduced by Ross,[19,20] suggests that atherosclerotic lesions develop through a response-to-injury mechanism. In this hypothesis, an inflammatory response of the endothelium to various injuries (metabolic, mechanical, chemical, or microbial) causes atherosclerosis, endothelial dysfunction being the first step of the process. Thereafter, characteristic atherosclerotic lesions represent different stages in a chronic inflammatory process. First, the endothelium encounters alterations that increase its

adhesiveness and permeability, and induce endothelial cells to form vasoactive molecules, cytokines, and growth factors. Subsequently, the inflammatory response stimulates the accumulation of macrophages and lymphocytes in the arterial intima, following the migration and proliferation of smooth muscle cells, leading to thickening of the arterial wall. With time, this inflammatory response continues, the accumulation of mononuclear cells increases and smooth muscle cells continue to proliferate resulting in the formation of early, intermediate and advanced lesions of atherosclerosis.

The American Heart Association has classified the atherosclerotic lesions to 6 histological stages.[35,36] Early lesions (types I and II) and intermediate lesions (type III) can be considered as precursors of advanced lesions (types IV and V) and complicated lesions (type VI). Type I lesions represent the initial changes in the arterial wall with an increase in intimal macrophages and the transformation of macrophages into cholesterol containing foam cells. Type II lesions are formed of the layers of macrophage foam cells and lipid laden smooth muscle cells. These lesions are also called fatty streaks, and they may continue to develop to type III lesions with small pools of extracellular lipid droplets and particles that interfere with the coherence of intimal smooth muscle cells. In more advanced lesions, the core of extracellular lipids starts to develop (atheroma), and the process continues with a fibrous cover enclosing the lipid core (fibroatheroma). Ultimately, at the complicated lesion stage, fibroatheromas become more complex with calcification, luminal surface defects, hemorrhage, and thrombotic deposits.

At present, it is not known whether the aforementioned lesion types are successive steps in the atherosclerotic process. However, each type of lesion tends to occur and predominate at certain ages: fatty streak formation has been observed already in the fetal period in the aorta,[25] and in every child over the age of 3 years.[37] Intermediate lesions have been found in the arteries of almost every teenager,[17,38] and young adults have already had advanced atherosclerotic lesions in coronary arteries.[39] Thus, these data and the fact that advanced lesions mainly occur at the lesion-prone sites where early lesions are also situated are suggestive of the progression of precursor lesions to advanced lesions.[17]

2.2. Risk factors for atherosclerosis

Numerous environmental and genetic factors and their interactions are related with atherosclerosis.[40] The major traditional risk factors include high serum total and LDL cholesterol,[41] hypertension,[42] and smoking.[43,44] Other important risk factors are diabetes,[45] obesity,[46,47] low high-density lipoprotein (HDL) cholesterol,[48] and hypertriglyceridemia.[49] Recently, the INTERHEART study showed that worldwide abnormal lipids, smoking, hypertension, diabetes, abdominal obesity, psychosocial factors, irregular consumption of fruits and vegetables, no alcohol intake, and avoidance of any regular exercise accounted for most (more than 90%) of the risk of myocardial infarction.[50] Of the genetic factors, male sex[51] and a family history of premature CHD[52] are the most important. In addition, ageing reflects the length of exposure to all risk factors.[51,53]

The risk factors of atherosclerosis in childhood are similar to adults. Additional to the lipid risk factors and smoking, multiple other risk factors in children and young adults have been identified and related with markers of subclinical atherosclerosis. These include elevated blood pressure,[24,54] diabetes,[55] obesity,[24,56] C-reactive protein (CRP),[57] some infectious agents (e.g. Chlamydia pneunoniae),[58] and a sedentary lifestyle.[59]

2.2.1. Lipid risk factors

An important event in atherosclerosis development is the accumulation of LDL in the subendothelial matrix.[60] Accumulation of LDL in the arterial wall is greater with raised blood levels of LDL. Then, endothelial injury, foam cell formation and inflammation are a result of different kinds of modifications (oxidation, glycation, aggregation) to the LDL particles.[61] On the other hand, HDL particles are capable of receiving accumulated cholesterol from the peripheral cells, such as macrophages, and transport it back to the liver for excretion (the reverse cholesterol transport pathway).[62] In addition, HDL has anti-inflammatory and antithrombotic properties, and it is antiatherogenic by inhibiting lipoprotein oxidation. Triglyceride-rich lipoproteins may have direct atherogenic effects, or hypertriglyceridemia may be associated with other atherogenic lipoprotein profiles such as low HDL cholesterol.[63]

More recently, disturbances in apolipoproteins have been acknowledged as risk factors of atherosclerosis. The concentration of apolipoprotein B (ApoB) provides a direct measure of the number of circulating atherogenic lipoproteins, as each of the atherogenic lipoprotein particles, i.e. very low-density lipoprotein (VLDL), intermediate-density lipoprotein, LDL, lipoprotein(a) [Lp(a)], and chylomicrons contain a single molecule of ApoB.[64] Apolipoprotein A-I (ApoA-I) is the main structural protein in antiatherogenic lipoproteins, and its plasma concentration correlates strongly with that of HDL cholesterol.[65] Thus, the ApoB/ApoA-I ratio represents the balance of proatherogenic and antiatherogenic lipoproteins.[64] In middle-aged adults, the ApoB/ApoA-I ratio is related to carotid artery intima-media thickness (cIMT),[66,67] and is a strong predictor of coronary events.[68,69] Thus, the ApoB/ApoA-I ratio may be the best single lipoprotein variable related to coronary risk.[64,69] The serum concentration of Lp(a) is mainly determined genetically, and apolipoprotein(a) in Lp(a) is a highly polymorphic molecule.[70] Lp(a) may have a important role in the initiation and progression of atherosclerotic disease due to its ability to activate macrophages and smooth-muscle cells, and its effects on fibrinolysis.[71]

In children and young adults, post-mortem studies have shown that elevated LDL cholesterol and low HDL cholesterol concentrations are associated with early and advanced atherosclerotic lesions.[24,72] Childhood cholesterol level has a predictive value for adult cIMT,[56,73] and serum cholesterol concentration in early adult life associates with cardiovascular mortality in midlife.[74] Moreover, LDL cholesterol is related to decreased arterial distensibility and decreased brachial flow-mediated dilation (FMD) in children.[75,76] Low HDL cholesterol levels have been related with decreased brachial FMD and increased cIMT in childhood,[77,78] and coronary artery calcification in young adults.[79] Hypertriglyceridemia is related with endothelial dysfunction of the brachial artery and increased intima-media thickness (IMT) in childhood.[77] Childhood levels of

ApoB/ApoA-I ratio predict cIMT and endothelial function in adulthood,[80] and children with a parental history of myocardial infarction have had lower ApoA-I levels and a higher ApoB/ApoA-I ratio than control children.[81] Lp(a) levels have been related with decreased FMD in hypercholesterolemic children.[82]

2.2.2. The role of smoking

The relationship between cigarette smoking and CHD was first reported in 1940.[3] Since then, hundreds of studies have confirmed the association.[43,83-85] According to epidemiological studies, active smoking increases the risk of CHD by 50% to 250%. Active smoking also increases the risk of cerebrovascular disease,[86] aortic aneurysms,[87] and peripheral vascular disease.[88] Acting synergistically with other risk factors,[83,89] cigarette smoking promotes the disease progression[90] and the incidence of its acute clinical manifestations.[83,86] The risk of CHD reduces after the smoking cessation.[84,91] According to the INTERHEART study,[84] the excess risk of CHD fell substantially during year 2 after stopping smoking, but the excess risk may not have completely disappeared even 20 years after quitting in moderate or heavy smokers.

In healthy subjects, active smoking is associated with carotid atherosclerosis.[92] Both past and current active smoking has been related with increased cIMT.[44,73,93-95] The PDAY study showed that smoking is related with autopsy findings in adolescents and young adults.[96] In addition, cigarette smoking is connected to arterial stiffness[97-99] and impaired endothelium-dependent dilation already in young adults.[95,100]

Adult and adolescent smokers show dose-dependent elevations of serum total cholesterol, VLDL cholesterol, LDL cholesterol, triglycerides, and ApoB, as well as decreases of HDL cholesterol, its antiatherogenic subfraction HDL_2, and ApoA-I as compared with non-smokers.[101-105] The ApoA-I/ApoB ratio has also been reported to be lower in smokers than in non-smokers.[106]

In addition, active smoking is associated with higher plasma glucose concentrations in lean young adults,[104] high plasma insulin levels in every body mass index (BMI) tertile,[107] metabolic syndrome in young men,[108] development of glucose intolerance,[109] and acute insulin resistance in lean young adults.[110] However, there are controversial results relating to the association between active smoking and the development of diabetes mellitus.[111,112]

Smoking is a potent inflammatory stimulus.[10] Active smoking promotes atherogenesis affecting many elements in the interface of the blood with the arterial wall and the cellular elements of the artery. Active smoking is associated with dysfunctional endothelial cells and inactivation of nitric oxide affecting the regulation of vascular tone, and leading to secretion of inflammatory cytokines, adhesion of leukocytes, and the reduction of normal antithrombotic properties. Indeed, chronic cardiovascular effects of smoking include increased plasma fibrinogen and leukocyte count, and activation of platelets.[113-115] In addition, prothrombotic genetic factors may interact with smoking in the risk of stroke.[116]

Smoking increases heart rate, blood pressure, and myocardial oxygen demand while reducing oxygen-delivering capacity, thus resulting in impaired cardiac

performance.[117-119] These acute effects of smoking are induced by increased blood carboxyhemoglobin level and adrenergic stimulation, triggered by nicotine.[120]

2.2.3. The role of passive smoking

Epidemiology

Epidemiological data on the relationship between passive smoking and heart disease have been accumulating since the mid 1980s, and multiple studies outline this relationship.[5-8] Accordingly, exposure to tobacco smoke at home or in the workplace results in a 25 to 35% increase in the risk of death from CHD. However, in the more recent study using cotinine as a biomarker of exposure, passive smoking increased the risk of CHD by 45% to 57%,[9] suggesting that earlier studies based on self-reported exposure may have underestimated the true effect size. Indeed, passive smoking may lead to 68% to 86% of the CHD risk of light active smoking, depending on the levels of exposure.[12] ETS is also associated with increased risk of stroke[121,122] and peripheral artery disease.[122]

The dose of smoke inhaled by passive smokers is approximately 1% of that delivered to active smokers. However, the cardiovascular effects of even brief (minutes to hours) ETS exposure are often nearly as large (80% to 90%) as those from chronic active smoking.[12] The reason for this difference is probably that the non-smoker's vascular system is not adapted to the chemicals in tobacco smoke.[123] Indeed, many important responses of the cardiovascular system are exquisitely sensitive to the toxins in the ETS.[12]

The pathophysiology behind the connection between passive smoking and atherosclerosis has not been entirely identified. However, many of the mechanisms involved in the effects of active smoking have also been described in passive smokers. The mechanisms by which passive smoking increases the risk of heart disease are multiple and interact with each other. Exposure to cigarette smoke increases platelet aggregation, inflammation, endothelial dysfunction, arterial stiffness, oxidation of LDL cholesterol, oxidative stress, and atherosclerosis.[11,12]

Acute cardiovascular effects of passive smoking

Acute exposure to ETS reduces exercise tolerance and impairs cardiac performance in heart disease patients and healthy non-smokers.[124,125] Children with parents who smoke may suffer from chronic tissue hypoxia, as they have been found to have elevated levels of 2,3-diphosphoglycerate, a physiological modulator of hemoglobin oxygen affinity.[126] ETS not only increases the demand and compromises the supply of oxygen to the heart, but also diminishes the myocardium's ability to use the oxygen.[127]

There are no consistent data on the changes in plasma catecholamines, heart rate or blood pressure after short-term exposure to ETS,[125,128] but increased muscle sympathetic nerve activity has been reported.[129] In addition, acute exposure to tobacco smoke reduces heart rate variability,[130] possibly by decreased parasympathetic input to the heart, thus possibly promoting tachyarrhythmia.

Platelet function

Exposure to ETS causes activation of blood platelets, thereby increasing the likelihood of thrombus formation and damage to the endothelium of the arteries.[123,131,132] Experimental studies indicate that nonsmokers are more sensitive to secondhand smoke than active smokers, and even very low levels of smoke exposure can have a major impact on platelet function in non-smokers.[131,132] In addition, animal studies have shown the effect of smoke exposure on platelet function, as bleeding time has been significantly shortened in association with smoke exposure.[133,134]

Passive smoking has been related to increased levels of fibrinogen both in adults[135,136] and adolescents,[137] and elevated levels of plasma thromboxane B_2.[138]

Inflammation

Both children and adults exposed to tobacco smoke have higher levels of inflammatory markers. Acute-phase proteins (CRP, haptoglobin, component of the complement [C3c]) are increased in children exposed to ETS.[139,140] As little as 3 hours of ETS exposure increases activated neutrophils and the leukocyte count.[141] In addition, chronic ETS exposure increases inflammatory markers, such as CRP,[142] homocysteine,[135,142,143] and white blood cell count.[142] Results from animal data have shown an increase in interleukine-6, a proinflammatory cytokine, after exposing mice to secondhand smoke.[144] The fine particulate matter in cigarette smoke probably has a role in mediating these effects.[145] Moreover, an interaction between passive smoking and chronic infections in atherogenesis has been reported.[146]

Oxidative stress and antioxidants

The oxidative stress from ETS affects the cardiovascular system both by directly delivering free radicals and by consuming antioxidants.[147] The free radicals from cigarette smoke decrease nitric oxide (NO) production by the endothelial cells without any effect on mitochondrial respiration.[148] Enzymes used as markers of oxidative stress (glutathione peroxidase, glutathione reductase) have been elevated in passive smokers, and increased DNA damage resulting from oxidative stress has been observed.[149] In addition, animal studies have shown increased oxidative stress and DNA damage in the mitochondria resulting from ETS exposure.[150]

Antioxidant depletion indicated by vitamin C (ascorbic acid) or carotenoid levels have been used as a marker of oxidative stress. Both long-term[151,152] and short-term[153,154] ETS exposure leads to alterations in vitamin C levels. In addition, children and adolescents who are passive smokers have had lower blood levels of vitamin C, independently of vitamin C intake.[155,156] Plasma levels of plant pigment ß-carotene has been shown to be lower in adults as a result of ETS exposure.[157,158] Moreover, both red blood cell folate and serum folate levels are decreased in association with passive smoking.[159] Thus, ETS leaves the vascular system without its endogenous antioxidant protection against oxidative stress.

Lipid profile and diet

Workplace exposure to ETS leads to lower levels of HDL cholesterol in adults.[106,160] Excessive exposure to secondhand smoke in the workplace can also have deleterious effects on HDL_2 and ApoA-I in female nonsmokers.[106] Acute 6-hour exposure to ETS reduces HDL cholesterol and HDL_2, and these levels remain depressed for at least 24 hours.[161] Studies in healthy children[126,162] and adolescents,[163,164] as well as in dyslipidemic children[165] have shown an association between decreased HDL cholesterol and passive smoking. Recently, it was indicated that maternal smoking in pregnancy is associated with an increase in total cholesterol levels and trends toward adverse lipoprotein profiles in the young adult offspring.[166]

The mechanisms of the effects of smoke exposure on lipid profiles have not been clearly elucidated. However, cigarette smoking has been shown to increase hepatic lipase activity,[167] inhibit lecithin cholesterol acyl-transferase activity,[168,169] and decrease lipoprotein lipase activity.[169] In nicotine-treated rats, increased synthesis and secretion of the ApoB containing lipoproteins was found.[170] Thus, nicotine may exert hypelipidemic effects particularly by increasing the synthesis and secretion of triglyceride-rich lipoproteins.

Exposure to tobacco smoke contributes to the progression of atherosclerosis also through increased susceptibility to lipid peroxidation and LDL cholesterol modification leading to increased lipid uptake by macrophages.[154]

Exposure to tobacco smoke at home has been associated with unfavorable diet quality in children from low-income families,[171] and even adults living with smokers have had less healthy dietary habits than those living with nonsmokers.[172]

Metabolic syndrome, glucose intolerance, and insulin resistance

There is some evidence that passive smoking is associated with metabolic syndrome in adolescents,[173] development of glucose intolerance in young adults independently of energy and saturated fat intake,[109] and insulin resistance in middle-aged women.[174]

Endothelial function

Acute tobacco smoke exposure has been associated with endothelial dysfunction in adults.[128,175,176] Acute smoking of one cigarette was associated with significant impairment of flow-mediated arterial dilation persisting at least 60 minutes.[176] Furthermore, 30 minutes of passive smoke exposure in non-smoking healthy young adults led to impaired endothelium-dependent vasodilation in coronary arteries measured by coronary flow velocity reserve using transthoracic Doppler echocardiography, similar to the effect seen in smokers.[128]

Long-term tobacco smoke exposure impairs vascular function in passive smokers.[177-179] Celermajer et al.[179] showed that passive smoking with an exposure level daily for at least one hour during three years is associated in a dose-dependent manner with significant endothelial dysfunction in teenagers and young adults. In addition, Woo et al.[177] found that in young adults, heavy passive smoking (over 8 hours daily for at least two years) in the workplace is related with impaired endothelial function. However,

endothelial function partially recovers in healthy adults after long-term exposure to ETS ends.[180]

Studies in animals have confirmed the association between exposure to ETS and endothelial dysfunction, as decrease in endothelium-dependent vasodilation in rabbits,[117] the synergistic effect of high cholesterol and cigarette smoke exposure on endothelial dysfunction in rabbits,[181] and impaired endothelium-dependent relaxation in newborn rats after in utero exposure have been reported.[182]

The brachial artery dilation caused by a sudden increase in flow is endothelium-dependent and largely mediated by NO secreted from endothelial cells.[183] In reaction to tobacco smoke, production of NO is decreased,[184,185] providing one mechanism by which the risk of CHD is increased. Moreover, Heiss et al.[185] showed that short-term exposure to ETS damages the vascular endothelium and its repair mechanism, as endothelial progenitor cell activity was depressed. Animal studies have shown that passive smoking reduces endothelium-dependent vasorelaxation, and dietary supplementation of NO precursor L-arginine improves endothelium-dependent dilation.[186,187] In addition, antioxidant diet[188] and consumption of flavanol-rich cocoa[189] may improve endothelial dysfunction caused by tobacco smoke.

Exposure to ETS has been reported to cause direct endothelial cell injury[190] and disruptions in actin filament organization, which is part of the endothelial cell cytoskeleton repair mechanisms.[191] Cigarette smoke extract, especially gas-phase oxidant acrolein in ETS, increases production of superoxide anion in isolated endothelial cells reducing NO-mediated endothelial function.[148,192]

Arterial elasticity

Only one study has reported the association between arterial stiffness and chronic tobacco smoke exposure in non-smokers.[193] In addition, experimental studies in adults have shown that acute passive smoking leads to a short-term increase in arterial wall stiffness in non-smokers.[98,194,195] In children and adolescents with type 1 diabetes, exposure to tobacco smoke assessed through a questionnaire was associated with decreased carotid distensibility, but not with cIMT.[196]

These acute and substantial effects of tobacco smoke on arterial mechanical properties may be caused by multiple mechanisms, including an increase in circulating catecholamines, impaired nitric oxide production, endothelial dysfunction, platelet activation, impaired endothelial release of prostacyclin and increased vasopressin release.[197,198]

Progression of atherosclerosis

Exposure to tobacco smoke contributes to the progression of atherosclerosis. In adults, both past and current passive smoking have been associated with increased cIMT.[93,94] Results from the Atherosclerosis Risk in Communities (ARIC) study have also shown a longitudinal association between secondhand smoke and the progression of cIMT.[199] Moreover, tobacco smoke exposure in pregnancy led to increased aortic intima-media thickness (aIMT) in neonates,[200] and increased cIMT in young adulthood.[201] Finally, the number of stenotic coronary arteries increased in a dose-dependent manner in

Chinese women exposed to their spouse's smoking.[202] In animal studies, carotid intimal thickening was significantly increased after 5 weeks of ETS exposure in mice.[203]

Multiple animal studies have shown increased LDL accumulation in the arterial wall after ETS exposure,[187,190,204,205] as well as increased atherosclerotic lesion size after breathing tobacco smoke.[133,150,206,207] Arterial lipid lesions have increased in experiments using ETS from nicotine-free cigarettes, suggesting that other components of tobacco smoke than nicotine are important in promoting atherosclerosis.[208] Another animal study showed that butadiene, a vapor phase component of ETS, accelerated atherosclerosis.[209]

Exposure to tobacco smoke contributes to atherosclerotic plaque instability by upregulation of matrix metalloproteinases in smooth muscle cells.[210] These degrading enzymes possibly weaken the arterial wall and contribute to the destabilization of atherosclerotic plaque.[211]

To summarize, the suggested mechanisms by which cigarette smoke promotes atherosclerosis progression are increased lipid peroxidation, and accumulation of cholesterol esters in atherosclerosis plaque. Decreased HDL cholesterol, increased platelet activation, and endothelial dysfunction also contribute to disease progression. Finally, stenosis of the coronary arteries and plaque instability caused by cigarette smoke are responsible for acute clinical manifestations of atherosclerosis.

2.3. Exposure to tobacco smoke in children

2.3.1. Contents of tobacco smoke

Exposure to ETS is defined as the exposure of a person to tobacco combustion products from smoking by others.[10] Tobacco smoke contains more than 4500 compounds found in both vapour and particle phases. These compounds include addictive nicotine, toxic substances such as carbon monoxide, acrolein, ammonia, and nitrogen oxides, at least 40 carcinogens such as benzene, nickel, polonium, formaldehyde, benzo[a]pyrene, and N-nitrosoamines. Radioactive substances, cyanide compounds, phenols, and toxic metals such as cadmium, arsenic, lead, and mercury are also found in cigarettes.[10,14]

Burning cigarettes emit two types of smoke: mainstream smoke, which is inhaled directly by the smoker before it is released into the surrounding air, and sidestream smoke which is the smoke emitted into the air from the burning cigarette between puffs, smoke escaping into the surrounding air during puffs, and smoke components diffusing through cigarette paper.[212] ETS is about 85% sidestream smoke and 15% exhaled mainstream smoke.[16] Although sidestream and mainstream smoke are qualitatively similar in chemical composition, sidestream smoke contains many toxic substances in higher concentrations, due to differences in the burning conditions. The cigarette burns at a higher temperature during inhalation leading to more complete combustion in mainstream smoke, and a filter also can produce reduced mainstream smoke emissions.[16]

2.3.2. Assessment of exposure to tobacco smoke

Since ETS is a complex mixture of gases and particulate matter, and relatively little is known about the importance of individual constituents in causing adverse health effects, and possible interactions between different compounds, the assessment of exposure to the entire ETS mixture is relevant.[14] Thus, it is important to identify a marker of ETS that can be measured, and that represents the magnitude, duration, and frequency of ETS exposure. A marker may be a metabolite measured in biological samples, a chemical compound measured in the air, a variable derived from questionnaires, or an estimate derived by modeling.[14,213]

A valid marker of ETS should be unique to tobacco smoke, easily detectable at low concentrations, should be emitted at similar rates for a variety of tobacco products, and should have a fairly constant ratio to other ETS components of interest.[16] Furthermore, the validity of a biomarker depends on the accuracy of the biologic fluid measurement in quantifying the intake of the marker chemical, which in turn may be influenced by individual differences in patterns of metabolism or excretion, the presence of other sources of the chemical (for example diet), and the sensitivity and specificity of the analytical methods used to measure the chemical.[13] Nicotine in the air and measurement of its metabolite cotinine in biological fluids meet these criteria reasonably well.

Multiple biomarkers of tobacco smoke exposure have been reported. These include nicotine, cotinine, carbon monoxide, thiocyanate, 4-aminobiphenyl-hemoglobin adducts, adducts of polycyclic aromatic hydrocarbons bound to plasma albumin, and metabolites of nicotine-derived nitrosoamines in the urine.[13] The last three of these biomarkers mainly reflect exposure to carcinogens in tobacco smoke. The advantages of nicotine and cotinine in body fluids as biomarkers of ETS include their relatively high sensitivity and specificity for tobacco combustion and the availability of accurate measurement methods at low concentration. There has been some debate of specificity of these biomarkers,[214] as plant sources other than tobacco have been identified (e.g. tomato, potato, cauliflower, black tea).[215] However, the contribution of dietary sources of nicotine to cotinine levels is estimated to be small compared to ETS exposure.[216] The usefulness of nicotine as a biomarker is limited, since it has a very short half-life of approximately 2 hours in the blood.[14]

At present, cotinine appears to be the most specific and sensitive biomarker for exposure to nicotine from ETS,[13] and the measurement of cotinine concentration in biological fluids has been widely used to evaluate ETS exposure. Cotinine is the major proximate metabolite of nicotine,[10] synthesized in the liver, and excreted in the urine. Cotinine can be measured in urine, plasma, serum, saliva or hair. Saliva and blood cotinine levels are highly correlated with a saliva-to-blood ratio of 1.1 to 1.4.[13,217] Urine concentrations are also highly correlated with blood concentrations, with urine levels being about 6 times higher than those in the blood. The half-life of cotinine in the blood may vary between 7-40 hours among adult nonsmokers exposed to ETS, and may be somewhat longer among children (32-82 hours), and even up to 160 hours in neonates.[14,217] The majority of the adult studies have reported cotinine half-life to be about 16 to 18 hours.[10] Thus, cotinine reflects the exposure to nicotine during the past

2-3 days. The possible limitations related to cotinine use are a relatively short half-life in the body fluids, intersubject variability due to differences in uptake, metabolism and elimination, and the likelihood that it is not necessarily the agent causing adverse health effects.[13] On the other hand, cotinine measurement can be helpful in distinguishing passive smokers from active smokers, and heavily exposed passive smokers from those exposed only moderately.[13,16]

Cotinine can be measured by a variety of techniques, and there is no standard method for determining nicotine or its metabolites in biological fluids.[218] The most commonly used methods are gas chromatography with nitrogen-phosphorus-specific detectors[219] or coupled to a mass spectrometer,[220] radioimmunoassay,[221] and high-performance liquid chromatography using either ultraviolet or mass spectrometric detection.[222] There is much variation in sensitivity, specificity, and the cost of these analytical procedures.[218] The gas chromatographic method reported by Feyerabend and Russell[219] has a sensitivity of 0.1 ng/ml, good specificity, and a moderate cost.

A questionnaire-based assessment of exposure to ETS is the most widely used method to evaluate exposure. The advantages of questionnaires are low cost, feasibility in administration of questionnaires in a variety of ways, and the possibility to assess both current and past exposure.[14] The disadvantages include difficulties in validation, particularly of past exposure, and the potential for misclassification, due to lack of knowledge about exposure, difficulties in characterizing exposure in complex indoor environments, and intentionally or unintentionally biased recall.[10] Thus self-report measures such as hours per day exposed to ETS by non-smokers are likely to be imprecise indicators of intake of tobacco smoke.[218]

To summarize, cotinine, a major metabolite of nicotine, appears to be the most specific and sensitive biomarker to measure ETS exposure. Gas chromatography is a valid method to determine cotinine concentrations. Cotinine reflects exposure to nicotine during the past few days.

2.3.3. Epidemiology

Smoking of the adults in Finland

In Finland, until 2004, smoking among men has decreased since the late 1970s, while women's smoking has remained almost unchanged since the mid 1980s.[223,224] According to the annual postal survey entitled "Health Behaviour and Health among the Finnish Adult Population", 31% of men and 20% of women in Finland were daily smokers in 1998,[225] and the respective percentages were 27% and 20% in 2004.[224] However, among young adults (aged 15 to 24 years), women's smoking was more prevalent (24%) than men's smoking (21%).[224] The most recent data in 2007 showed a slight reduction in smoking among women, as the proportions of daily smokers were 26% and 17% in men and women, respectively.[226] In all of these Finnish studies, adults who had ever smoked at least 100 times, and had smoked daily for at least one year, and had last smoked at the same or previous day were defined as daily smokers.[226]

Questionnaire data

The prevalence and levels of exposure to ETS among children and adolescents has been widely studied, but there are no reports on tobacco smoke exposure in children measured with biomarkers from Finland. According to a mailed questionnaire study launched in 1995 in the Nordic countries,[15] children in Finland were exposed to ETS weekly in 7% of all households, and the percentage of exposed children in households containing smokers was 23%. The number of exposed children was lowest in Finland, as the prevalence of all households in which children were exposed to ETS varied between 15-47% in other Nordic countries. In an other questionnaire study executed in 1991 in Finland,[227] the prevalence of child's exposure to ETS at home was estimated to be 9.9% among all children aged 1 to 6 years, and 25.2% in families where either or both parents were current smokers. The home as the major ETS source in 3-year-old children was clear in a Swedish study,[228] because only 12% of the children were in smoky environments outside the home every week and just 2% every day.

According to the National Health Interview Survey from the U.S., in 1994 35% of children lived in homes where they had contact with a smoker at least one day per week.[229] In another U.S. Survey, the National Health and Nutrition Examination Survey (NHANES) 1999-2002, the proportion of children aged 3 through 11 years living with one or more smokers in the household was 24.9%.[10]

The questionnaire data from the Global Youth Tobacco Survey indicates that during 2000-2007 nearly half of never smoking 13- to 15-year-old students worldwide were exposed to ETS at home (46.8%), and in places other than the home (47.8%). Respective proportions in Europe (n=154750) were 71.5% and 79.4%, indicating extensive exposure among adolescents.[230]

Biomarker data

Exposure of children and adolescents to ETS measured by biomarker in some Western countries is shown in Table 1. According these studies, tobacco smoke exposure of children has been highly prevalent. In the NHANES III study,[216] examining the exposure of the U.S. population to tobacco smoke in 1988 to 1991 using serum cotinine concentration as a measure, 87.9% of non-smoking children and adults had detectable (detection limit 0.05 ng/ml) serum cotinine values. In that study, 43% of the children lived with at least one smoker. The geometric means of serum cotinine concentrations among all 4- to 11-year-old children, among those not exposed at home, and among children exposed at home were 0.297 ng/ml, 0.119 ng/ml and 1.14 ng/ml, respectively.[216] Cook et al.[231] reported in 1994 that only 6.0% of children (n=2721) aged 5-7 years in England had nondetectable (detection limit 0.1 ng/ml) concentrations of cotinine in saliva. These children's geometric mean salivary cotinine rose from 0.29 ng/ml in children with no identified exposure to 4.05 ng/ml in households where both parents smoked. The authors estimated that 53% of the children lived with at least one smoker.

According to more recent reports, in children aged 11-15 in England, the overall saliva cotinine concentration decreased from 0.96 ng/ml in 1988 to 0.52 ng/ml in 1998 (Table 1).[232] In the NHANES 2001-2002 study, the 50th, 75th and 90th percentile points for

serum cotinine values were 0.067 ng/ml, 0.495 ng/ml, and 2.03 ng/ml in children aged 4 to 11 years (n=1278), and respective percentile points for adolescents aged 12-19 years (n=1902) were 0.051 ng/ml, 0.352 ng/ml and 1.53 ng/ml.[233]

Table 1. Exposure of children and adolescents to tobacco smoke measured by biomarker in some Western countries.

Source	Study year	Country	Age	n	Biomarker	Geometric mean cotinine concentration
Cook et al., 1994 [231]	1990	England	7 years	647	Salivary cotinine	0.83 ng/ml
Pirkle et al., 1996 [216]	1988-1991	U.S.	4-11 years	1071	Serum cotinine	0.297 ng/ml
Pirkle et al., 1996 [216]	1988-1991	U.S.	12-16 years	713	Serum cotinine	0.248 ng/ml
Jarvis et al., 2000 [232]	1998	England	11-15 years	992	Salivary cotinine	0.52 ng/ml
Pirkle et al., 2006 [233]	2001-2002	U.S.	4-11 years	1278	Serum cotinine	0.1 ng/ml
Akhtar et al., 2007 [235]	2006	Scotland	11 years	2339	Salivary cotinine	0.35 ng/ml

Indeed, reports from the U.S,[234] England,[232] and Scotland,[235] have shown that exposure to ETS has declined in many populations due to widespread implementation of laws and policies prohibiting smoking in indoor workplaces and public places. Accordingly, the percentage of nonsmokers with detectable serum cotinine concentrations decreased for all age groups in NHANES between 1988-1994 and 1999-2004, and remained highest for those aged 4-11 years (60.5%) and 12-19 years (55.4%) compared with those aged ≥20 years (42.2%).[234] The decline in the prevalence of detectable serum cotinine was 28.1% in 4-11-year-old children, 35.1% in 12-19-year-old adolescents, and 49.5% in adults. The study from Scotland provides evidence of a population level change in exposure to ETS with exposure falling by 39% in one year among 11-year-old children after the introduction of smoke-free legislation in many public places.[235] These data suggest that legislation has made initial and rapid progress toward promoting health in children by reducing exposure to secondhand smoke. However, reduction in exposure occurred particularly among groups with lower ETS exposure in the home, and not in children whose parents smoke,[235] and parallel results were found in England.[232]

2.3.4. Determinants

The main sources of exposure to ETS among children are domestic, usually in the home or in the car.[236,237] The levels of children's exposure correlate with the prevalence of parental smoking, particularly maternal smoking.[231,232,235,238-242] For instance, in 11- to 15-year-old non-smoking English children, geometric mean salivary cotinine concentrations were 0.28 ng/ml in children with non-smoking parents, 0.71 ng/ml in children with the father smoking, 1.47 ng/ml among children whose mother smoked,

and 2.25 ng/ml in children whose both parents smoked.[232] On the other hand, it has been shown that the father's smoking in the living room and also on the patio/balcony increases the urinary cotinine levels in children to the higher level than respective behaviors of the mother.[243]

Clearly the number of cigarettes smoked in the room, exposure time, and proximity of the child to the smoking adult determines the amount of exposure of the child to tobacco smoke.[244,245] In the Swedish study, urine cotinine values were highest in 3-year-old children, whose parent's smoked indoors, and outdoor smoking with the door closed seemed to be the best precaution to protect children from ETS.[228] However, there was still a significant difference in children's cotinine values between the outdoor smoking group and the non-smoking control group.[228] Another study reported that 3- to 10-year old children from households allowing smoking at home had higher salivary cotinine levels (2.50 ng/ml) compared with children from households with complete smoking bans (0.63 ng/ml), and significant differences were also found regarding car smoking bans.[246] On the other hand, no significant difference in hair cotinine levels was found in small children who were exposed to maternal indoor vs. outdoor smokers.[241]

Smoking by multiple individuals, other than the parents, has been shown to contribute to the ETS exposure of infants.[247,248] The influence of other people on children's exposure becomes increasingly important when children grow older and more time is spent with friends and outside the home.[249] Indeed, children can also be exposed in other environments, including public places where smoking is allowed,[236] yet this is a little studied area.

Several studies have reported that there is no difference between genders in exposure to ETS,[240,247,250] but there are also reports where exposure has been greater in girls than in boys.[231,245,251] Lower age has been associated with higher cotinine levels.[244,250] Young children have had higher cotinine levels than older children despite similar exposure, suggesting a higher relative nicotine dose.[252] However, some studies have not observed the effect of age.[231,253] Race/ethnicity is a known predictor of cotinine levels. Mexican-American children have lower serum cotinine levels than Non-Hispanic whites or Non-Hispanic blacks, whereas cotinine concentrations in Non-Hispanic blacks are the highest.[233] Suggested mechanisms for higher cotinine levels among African-American children are the higher exposure levels in that population and the slower metabolism of cotinine.[254]

Exposure to tobacco smoke has also depended on weekday and season. Higher cotinine concentrations in winter than in summer[255,256] no doubt reflect lower rates of room ventilation in colder months and more time spent inside. Similarly, higher concentrations in children on Mondays than other days of the week[245,251] show the importance of increased time spent with parents at the weekends.

Cotinine concentrations have been observed to be higher in children from more disadvantaged backgrounds. Exposure to tobacco smoke has been related to the size of dwelling,[245,257] crowding in the home,[244,251,253] parent's educational level,[227,253,257] and occupational class.[231,251]

In the NHANES III study,[250] factors that predicted serum cotinine levels were similar among 4- to 16-year-old children regardless of whether there was a reported smoke exposure in the home, although the relative importance of the predictive factors in these groups varied. In that study,[250] significant predictors of cotinine levels among smoke-exposed children included age, education level of the parents, race/ethnicity, the number of rooms in the home and the number of cigarettes smoked in the home, the latter being the most important predictor. These variables explained 36% of the variability in children's cotinine levels. In the same study,[250] among unexposed children, the significant predictors were age, region of the U.S., education level of the parents, race/ethnicity, the number of rooms in the home, family poverty index, and family size. These variables explained only 14% of the variability in cotinine levels, suggesting that also other individual or societal factors may be important.

2.4. Detection of subclinical atherosclerotic vascular changes

Noninvasive ultrasonic methods have increasingly gained acceptance in the determination of early vascular changes related to atherosclerosis, especially in young subjects and healthy populations. Impaired arterial vasodilatory function, loss of arterial elasticity, and thickening of the arterial walls are the main markers of subclinical atherosclerosis that can be measured using ultrasound. These methods have proved to be safe, reliable and reproducible, and may be useful in identifying those apparently healthy individuals at risk of vascular disease later in life.[18]

2.4.1. Endothelial function

The endothelium is a large endocrine organ with multiple functions. As a major regulator of local vascular homeostasis, the endothelium not only allows selective infiltration, but is responsible for synthesis and secretion of numerous substances including vasoactive peptides. The intact endothelium maintains the local balance between vasodilation and vasoconstriction, controls thrombogenesis and fibrinolysis, regulates platelet and leukocyte interactions with the arterial wall, and helps to control smooth muscle proliferation and migration.[258,259] Endothelial dysfunction is an important early event in atherosclerosis progression preceding structural atherosclerotic changes in the vascular wall.[260]

A noninvasive ultrasound technique first described by Celermajer et al. in 1992[261] to measure endothelial function by flow-mediated dilation of the brachial artery is currently widely used. The method is based on the measurement of brachial artery diameter at baseline and after increased blood flow induced by inflation of a pneumatic tourniquet placed around the forearm to a suprasystolic pressure of over 250 mmHg for up to 5 minutes, followed by acute release. Then percentual increase in luminar diameter after hyperemia is used as a marker of systemic endothelial function. The dilation response is mainly mediated by increased NO release by arterial endothelial cells.[262] To ascertain that attenuated FMD in not due to smooth muscle insensitivity to NO but induced by true endothelial dysfunction, the ultrasound method also includes

the determination of endothelium-independent dilation by measuring peak dilation of the artery after administration of nitroglycerin.

Sørensen and coworkers[263] have studied the accuracy and reproducibility of the brachial ultrasound method and shown that although the day to day within subject variation in FMD (2.8%) is rather substantial, the long-term variation in FMD between weeks (0.1%) and months (1.3%) is acceptable. Multiple factors such as temperature of the room, time of day, diet, medication, and caffeine may influence the FMD. Optimizing the study conditions and large sample size helps to minimize these confusing factors. Recent data suggest that strict requirements of fasting are not essential when measuring endothelial function from the peripheral artery.[264] Other methodological aspects are also important, as in children the time to reach the peak FMD response may vary considerably, thus emphasizing the use of several brachial artery measurements up to 120 seconds after cuff release to determine the true FMD peak response.[76]

Noninvasive assessment of endothelial function from the brachial artery correlates with invasive methods testing coronary and brachial endothelial function.[265,266] Traditional cardiovascular risk factors in children and in adults are related with impaired FMD.[261,267,268] In addition, nitrate-mediated dilation has been shown to be attenuated in the atherosclerotic process.[269] Endothelial dysfunction associates with the extent of coronary atherosclerosis[270] and predicts cardiovascular events in patients undergoing nonemergent vascular surgery.[271] Moreover, endothelial function can be modified by a variety of interventions such as diet,[272] exercise,[273] and statin therapy.[274]

2.4.2. Arterial elasticity

Decreased elasticity of large arteries is considered to be a risk factor for cardiovascular diseases, as well as an early marker of subclinical atherosclerosis. Ageing is the most prominent factor related to decreased arterial elasticity.[275] Various structural and functional changes of the vascular tree leads to stiffening of the arteries.[276] Phenomena responsible for arterial stiffening with age and with the presence of cardiovascular risk factors include breaks in elastic fibers, accumulation of collagen, fibrosis, inflammation, medial smooth muscle necrosis, and calcification.[277] More acutely, arterial stiffness is affected by mean arterial blood pressure and smooth muscle tone.[278]

Arterial elasticity can be determined by different non-invasive methods.[276] Pulse wave velocity (PWV) is the most recognized measure of arterial stiffness, determined as the difference between two recording sites in the line of pulse travel, and the delay between corresponding points on the wave. Arterial elasticity can also be measured by applanation tonometry assessing arterial pressure waveforms. In addition, ultrasound can be used to evaluate the mechanical properties of arteries, and these indices of aterial stiffness are based on the measurement of blood pressure and arterial diameter changes during the cardiac cycle. Several indices of arterial stiffness can be defined. Of these, the stiffness index, Young's elastic modulus, and distensibility are measures of local stiffness reflecting different aspects of mechanical properties of arteries.[279,280] The stiffness index characterizes the elastic properties of the arterial wall relatively independently of blood pressure. Young's elastic modulus is a parameter independent

of the arterial geometry and may be considered as a measure of intrinsic stiffness of the arterial wall material. Distensibility measures the ability of the arteries to dilate during the cardiac cycle, and evaluates the function of the artery as a hollow structure. It has been shown that ultrasonically assessed indices of arterial stiffness and PWV correlate.[281]

Arterial stiffness has been associated with a number of cardiovascular risk factors in adults.[282,283] Loss of arterial elasticity is an independent predictor of cardiovascular events in high-risk individuals.[284,285] In addition, the decreased elasticity of the large arteries has been observed in certain pediatric patient groups, i.e. in children with hypercholesterolemia,[286,287] diabetes mellitus,[288] and severe obesity.[289] However, there is conflicting evidence as to the influence of hypercholesterolemia and arterial stiffness in children.[75,286,287] Increased systolic blood pressure and sedentary lifestyle have been associated with decreased arterial elasticity in children.[290,291] Moreover, accumulation of risk factors in childhood leads to decreased arterial elasticity in adulthood.[283]

2.4.3. Intima-media thickness

To assess early structural atherosclerotic changes ultrasonic measurement of arterial wall thickness has been shown to be an accurate, safe, and reproducible method.[292] Measurement of intimal plus medial thickness of the arterial wall, i.e. intima-media thickness (IMT) was first presented in 1986 when Pignoli and coauthors showed a close relation between ultrasound measurements and histological measurements of IMT using post-mortem arterial material.[293]

The carotid arteries have been shown to be ideal objects for ultrasonic IMT measurement because of their size and easily attainable location. cIMT correlates with vascular risk factors,[44,92,93] relates to coronary atherosclerosis,[294] and CHD severity,[270] as well as predicts the likelihood of cardiovascular events.[295-298] It has been found that an 0.03 mm increase per year in cIMT associates with a 2.2-fold increase in coronary event risk.[299] Several cardiovascular risk factors such as hypercholesterolemia, hypertension, diabetes mellitus, and obesity have been related to early structural vascular wall changes in carotid arteries already in childhood,[55,300-302] and it has been demonstrated that exposure to cardiovascular risk factors in childhood predicts adult cIMT.[56,73,303]

At present, cIMT has been recognized as a surrogate measure of atherosclerosis, and both the American Heart Association[304] and the Third Joint Task Force of European and other Societies on Cardiovascular Disease Prevention in Clinical Practice[305] have acknowledged the usefulness of cIMT in predicting cardiovascular risk in adults. In contrast, measurement of aIMT has not been as extensively examined as cIMT, and utility of the aIMT measurement remains insufficiently asserted. It has been claimed that with the ultrasonic technique, the aorta is beyond proper reach, especially in adults. However, it has been shown that using common carotid, common femoral and popliteal arteries as a reference data, far wall aIMT can be satisfactorily studied with the B-mode ultrasound technique.[306] In addition, aIMT is associated with CHD.[307] Indeed, measuring aIMT may provide an even better index of subclinical atherosclerosis than cIMT, as autopsy studies have shown that the earliest structural

alterations in the vascular tree appear in the abdominal aorta.[17] The ultrasonically measured aIMT may be particularly useful in children and adolescents, as aIMT identifies subjects with increased atherosclerosis risk factor load even more efficiently than cIMT,[58,308] and thus aIMT has proven to be a feasible, accurate and sensitive marker for risk of atherosclerosis.

3. AIMS OF THE STUDY

The purpose of the present study was to evaluate exposure to tobacco smoke with an objective biomarker, serum cotinine concentration, and its associations with the development of atherosclerosis in school-aged children and adolescents participating in an atherosclerosis prevention trial, STRIP. The more specific aims were:

1. To examine the possible impact of frequent lifestyle intervention on the active smoking of parents and the tobacco smoke exposure of their children, and to evaluate the association between parental smoking habits and child exposure (I).

2. To investigate the association of exposure to tobacco smoke with arterial reactivity and arterial elasticity at the age of 11 years (II, III).

3. To study the relationship between exposure to tobacco smoke and serum lipid profile (IV).

4. To investigate the association of frequent exposure to tobacco smoke with arterial wall thickness and endothelial function in 13-year-old adolescents (IV).

4. SUBJECTS AND METHODS

4.1. Study design

The STRIP project is a randomized, prospective intervention trial aimed at decreasing the exposure of children to known environmental cardiovascular risk factors.

The initial recruitment was done by nurses of the well-baby clinics of the city of Turku. Parents of 1880 eligible 5-month-old infants were informed about the STRIP project and offered the possibility to participate. A total of 1105 families were interested in participating, to whom the STRIP study design, purpose and the goals were explained when the infant reached the age of 6 months at the Research Centre of Applied and Preventive Cardiovascular Medicine of Turku University. Finally, the original STRIP cohort comprised 1062 infants from 1054 families (56.5% of the eligible cohort, 8 twin pairs) (Figure 1). These 1062 infants were allocated to an intervention group (n=540) or a control group (n=522) by random numbers. The first actual study visits at the infant age of 7 months occurred between February 1990 and June 1992.

The families of the intervention and control groups met a pediatrician and a dietitian first at 1- to 3-month intervals and at 4- to 6-month intervals, respectively. After the age of 2 years, the visits of both groups took place at 6-month intervals. When the children of the control group reached the age of 7, they visited the research personnel only once a year.

At each visit, the child's health history was recorded, and the physician carried out a clinical examination, including measurements of height, weight and blood pressure. Food records for 3-4 days were collected at the ages of 8 and 13 months, twice a year between ages 2 and 7 years, and thereafter twice a year in the intervention group and once a year in the control group. Non-fasting venous blood samples were drawn at the ages of 8 and 13 months, and 2, 3, and 4 years. Since the age of 5 years, fasting blood samples were drawn annually, except at the age of 6 years, when no blood was drawn, and at the age of 8 years, when blood samples were non-fasting. Ultrasound studies were performed at the ages of 11 and 13 years.

4.2. Intervention

The intervention families received individualized and detailed, child-targeted lifestyle counseling at each visit, given by a pediatrician or physician, a dietitian, and a registered nurse.

During the first years, a pediatrician generally discussed atherosclerosis risk factors such as smoking, cholesterol, sedentary lifestyle and being overweight, and encouraged the families to change both the child's and parent's habits towards a healthier lifestyle. In families with smoking parent(s), the children's possibility to be exposed to tobacco

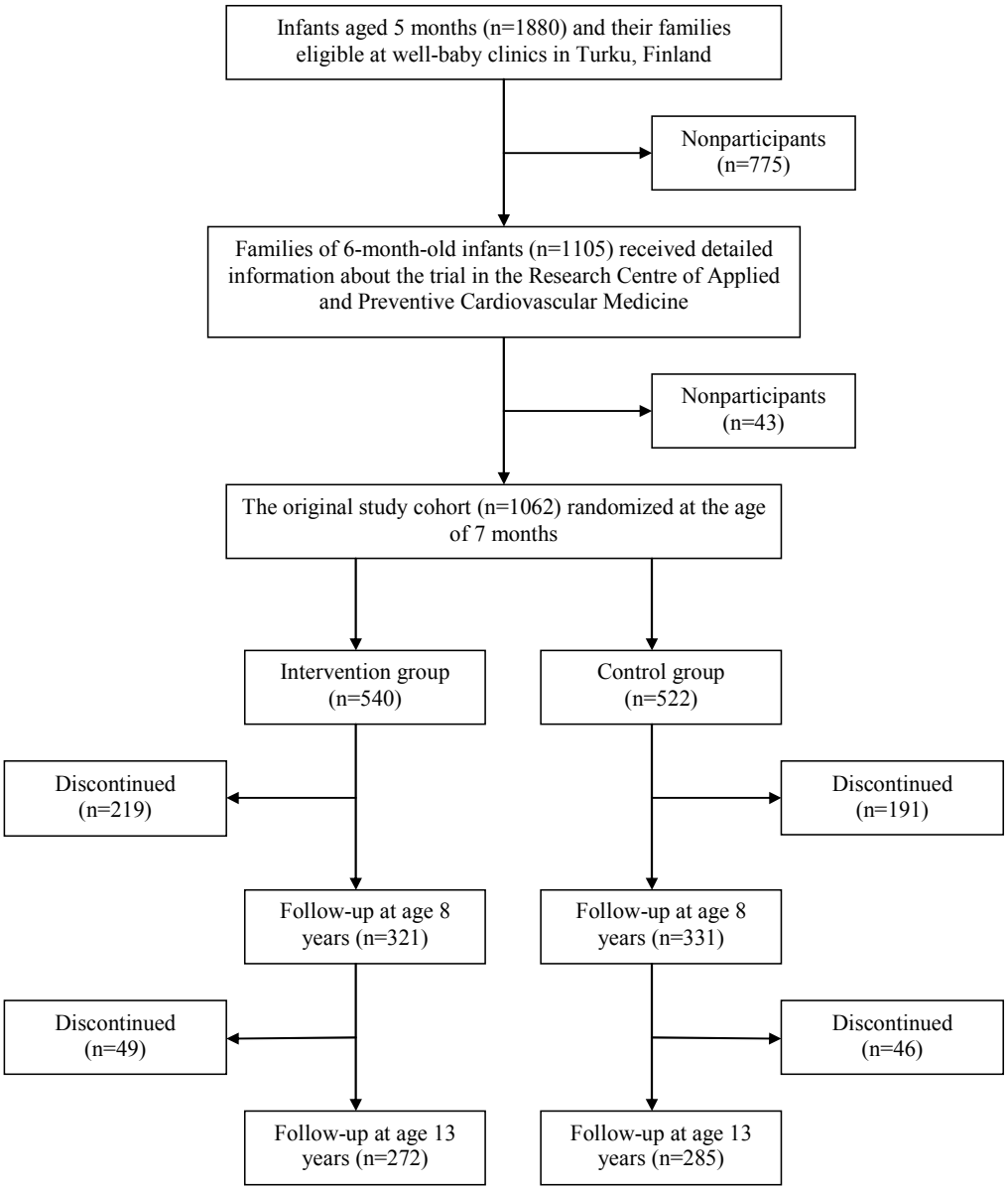

Figure 1. Flow diagram of the Special Turku coronary Risk factor Intervention Project (STRIP) for children.

smoke at home was discussed with the parents. At the child's age of 5 years, intervention parents received a booklet about the adverse health effects of smoking. If the family history was positive for premature heart disease, the importance of cessation of smoking was repeatedly discussed with the intervention parents who smoked. The dietitian had the main responsibility for dietary counseling of the intervention families. The dietary counseling was individual and based on the food records of the child and dietary history of the family. A fixed diet was never ordered, but suggestions were made toward a better composition of diet. The intervention was aimed at reducing the intake of saturated and total fat and cholesterol in the child's diet. The recommended intakes of fat were 30-35% of daily energy (E%) until the age of 3 years, and 30 E% thereafter, with a ratio of saturated to (monounsaturated plus polyunsaturated) fatty acid of 1:2 and a cholesterol intake of less than 200 mg/day. In addition, families were encouraged to use vegetables, fruits, low salt and whole-grain products. After 7.5 years of age, the child met the dietitian first alone and then with the parents, and more information was given directly to the child. Food consumption data were obtained through annual 3-4 day food records, and the dietitian checked the records for accuracy on each visit. The nutrient intakes were calculated with Micro Nutrica® software (Research and Development Centre of the Social Insurance Institution, Turku, Finland) based on the Food and Nutrient Database of the Social Insurance Institution.[309]

Child-oriented counseling aiming at prevention of active smoking began at the child's age of 9.5 years. A registered nurse met the intervention child alone twice a year. Counseling sessions included paper-pencil, picture based, or computer assisted tasks. Most of the counseling material used has been particularly developed for the project because ready-made counseling materials for children are sparse. Counseling covered cardiovascular physiology and pathophysiological effects of smoking. It was based on supporting the self-image of the non-smoking children and on understanding the risks associated with smoking. Attitudes towards smoking, avoiding passive smoking, and the development of addiction were discussed with the child, and suggestions were made how to refuse offered tobacco. The parents were informed about the contents of the child's counseling session and were encouraged to further discuss the smoking-related topics with the child at home.

The families of the control group received the basic health education given to all Finnish families at the well-baby clinics and school health care. The control children received no detailed dietary counseling or smoking prevention.

4.3. Subjects

The subjects consisted of children aged 8 – 13 years who participated in the STRIP study. Visits of the 8-year-old children began in September 1997, and all 13-year-old children had visited the Research Centre by November 2004. At the ages of 8 and 13 years, a total of 652 (61% of the original cohort) and 557 (52% of the original cohort) children still remained in the study (Figure 1).

To study the exposure of the children to tobacco smoke with an objective biomarker, the serum cotinine concentration of the children was measured annually from every obtained blood sample with sufficient serum volume for analysis from 8 years of age onwards. The serum cotinine concentration of the parents was measured only at the child's age of 8 years.

To longitudinally analyze parent's smoking, smoking status recorded annually by a physician during the STRIP visits was used. These interviews were made between the child's age of 8 months and 13 years. The number of attending mothers and fathers was 1047 and 1015 at the beginning of the project and respective numbers were 397 and 287 at adolescent's age of 13 years.

Study I

In study I, the impact of 8 years of lifestyle intervention on the active smoking of the parents and passive smoking of their 8-year-old children was studied.

At the child's 8-year visit, the attending parents answered a detailed smoking questionnaire (comprising 425 mothers and 282 fathers). At least one parent of each of the 475 children answered the questions concerning tobacco smoke exposure of the child. The detailed smoking status of the parents was a combination of the parents' questionnaire responses at the child's age of 8 years, or responses at the child's age of 9 years if the parent had missed the 8-year visit. If the parents had not returned the questionnaires but the smoking history had been asked about and registered by the physician, this information was used. Altogether, the smoking status of 585 mothers and 457 fathers was obtained at child's age of 8 years. For 419 study children, the smoking status of both parents was available. Serum samples of 561 mothers and 376 fathers were available for analysis.

The study group of children consisted of 625 8-year-old children with successful serum cotinine measurement (comprising 96% of the total participating age cohort). A total of 306 children from the intervention group (160 boys and 146 girls) and 319 from the control group (168 boys and 151 girls) participated in study I.

Study II

For the purpose of study II, exposure to tobacco smoke of the study children was evaluated between the ages 8 and 11 years. At the ages of 8, 9, 10, and 11 years, serum cotinine values of 625, 561, 555 and 548 healthy children were available, comprising 96%, 91%, 94%, and 95% of the participating age cohort, respectively. Serum cotinine values from all four age points were available from 441 children. None of the children reported active smoking during follow-up, but one 10-year-old child who had a cotinine concentration of 100.8 ng/ml was excluded. The study children who had a chronic disease (familial hypercholesterolemia, type 1 diabetes) that might affect the outcome measures were excluded.

In study II, the associations between present and prior exposure to tobacco smoke and arterial reactivity at the age of 11 years were studied among 418 children who volunteered to participate in the ultrasound study at age 11 years and had serum cotinine measured. Successful brachial artery measurements were available in 402 of

these 418 children. For the longitudinal analyses, all children having cotinine values at all four age points and brachial artery measurements at age 11 were included (n=327).

The distribution of the STRIP children's serum cotinine concentrations was highly skewed, the majority of the children having an undetectable cotinine concentration (<0.16 ng/ml), and a large amount of the children having very low cotinine concentrations indicative of scarce exposure to tobacco smoke. Children with the highest cotinine concentrations were also only modestly exposed. Thus, to analyze the effect of passive smoking on the ultrasound measures, children were divided into three groups. The top decile cotinine group (n=39) was constituted of 11-year-old children representing the highest 10th percentile of cotinine concentrations (serum cotinine ≥1.7 ng/ml), because the functional sensitivity of the chromatographic method [inter-assay coefficient of variation (CV) <20%] was between 1 and 2 ng/ml. The noncotinine group (n=229) comprised children with a cotinine concentration under the detection limit of the assay (57% of the 11-year-old children), and the remainder of the children formed the low cotinine group (n=134).

To study whether prolonged exposure of children to tobacco smoke is associated with endothelial function, longitudinal data from a subgroup of the children with all four cotinine measurements between the ages 8 to 11 years (n=327) were analyzed. In line with the afore-mentioned grouping, children with a serum cotinine concentration in the highest decile in at least two of the annual measurements (n=20) constituted the group of children with repeatedly high cotinine values and the other extreme group comprised children with a cotinine concentration never in the highest decile (persistently low cotinine values, n=211). The remainder of the children constituted the group of children with remittent cotinine values. For comparison, only one child had cotinine value in the highest decile four times during follow-up.

The smoking questionnaire was completed by 331 mothers and 239 fathers at the child's age of 11 years among families where the child participated in ultrasound studies.

Study III

In study III, the association between exposure to tobacco smoke and arterial elasticity was studied in those STRIP children who underwent ultrasound scanning at the age of 11 years with successful aortic and carotid elasticity measurements and who had their serum cotinine concentration measured at the age of 11 years (n=386). The study children who had a chronic disease (familial hypercholesterolemia, type 1 diabetes) that might affect the outcome measures were excluded.

For the purpose of study III, 11-year-old children were divided into three cotinine level groups. The top decile cotinine group (n=39) was constituted of children representing the highest 10th percentile of cotinine concentrations (serum cotinine ≥1.7ng/ml). The noncotinine group (n=220) comprised children with a cotinine concentration under the detection limit of the assay. The remainder of the children formed the low-cotinine group (n=127).

Study IV

In study IV, the study children's serum cotinine concentrations were determined annually from every blood sample obtained between the ages 8 and 13 years. To evaluate the children's longitudinal exposure to tobacco smoke, the serum cotinine values of the children with cotinine values available 2 to 6 times between the ages 8 and 13 years were averaged for the analyses. Study children were then divided into tertiles according these averaged cotinine values. At the age of 13 years, the number of the adolescents was 545 (98% of the participating age cohort), and the majority of the subjects (93%) had cotinine values available 5 or 6 times during follow-up.

Of the 545 adolescents with longitudinal cotinine measurements, 494 (91%) had appropriate ultrasound measurements available at the age of 13 years: cIMT measurements were available in 494, aIMT measurements in 487, and brachial artery measurements in 482 adolescents. The serum lipid profile was available for 494 adolescents. Of the 494 study subjects with ultrasound data, 163 belonged to the high tobacco smoke exposure group (the highest tertile of averaged cotinine values), 171 to the intermediate exposure group (the midmost tertile), and 160 to the low exposure group (the lowest tertile of averaged cotinine concentrations).

The study children who had a chronic disease (familial hypercholesterolemia, type 1 diabetes) that might affect the outcome measures were excluded. Children with a serum cotinine concentration of ≥15 ng/ml were defined as active smokers, and excluded from all analyses. The number of active smokers was 1 and 5 in 10- and 13-year-old children, respectively.

The smoking questionnaire was completed by 386 mothers and 313 fathers at the adolescent's age of 13 years among families where the adolescent participated in ultrasound studies.

4.4. Physical examination

At each STRIP visit, weight was measured using an electronic scale to the nearest 0.1 kg (Soehnle S10; Soehnle, Murrhardt, Germany), and standing height to the nearest 0.1 cm using a wall-mounted Harpenden stadiometer (Holtain, Crymych, U.K.). Both of the measurements were taken with the child wearing only light underwear. The BMI was calculated as kilograms per meter squared.

Pubertal status was recorded beginning at the age of 9 years. Children's sexual maturation was classified using Tanner staging.[310] Breast tissue diameter and pubic hair development were estimated visually. Testicular length was measured with a ruler. The children were classified as either prepubertal (Tanner stage M or G=1), pubertal (Tanner stage M/G ≥2) (studies II and III), or late pubertal (Tanner stage ≥3) (study IV).

4.5. Interview and questionnaires

From the beginning of the STRIP project, the pediatrician asked about the smoking habits of the parents during the annual office visit, and the parents were classified as smokers or non-smokers. During the child's 8-year visit the attending parents answered, for the first time, a detailed questionnaire about smoking. Parents were asked whether they had ever smoked, had smoked at least 100 cigarettes in their lifetime, how many years they had smoked, whether they were currently smoking, and the amount of tobacco products they consume daily. The parents were also asked about intentions and attempts to quit smoking. To estimate the exposure to tobacco smoke of the study children by questionnaire, parents were asked whether anyone smoked indoors at their home, and how many hours they estimated their 8-year-old child was exposed to tobacco smoke during the three consecutive days prior to the visit. If the parent had missed the 8-year visit, but participated at the child's age of 9 years, a similar detailed questionnaire was asked to be completed. Otherwise, from the 9-year visit onwards, parents annually answered a brief questionnaire about their current smoking status.

The smoking experiments and smoking habits of the study children were queried with a detailed questionnaire completed by a registered nurse. Children of the intervention group answered the questionnaire first at the age of 9.5 years and children of control group at their 10-year visit. Thereafter, the smoking status of the children was enquired by a questionnaire annually. Both parents and children were informed that their answers were confidential.

4.6. Laboratory methods

Venous blood samples drawn from an antecubital vein were non-fasting at the age of 8 years and fasting (10 to 12 hours) in 9- to 13-year-old children.

Serum cotinine analyses were performed in the Joint Clinical Biochemistry Laboratory of the University of Turku, Turku University Central Hospital and Wallac Oy, in Turku, Finland. All serum lipid, lipoprotein, apolipoprotein, and high-sensitivity C-reactive protein (hsCRP) measurements were performed in the laboratory of the Research and Development Unit of the Social Insurance Institution in Turku, Finland. The results were regularly crosschecked by Labquality, Helsinki, Finland.

4.6.1. Cotinine measurements

The venous blood samples were centrifuged, serum was separated and stored at –70°C until analyzed.

Cotinine was extracted into dichloroethane from 0.2 ml of serum to which 0.2 ml of 5-methylcotinine (0.1 μg/ml in 0.01M HCl) was added.[219] Concentrated extract (2.0 μl) was injected into the Hewlett Packard FFAP (nitroterephtalic acid modified polyethylene glycol) silica capillary column (13 m, i.d. 0.32 mm, film thickness 0.52 μm) (Agilent Technologies, Palo Alto, CA) of the Shimadzu model GC-17 gas

chromatograph, equipped with a nitrogen-sensitive Shimadzu FTD-17 flame-thermoionic detector and a Shimadzu AOC-20 auto-injector (Shimadzu, Kyoto, Japan). The injector and detector temperatures were 220°C and 300°C, respectively. The retention times for nicotine, cotinine and 5-methylcotinine were 2.9 min, 10.8 min and 12.8 min, respectively. The peak areas were analyzed using Shimadzu Class-VP™ chromatography software.

Cotinine recovery from serum was 76-112% depending on its concentration (Table 2). Gas-chromatographic analysis of cotinine was linear (y=1.06x-3.47, r=0.998) at the studied 5-100 ng/ml concentrations. The analytical sensitivity of the assay was determined from 15 serum samples without cotinine. The detection limit mean + 2 standard deviation (SD) was 0.08915+2x0.03545=0.16 ng/ml. The intra-assay and inter-assay CV at a cotinine concentration of 22 ng/ml were 4.4% and 11.7%, respectively (n=20). The inter-assay CV at a cotinine concentration of 1 ng/ml was 23.3% (n=9) (Table 2).

The method was validated by cotinine analyses of 20 serum samples both in our laboratory and in ABS Laboratories (Medical Toxicology Unit, London, UK). The correlation coefficient of these determinations was 0.994.

Table 2. Inter-assay coefficient of variation (CV), functional sensitivity of the assay (CV<20%), and recovery in gas-liquid chromatographic measurement of serum cotinine concentration. Mean±SD values are shown for observed cotinine concentrations.

Number of serum samples	Serum cotinine concentration, ng/ml	Cotinine observed, ng/ml	Inter-assay CV %	Recovery %
9	0.5	0.56±0.33	58.9	112
9	1.0	1.03±0.24	23.3	103
9	2.0	1.64±0.26	15.8	82
9	4.0	3.03±0.33	10.9	76
20	22.0	18.48±2.17	11.7	84

4.6.2. Lipid, lipoprotein and apolipoprotein measurements

After centrifugation, serum was separated and stored at –20°C for up to a month before lipid measurements. Serum cholesterol concentration was measured with a fully enzymatic cholesterol oxidase-p-aminophenazone method (CHOD-PAP, Merck, Darmstadt, Germany)[311] in an AU 510 automatic analyzer (Olympus, Hamburg, Germany) until January 10, 2001, or, thereafter, in an AU 400 Analyzer.

Serum HDL cholesterol concentration was measured after precipitation of LDL and VLDL with dextran sulfate 500 000.[312]

Serum triglyceride values were measured with the colorimetric GPO-PAP method (Merck, Darmstadt, Germany) in automatic Olympus AU 510 (until January 10, 2001) and AU 400 (since January 11, 2001) analyzers.

Serum ApoA-I and ApoB were determined immunoturbidimetrically with ApoA-I and B kits (Orion Diagnostica, Espoo, Finland)[313] in automatic Olympus AU 510 (until January 10, 2001) and AU 400 (since January 11, 2001) analyzers. The apolipoprotein measurements were standardized against WHO International Reference Materials SP1-01 for ApoA-I and SP3-07 for ApoB.

Due to the changes in determination methods and kits during the study years, lipid levels determined with the Olympus AU510 analyzer were corrected by using the following correction factor equations, determined in the laboratory of the Research and Development Unit of the Social Insurance Institution, Turku, Finland:

Total cholesterol (new) = 1.0207 x (old total cholesterol) + 0.0526

HDL cholesterol (new) = 1.031 x (old HDL cholesterol) – 0.0083

Triglycerides (new) = 1.0247 x (old triglycerides) + 0.0191

ApoA-I (new) = 1.0239 x (old ApoA-I) + 0.0991

ApoB (new) = 0.8725 x (old ApoB) + 0.1812

The inter-assay CV for AU510 analyzer (for AU400 analyzer) of total cholesterol, HDL cholesterol, triglycerides, ApoA-I, and ApoB determinations were 2.0% (2.2%), 1.9% (2.3%), 4.3% (3.8%), 3.0% (3.2%), and 4.5% (2.8%), respectively.

As serum triglyceride values were <4 mmol/l in all children, serum LDL cholesterol values were calculated using the Friedewald formula[314]: LDL cholesterol=Total cholesterol–HDL cholesterol–0.45 x triglycerides.

Serum Lp(a) was determined using a solid-phase immunoradiometric assay (Mercodia. Apo(a) RIA; Mercodia AB, Uppsala, Sweden) based on the direct sandwich technique.[315] The inter-assay CV of Lp(a) was 7.4% at the mean Lp(a) level of 50.9 mg/l.

4.6.3. Determination of C-reactive protein

Serum hsCRP concentration was determined using an immunoturbidimetric method (Olympus Diagnostica, Lismeehan, Ireland) with an automatic clinical chemistry analyzer (Olympus AU400). The sensitivity of the method was 0.02 mg/l. The inter-assay CV at the mean CRP level of 1.66 mg/l was 1.07%.

4.7. Ultrasound studies

All ultrasound studies were performed using an Acuson Sequoia 512 mainframe (Acuson, Mountain View, CA, USA) with a 13.0 MHz linear array transducer. The studies were carried out in a silent, dimmed clinical research laboratory in the morning on fasting children. A single experienced vascular sonographer blinded to the subjects'

details performed ultrasound scanning and the offline analysis of the scans at the age of 11 years, and another single operator performed the studies at age 13 years. Ultrasound studies of the 11-year-old and 13-year-old children were performed between October 2000 and November 2002, and between March 2002 and November 2004, respectively.

Blood pressure was measured in a supine position after 10 minutes' rest 3 times from the right arm using a standard sphygmomanometer (Omron M4, Omron Matsusaka, Matsusaka, Japan), and the average of the three measurements was used in the calculations.

4.7.1. Brachial artery physiology

The study subjects lay quietly for 10 minutes before the first scan. The left brachial artery was scanned in a longitudinal section 5 to 15 cm above the elbow. Depth and gain settings were set to optimize images of the lumen-arterial wall interface, and the operating parameters were not changed during the study. When a satisfactory transducer position was found, the position was marked on the skin, and the arm remained in the same position throughout the study.

The brachial artery diameter was determined from B-mode ultrasound images at rest, during reactive hyperemia and after administration of sublingual nitroglycerin (Figure 2). First, a resting scan was performed and arterial flow velocity was measured using a Doppler signal. Increased flow was then induced by inflation of an adult size blood pressure cuff (14.8 x 44.8 cm) around the forearm to a pressure of 250 mmHg for 4.5 minutes, followed by release. An adult size cuff was used, as in our experience the use of a narrower pediatric cuff often causes unnecessary discomfort for children. Subsequent scans were taken continuously 40 to 180 seconds after cuff deflation. Repeated flow velocity recording was included for the first 15 seconds after the cuff release. The arterial diameter was measured offline at a fixed distance from an anatomic marker (e.g. a fascial plane) using ultrasonic calipers at end-diastole, incident with the R-wave on a continuously recorded electrocardiogram. The percentual dilation from baseline in 10-second intervals between 40 and 180 seconds was assessed. The maximal dilation response (peak FMD, %) and total dilation response, defined as the area under the dilation response vs. time curve between 40 and 180 seconds after hyperemia (AUC, % x s) were determined. FMD and AUC, reflecting endothelium-dependent dilation, significantly correlated ($r=0.9$, $P<0.001$).

Nitrate-mediated, endothelium-independent dilation capacity was tested by administration of two sublingual doses (50 μg + 200 μg) of glyseryltrinitrate, 5 minutes apart. Maximal artery diameter 5 minutes after cumulative nitrate administration was used to calculate the proportional increase in diameter from the baseline value (NMD, %). NMD measurements were performed only at the child's age of 11 years. The interobserver CV of FMD measurements was 8.6% and the between-study CV was 9.3%.

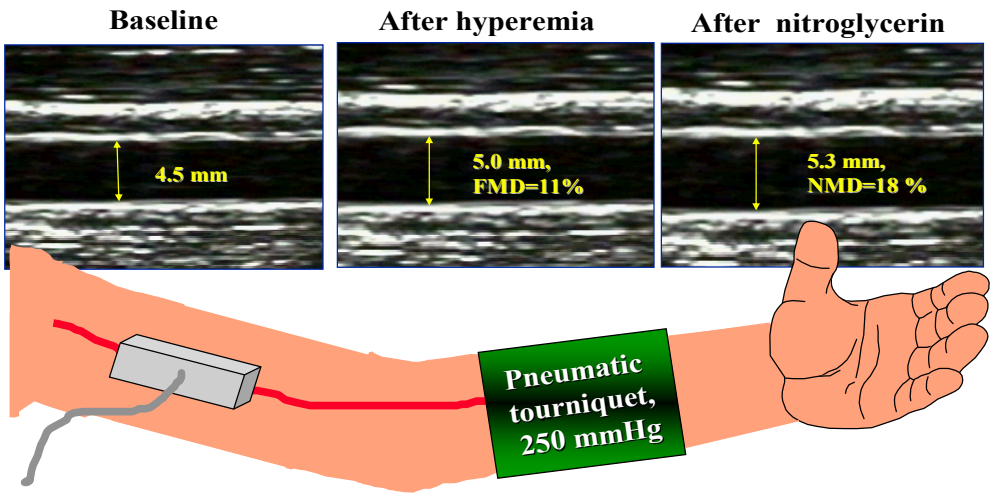

Figure 2. Brachial artery diameter was determined by scanning 3 times using ultrasound.

4.7.2. Arterial elasticity

Aortic and carotid elasticity were analyzed using M-mode ultrasound imaging (Figure 3), based on the measurement of blood pressure and arterial diameter changes during diastole and systole. The abdominal aorta and the left common carotid artery were scanned with a 7.0- or 13.0 MHz linear array transducer. The aorta was scanned approximately 1 cm distal from the bifurcation of the inferior mesenteric artery, and the carotid artery was scanned approximately 2 cm proximal to the carotid bulb. The diameters of the arteries were scanned using M-mode, and the images were stored in digital format for subsequent off-line analysis. The arterial diameters were measured with ultrasonic calipers twice in end diastole and twice in end systole. The mean of the measurements was used as the end-diastolic or the end-systolic diameter, respectively.

The aforementioned ultrasound measurements and simultaneously measured brachial blood pressure values were used to calculate the following aortic elasticity indices:

Stiffness index of the aorta (aSI) = ln(BPs/BPd)/([aDs–aDd]/aDd),

Young's elastic modulus of the aorta (aYEM) = ([BPs–BPd]·aDd)/([aDs–aDd]/aortic wall thickness),

Distensibility of the aorta (aD) = ([aDs–aDd]/aDd)/(BPs–BPd)·1000

In these formulas, aDs is the systolic diameter of the aorta, aDd is the diastolic diameter of the aorta, BPs is systolic blood pressure, and BPd is diastolic blood pressure.

Carotid artery stiffness index (cSI), Young's elastic modulus (cYEM), and distensibility (cD) were computed as the aforementioned aortic elasticity indices, but systolic diameter of the carotid artery, diastolic diameter of the carotid artery, and carotid wall thickness were used in the formulas.

There was a strong correlation between aortic elasticity indices as well as between carotid elasticity indices (P always <0.001), with a correlation coefficient of r=0.88 between aSI and aYEM, r=–0.94 between aSI and aD, r=–0.89 between aYEM and aD, r=0.83 between cSI and cYEM, r=–0.94 between cSI and cD, and r=–0.89 between cYEM and cD. Moderate correlation between aortic and carotid elasticity indices was found (r between 0.24 and 0.36, P always <0.001). FMD correlated weakly only with aSI (r=–0.11, P=0.043).

To study the reproducibility of elasticity measurements, 19 subjects underwent ultrasound studies twice (8-15 months apart). The CV for two consecutive measurements was 8.4% for diastolic diameter of the aorta, 26.9% for aSI, 23.3% for aYEM, and 23.3% for aD. Regarding the carotid artery, the CV was 6.2% for carotid diastolic diameter, 15.6% for cSI, 15.3% for cYEM, and 16.5% for cD.

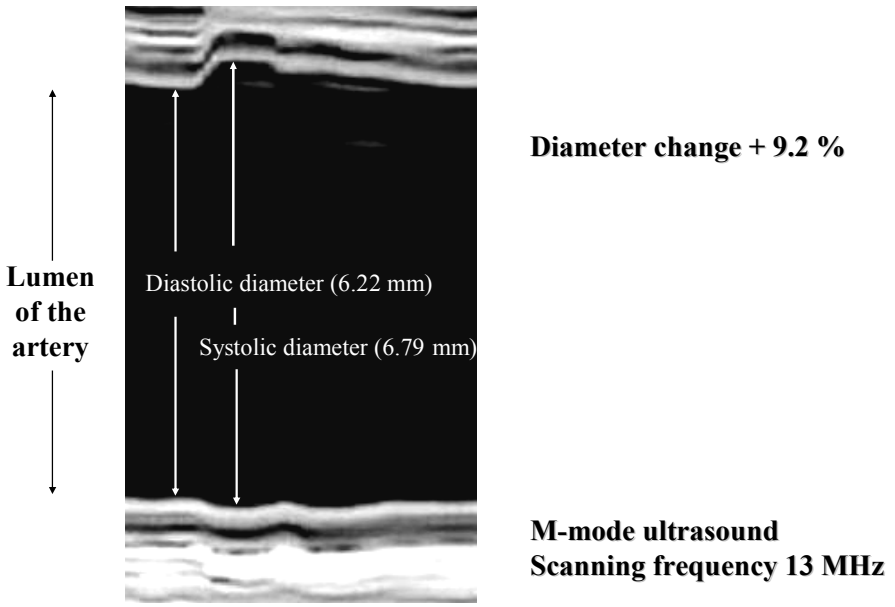

Figure 3. The elasticity of the abdominal aorta and common carotid artery were assessed by measuring the change in arterial diameter from diastole to systole from M-mode ultrasound images.

4.7.3. Carotid artery intima-media thickness

The posterior wall of the distal common carotid arteries on both sides was scanned. The cIMT measurements were performed following predetermined, standardized scanning protocols.[308] The place of the measurements was anatomically standardized in every study by identifying the proximal part of the carotid bulb and then by scanning the common carotid artery. The image was focused on the posterior (far) wall, and the resolution box function was used to magnify the arterial far wall. Several images of the common carotid wall segment from 1 to 2 cm proximal to the carotid bulb were acquired (a far wall segment of 10 mm in width). In each case, two interrogation angles were used: anterior oblique (~30 degrees from midline) and lateral (~100 degrees from midline). All scans were digitally stored for subsequent offline analysis. Two end-diastolic frames from both interrogation angles on both sides were selected and analyzed for mean and maximum IMT. Maximum cIMT, and the average of these measurements as mean cIMT were used. The interobserver CV of cIMT measurements was 3.0%, and the between-visits variation (CV) was 3.9%.

4.7.4. Abdominal aortic intima-media thickness

The most distal 15 mm of the abdominal aorta was scanned with a 7.0- or 13.0 MHz linear array transducer following a standardized protocol (Figure 4).[308] For the aIMT measurements, the image was focused on the far wall (dorsal arterial wall) and gain settings were used to optimize image quality. Images 15 mm in width were magnified using resolution box function. A scanning frequency of 13 MHz was preferred but, when necessary (to reach sufficient tissue penetration), scanning frequencies of 11.5 and 10 MHz were used. All images were taken at end-diastole, incident with the R-wave on a continuously recorded electrocardiogram. In every case, several images of the most distal 15 mm aortic far wall were captured. The images were stored for subsequent offline analysis. Two images of the best quality were chosen by the sonographer for analysis in each study subject. Using ultrasonic calipers, at least 4 to 6 IMT measurements covering the entire far wall segment of interest were taken for each image. The average of these measurements was used as mean aIMT, and the thickest of the measurements was used as maximum aIMT. The interobserver CV in aIMT measurements was 3.9%, and the between-visits CV was 4.9%.

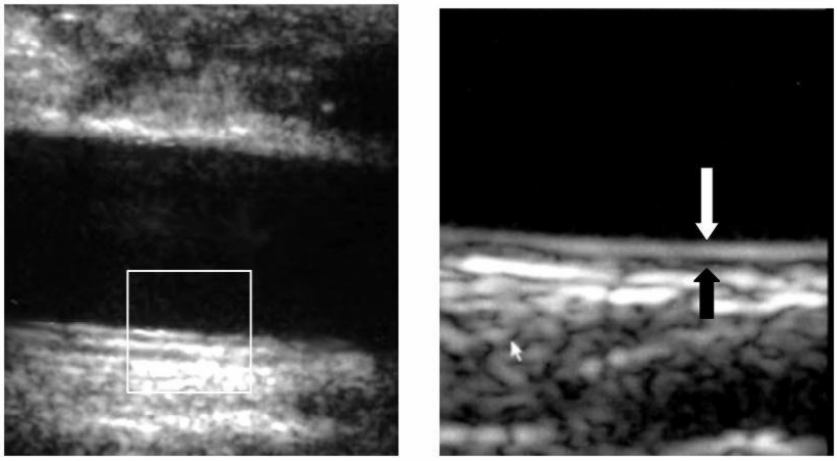

Figure 4. The far wall of the distal abdominal aorta was scanned (on the left), and the intima-media complex was measured from the far wall images, which were magnified using a resolution box function (inset on the left; on the right). The ultrasound visualizes the lumen-intima interface (white arrow) and the media-adventitia interface (black arrow), and the distance between these interfaces is termed intima-media thickness (IMT).

4.8. Statistical analyses

Variables with a skewed distribution were log-transformed for the analyses. Nondetectable serum cotinine concentrations were coded as 0.01 before transformation. The results are expressed as mean (SD), mean (standard error of mean [SEM]), or geometric mean (95% confidence interval [CI]), unless stated otherwise. Differences were considered to be statistically significant at a value of $P<0.05$. Statistical analyses were performed using the SPSS 11.0 package for Windows (SPSS, Chicago, IL, USA) and SAS 8.2 (I) and 9.1.3 (II-IV) (SAS Institute, Cary, NC, USA).

The longitudinal smoking status of the parents in STRIP study groups was studied using subject-specific logistic regression models with the smoking status at the child's age of 8 months as a covariate.

Study I

The Chi-square test was used to compare the proportions of smoking parents. Because the distribution of the serum cotinine concentrations was highly skewed, non-parametric models were used. Group differences were evaluated using the Kruskall-Wallis test and pairwise differences were assessed by the Wilcoxon two-sample rank sum test with Bonferroni adjustment. Correlation analyses were performed using Spearman correlations.

Study II

The nonparametric Wilcoxon two-sample rank sum test was used to compare serum cotinine values of children with and without ultrasound data. The Pearson correlation coefficient was used to study the association between ultrasound measures. The associations between serum cotinine levels and ultrasound variables were examined with single predictor regression models. The effect of possible confounding factors was controlled with multivariable analyses conducted in two phases. First, single predictor regression analysis was used to examine the relationships of background variables and cotinine levels or ultrasound measures. The Cochran-Mantel-Haenszel method for non-zero correlation was used for the association of categorical background variables with categorical cotinine levels. All variables showing considerable effect ($P<0.10$) in these primary models were included as covariates in the further analyses of ultrasound measures, conducted with multivariable regression models using a backward selection method with exclusion criteria $P>0.10$. Cotinine levels were entered in these regression models using consecutive values (nondetectable cotinine=0, low cotinine=1, top decile cotinine=2). To assess sensitivity of the results to the coding of the cotinine group variable, we also analyzed the cross-sectional data using geometric cotinine mean values of the groups (nondetectable cotinine=0, low cotinine=0.6, top decile cotinine=2.8) in regression models with similar results. Repeated-measures analysis of variance was used to test whether the magnitude of FMD responses measured between 40 and 180 seconds after cuff release differed between the cotinine groups.

Study III

The Pearson correlation coefficient was used to study the association between ultrasound measures. The associations between serum cotinine levels and ultrasound variables were examined with a single predictor regression model. The effect of possible confounding factors was controlled with multivariable analyses conducted in two phases. First, single predictor regression analysis was used to examine the relationships of background variables and cotinine levels or elasticity indices. The Cochran-Mantel-Haenszel method was used to study the association of categorical background variables with cotinine levels. All variables showing considerable effect ($P<0.10$) in these primary models were included as covariates in the further analyses of ultrasound measures, conducted with multivariable regression models using the backward selection method with exclusion criteria of $P>0.10$. Cotinine levels were entered in these regression models using consecutive values (nondetectable cotinine=0, low cotinine=1, top decile cotinine=2).

Study IV

Serum lipid profiles and ultrasound measures were compared between the longitudinal cotinine groups at the age of 13 years. The associations were first examined with single-predictor regression models. Cotinine levels were entered in these models with consecutive values of the exposure groups (low=0, intermediate=1, high=2). The effect of possible confounding factors was controlled with multivariable analyses conducted in two phases. First, single predictor regression analysis was used to examine the relationships of background variables and cotinine levels or ultrasound measures. The

Cochran-Mantel-Haenszel method for non-zero correlation was used for association of categorical background variables with categorical cotinine levels. All variables showing considerable effect ($P<0.10$) in these primary models were included as covariates in the further analyses, conducted with multivariable regression models using a backward selection method with exclusion criteria $P>0.10$. However, gender was included in every multivariable model. To examine whether the association between cotinine level and IMT was modified by endothelial function, FMD was forced into multivariable models of cIMT and aIMT. Regarding multivariable models of serum lipid values, gender, STRIP study group, pubertal status, saturated fat intake, diastolic blood pressure, and BMI were considered as possible confounding factors.

4.9. Ethics

The study was conducted according to the declaration of Helsinki. The study protocol of the main STRIP study and the ultrasound studies were approved by the Joint Commission on Ethics of the Turku University and Turku University Central Hospital. At the beginning of the STRIP study, all parents gave written informed consent.

5. RESULTS

5.1. Parents' smoking (I)

Interview data

At the beginning of the STRIP trial, 17.0% of the mothers and 33.2% of the fathers smoked (Figure 5). Although a slightly higher proportion of parents in the control group than in the intervention group were smokers at the beginning of the project, the difference was non-significant. According to interviews during STRIP visits, the proportion of smokers among the mothers and fathers declined in both groups during the child's infancy and toddler years. The decline then continued at a lower rate among fathers and stopped among mothers after the child's age of 5 years. When the study children reached the age 13, 10.8% of the mothers and 17.0% of the fathers smoked, with no difference between study groups.

In the longitudinal analysis using smoking at the child's age of 8 months as a covariate, no significant differences were found between STRIP study groups in smoking of the mothers (P=0.070) or fathers (P=0.22).

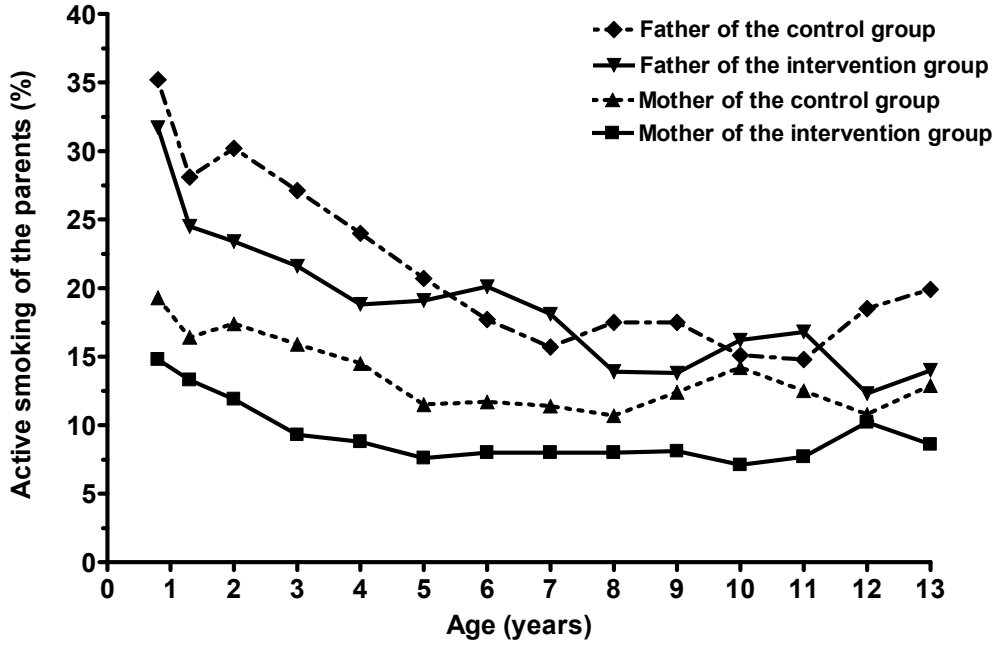

Figure 5. Proportion of actively smoking parents of the STRIP children between child's age of 8 months and 13 years.

Questionnaire data

At the child's age of 8 years, according questionnaires, of the mothers of the intervention and control group, 29 (10.1%) and 45 (15.1%), smoked regularly, respectively. An additional 5.9% (5.7%) of the intervention (control) mothers smoked occasionally. Regarding fathers, 43 (19.7%) of the intervention group and 60 (25.1%) of the control group smoked regularly (Table 3), and 8.3% (6.7%) of the intervention (control) fathers smoked occasionally. The smoking habits of the parents in the intervention and control groups did not differ at the child's age of 8 years. Those who had permanently stopped smoking did so early during the study.

According questionnaires at the child's age of 11 years, 10.5% of the mothers smoked daily and 6.3% smoked occasionally. Of the fathers, 15.1% smoked daily and 10.0% occasionally. The smoking frequencies of intervention and control parents showed no significant difference at the child's age of 11 years.

When the study children reached the age of 13, 10.9% (5.1%) and 16.1% (12.6%) of the mothers and fathers, respectively, smoked daily (occasionally). A significant difference was found between the study groups in mothers (P=0.033), but not in fathers.

Table 3. Parents' smoking habits at child's age of 8 years according to questionnaires. (Study I)

	Intervention mothers		Control mothers		All mothers	
	n	**%**	**n**	**%**	**n**	**%**
> 10 cigarettes daily	9	3.1	13	4.4	**22**	**3.8**
≤10 cigarettes daily	20	7.0	32	10.7	**52**	**8.9**
Occasional smoker	17	5.9	17	5.7	**34**	**5.8**
Ex-smoker for ≤ 6 months	4	1.4	3	1.0	**7**	**1.2**
Ex-smoker for > 6 months	56	19.5	56	18.8	**112**	**19.1**
Non-smoker	181	63.1	177	59.4	**358**	**61.2**
Total	287	100.0	298	100.0	**585**	**100.0**

	Intervention fathers		Control fathers		All fathers	
	n	**%**	**n**	**%**	**n**	**%**
> 10 cigarettes daily	20	9.2	22	9.2	**42**	**9.2**
≤ 10 cigarettes daily	23	10.5	38	15.9	**61**	**13.4**
Occasional smoker	18	8.3	16	6.7	**34**	**7.4**
Ex-smoker for ≤ 6 months	6	2.8	4	1.7	**10**	**2.2**
Ex-smoker for > 6 months	45	20.6	52	21.7	**97**	**21.2**
Non-smoker	106	48.6	107	44.8	**213**	**46.6**
Total	218	100.0	239	100.0	**457**	**100.0**

Cotinine data

In mothers and fathers of 8-year-old children, serum cotinine concentrations ranged from nondetectable to 308 ng/ml and 449 ng/ml, respectively. Serum cotinine values showed that parents reported their smoking habits accurately (Figure 6).

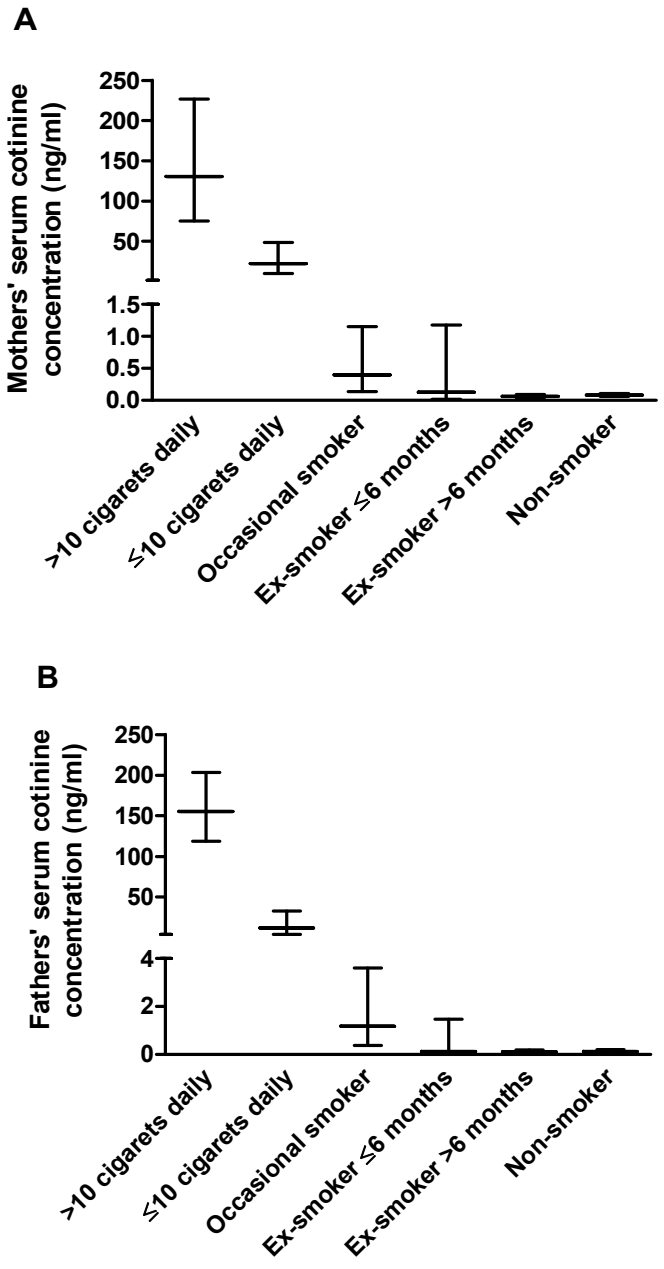

Figure 6. Serum cotinine concentrations of the mothers and fathers of the 8-year-old children according to the smoking habits of the parents. Geometric means (95% CIs) are shown.

5.2. Exposure to tobacco smoke in children and adolescents

5.2.1. Cross-sectional analyses (I)

At the age of 8 years, serum cotinine values of the study children ranged from nondetectable (detection limit 0.16 ng/ml) to 8.2 ng/ml (Figure 7). The geometric mean and median of the serum cotinine values of all children were below the detection limit of the assay (Table 4). The serum cotinine concentration of 289 (46.2%) children was above the detection limit. Their geometric mean serum cotinine concentration was 1.1 ng/ml and median 1.1 ng/ml.

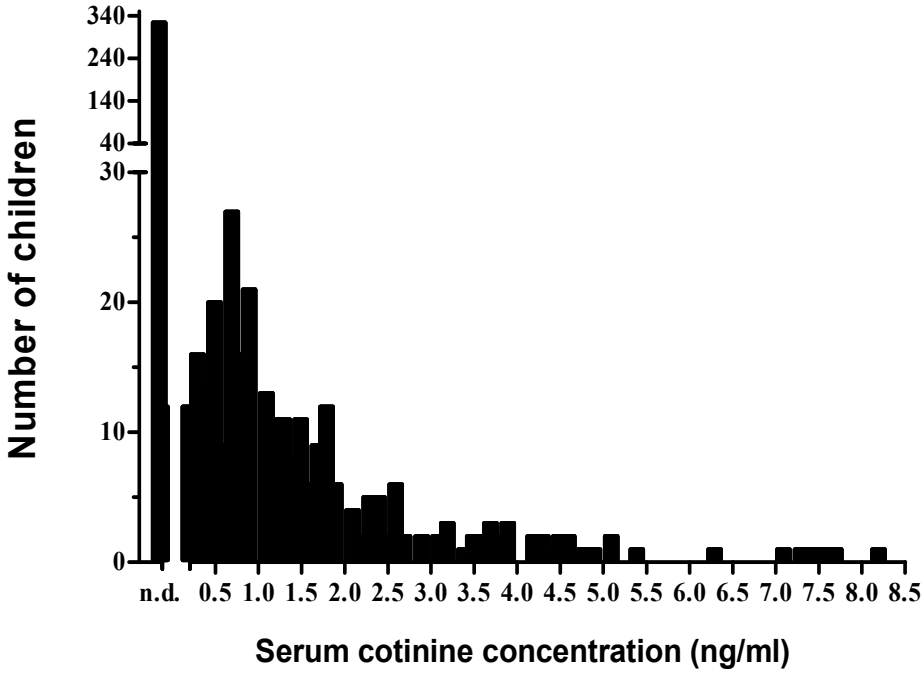

Figure 7. Distribution of serum cotinine concentrations of 8-year-old children (n=625). n.d. indicates nondetectable cotinine values (n=336).

The serum cotinine values of all study children whose cotinine had been analyzed between the ages 8 and 13 years (without suspected active smokers with serum cotinine of ≥15ng/ml) are shown in Table 4. Distribution of serum cotinine concentrations was highly skewed in every age group. About half of the children had nondetectable serum cotinine between the ages 8 and 11 years, and thereafter less than 20% of the children had nondetectable serum cotinine. The geometric mean of the cotinine values was between 0.07 and 0.7 ng/ml in different age groups. The number of children with a serum cotinine value between 8.3-14.9 ng/ml was 3 among 9-year-old children, one among 10- and 12-year-old children, and 2 among 13-year-old adolescents.

Table 4. Serum cotinine values of all STRIP study children between ages 8 and 13 years. (previously unpublished data)

	Age (years)					
	8	**9**	**10**	**11**	**12**	**13**
Number of children	625	561	554	548	539	538
Cotinine mean (SD), ng/ml	0.7 (1.3)	0.7 (1.3)	0.4 (0.8)	0.5 (1.0)	0.8 (0.9)	0.8 (0.9)
Cotinine median, ng/ml	u.d.	0.4	0.1	u.d.	0.6	0.7
Geometric mean (95% CI), ng/ml	0.08 (0.07-0.11)	0.1 (0.10-0.15)	0.08 (0.06-0.09)	0.07 (0.06-0.09)	0.4 (0.32-0.43)	0.7 (0.64-0.72)
Cotinine maximum, ng/ml	8.2	14.0	14.1	6.8	11.4	13.9

u.d.= under detection limit (0.16 ng/ml)

At the age of 11 years, comparison of serum cotinine concentration between children with and without ultrasound data revealed no significant difference (P=0.70). In 11-year-old children with ultrasound data, serum cotinine concentration was above the detection limit in 43% of the children. Cotinine values ranged from 1.7 to 6.8 ng/ml (geometric mean 2.8 ng/ml) in the top decile cotinine group, and from 0.2 to 1.6 ng/ml (geometric mean 0.6 ng/ml) in the low cotinine group.

5.2.2. Longitudinal analyses (II, IV)

Children with ultrasound data at age 11

At the age of 11 years, a subgroup of the children with ultrasound data and all four cotinine measurements between the ages 8 to 11 years (n=327) were divided into three tobacco smoke exposure groups. The geometric means (95% CIs) of the 4 cotinine concentrations measured during follow-up were calculated:

Persistently low cotinine values (n=211): serum cotinine concentration never in the highest decile. Geometric mean was nondetectable.

Remittent cotinine values (n=96): serum cotinine concentration once in the top decile during follow-up. Geometric mean was 0.2 (0.16-0.24) ng/ml.

Repeatedly high cotinine values (n=20): serum cotinine concentration in the highest decile 2-4 times during follow-up. Geometric mean was 0.7 (0.37-0.94) ng/ml.

Children with ultrasound data at age 13

To define longitudinal exposure to tobacco smoke at the adolescents' age of 13 years, serum cotinine values of the children with cotinine values available 2 to 6 times between the ages 8 and 13 years were averaged. Study children were then divided into tertiles according these averaged cotinine values. The majority of the subjects (93%) had cotinine values available 5 or 6 times during follow-up.

The geometric mean (95% CI) of 2 to 6 serum cotinine measurements between the ages 8 and 13 years was 0.28 (0.27-0.29) ng/ml in the low exposure group (n=160), 0.52

(0.51-0.53) ng/ml in the intermediate exposure group (n=171), and 1.05 (1.00-1.11) ng/ml in the high exposure group (n=163). The range of averaged serum cotinine values in the longitudinal cotinine groups are shown in Figure 8. Figure 9 shows geometric mean (95% CI) serum cotinine values at every age point between the ages 8 and 13 years according to the longitudinal cotinine groups.

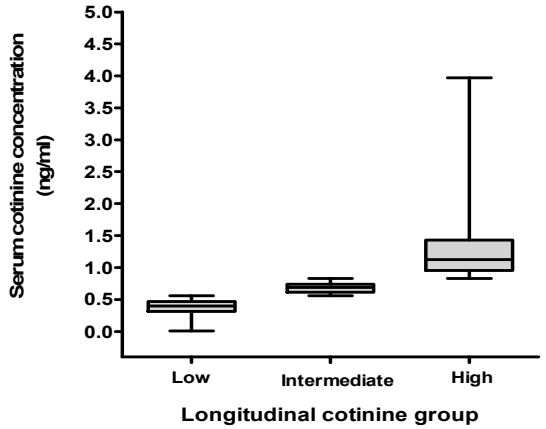

Figure 8. Averaged serum cotinine concentrations between ages 8 and 13 years in three longitudinal tobacco smoke exposure groups. Median (horizontal line inside the box), 25% - 75% percentiles (box), and minimum and maximum values (whiskers) are shown.

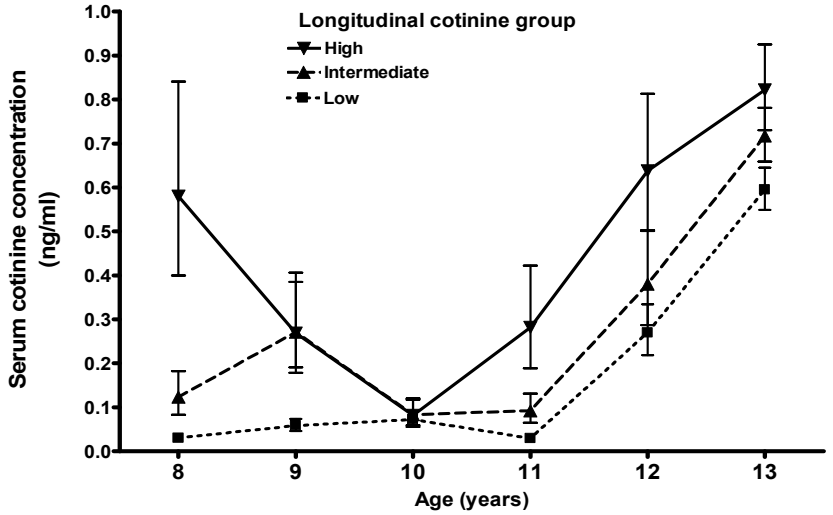

Figure 9. Serum cotinine concentrations at each age point according to longitudinal cotinine groups (mean of the 2 to 6 cotinine values measured between 8 and 13 years and divided into tertiles [exposure groups]: high, intermediate, low). Values are means (95% CIs).

5.2.3. Active smoking of the study children

Using the previously suggested cut-off point for active smoking (serum cotinine concentration ≥15 ng/ml),[216] one 10-year-old boy from the control group with a cotinine value of 100.8 ng/ml and five 13-year-old adolescents with cotinine values between 45.6 and 109.3 ng/ml were defined as active smokers. Of these 13-year-old smokers, 3 were boys and 3 belonged to the control group. None of the STRIP study children had serum cotinine between the values 15.0-45.5 ng/ml.

According to questionnaires, 13% of the 13-year-old adolescents reported that they had tried smoking, but only one 13-year-old adolescent reported active smoking (serum cotinine concentration 96.5 ng/ml). Active smokers were excluded from analyses examining the effects of exposure to environmental tobacco smoke.

5.3. Determinants of tobacco smoke exposure (I-IV)

Gender and STRIP study group

At age 8 years, of the 289 children with detectable serum cotinine 134 (46%) belonged to the intervention group and 155 (54%) to the control group. Serum cotinine concentrations did not differ between the genders (P=0.61) or between the children of the intervention and control groups (P=0.61).

At age 11, the serum cotinine concentrations of the children with ultrasound data showed no difference between the genders (P=0.45) or the intervention and the control groups of the project (P=0.20). No difference was found in the proportion of boys (P for trend=0.38) or the proportion of children belonging to the STRIP intervention group (P for trend=0.19) in three cotinine groups.

Serum cotinine values were similar between genders (P=0.95) and STRIP study groups (P=0.85) also at the age of 13 years in adolescents with ultrasound measurements. The proportion of boys (P for trend=0.46) or the proportion of adolescents belonging to the STRIP intervention group (P for trend=0.085) did not differ in longitudinal tobacco smoke exposure groups.

Parents' smoking habits

At the child's age of 8 years, both parents of 29 (6.9%) children smoked daily. The serum cotinine values of these children were higher (geometric mean 0.4 ng/ml) than those of children from the non-smoking families (geometric mean nondetectable, [P=0.007]), or from families where only the mother smoked (geometric mean nondetectable, [P=0.002]). In addition, the serum cotinine concentrations of the children were higher in the families where only the father smoked than in the families where only the mother smoked (P=0.02) (Figure 10).

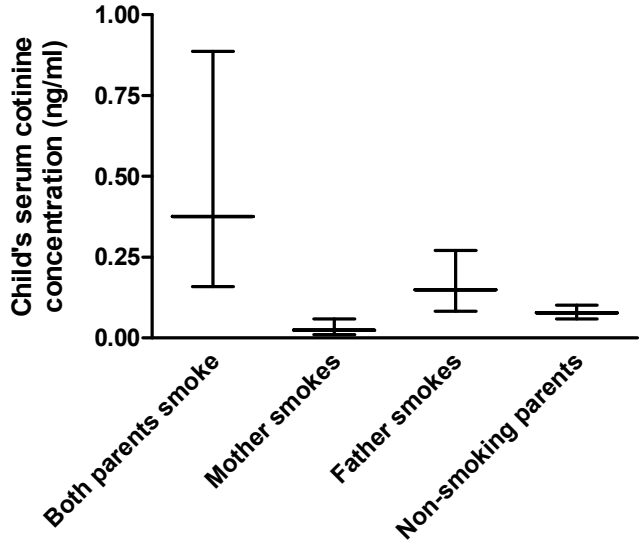

Figure 10. Serum cotinine concentration of 8-year-old children according to parents smoking. Geometric means (95% CIs) are shown. (Study I)

In 13-year-old adolescents belonging to the longitudinal low exposure group, 11.8% of the mothers and 24.8% of the fathers smoked daily or occasionally. The respective smoking frequencies were 17.9% and 29.0% in the longitudinal intermediate exposure group and 20.0% and 30.4% in the high exposure group.

Parents' estimation of children's exposure to tobacco smoke

At the child's age of 8 years, parents of the 39 (8%) children reported that the child was exposed to tobacco smoke during the three days prior to the STRIP visit. These children were equally divided into intervention and control groups. The serum cotinine values of these children with a mean (SD) exposure time of 3.7 (4.8) hours (range from 0.1 to 27) did not differ from those of the other 8-year-old children. Children's serum cotinine values correlated poorly with the reported amount of exposure (r=0.094, P=0.57).

Characteristics at child's age of 11 years

With increasing exposure to tobacco smoke children did not differ in terms of pubertal status, body size, blood pressure, or hsCRP concentration (Table 5).

Table 5. Characteristics of the 11-year-old study children in three cotinine groups.

	Nondetectable serum cotinine concentration (n=229)	Low serum cotinine concentration (n=134)	Top decile serum cotinine concentration (n=39)	P for trend
Pubertal, %*	59	54	59	0.56
Body mass index, kg/m²	17.9±2.8	18.0±2.8	18.1±2.4	0.58
Systolic blood pressure, mmHg	104±8	105±9	103±7	0.97
Diastolic blood pressure, mmHg	63±5	64±6	63±5	0.98
hsCRP, mg/l†	0.35 (0.30-0.42)	0.31 (0.25-0.38)	0.34 (0.23-0.51)	0.55

Values are means±SD, unless otherwise indicated

*Pubertal indicates Tanner stage M/G≥2

†Geometric mean (95% CI), logarithmic transformation used in statistical analyses

hsCRP indicates high-sensitivity C-reactive protein

Characteristics at adolescent's age of 13 years

We did not find any differences between longitudinal tobacco smoke exposure groups regarding pubertal status, body size, systolic blood pressure, hsCRP, energy intake, protein intake, carbohydrate intake, saccharose intake, fat intake, or sodium intake (Table 6). However, diastolic blood pressure differed across the exposure groups (P=0.002), and saturated fat intake tended to increase with increase in cotinine levels.

Table 6. Characteristics of the 13-year-old adolescents in longitudinal tobacco smoke exposure groups.

	Longitudinal exposure to tobacco smoke*			
	Low	**Intermediate**	**High**	**P for trend**
Number of subject	160	171	163	-
Late pubertal, %†	81	74	73	0.10
Body mass index, kg/m²	19.3±3.1	19.4±3.3	19.0±2.9	0.40
Systolic blood pressure, mmHg	107±8	107±8	107±9	0.69
Diastolic blood pressure, mmHg	61±4	61±5	63±5	0.002
hsCRP, mg/l‡	0.31 (0.27-0.37)	0.31 (0.26-0.36)	0.31 (0.26-0.35)	0.84
Energy intake, kcal	1936±443	1842±445	1873±471	0.23
Protein intake, E%	16.8±2.8	16.7±3.0	16.8±2.8	0.76
Carbohydrate intake, E%	52.1±5.4	51.9±5.6	51.5±5.7	0.38
Saccharose intake, E%	9.0±3.1	9.0±3.5	9.6±3.7	0.12
Fat Intake, E%	31.0±5.2	31.4±5.1	31.7±5.3	0.22
Saturated fat intake, E%	12.1±2.9	12.3±2.9	12.7±2.9	0.054
(P+M)/S	1.42±0.38	1.41±0.38	1.34±0.35	0.079
Sodium intake/1000 kcal, mg	1490±281	1522±330	1497±264	0.83

Values are means±SD, unless otherwise indicated

*Serum cotinine measurements (2-6) between the ages 8 and 13 years averaged and divided into tertiles

†Proportion of children in late puberty (Tanner stage M/G≥3)

‡Geometric mean (95% CI), logarithmic transformation used in statistical analyses

hsCRP indicates high-sensitivity C-reactive protein

(P+M)/S=(polyunsaturated+monounsaturated)/saturated fat intake

5.4. Exposure to tobacco smoke and serum lipid profile

In 11-year-old children (II)

The lipid profile of 11-year-old children in three serum cotinine groups is shown in Table 7. No differences across the cotinine groups were found in any of the lipid variables measured.

Table 7. Lipid profile of the 11-year-old study children in three cotinine groups.

	Nondetectable serum cotinine concentration	Low serum cotinine concentration	Top decile serum cotinine concentration	P for trend
Number of subjects	229	134	39	-
Proportion of boys, %	53	57	38	0.38
In STRIP intervention group, %	51	44	44	0.19
Total cholesterol, mmol/l	4.44±0.74	4.54±0.76	4.61±0.64	0.10
HDL cholesterol, mmol/l	1.30±0.27	1.34±0.29	1.25±0.21	0.95
LDL cholesterol, mmol/l	2.77±0.63	2.85±0.67	2.87±0.67	0.21
Triglycerides, mmol/l*	0.77 (0.73-0.82)	0.73 (0.67-0.78)	0.80 (0.71-0.91)	0.79
ApoB, g/l	0.82±0.18	0.84±0.19	0.85±0.18	0.18
ApoA-I, g/l	1.39±0.21	1.41±0.22	1.38±0.21	0.76
ApoB/ApoA-I	0.60±0.16	0.60±0.15	0.63±0.16	0.41
Lp(a), mg/l*	128.0 (115.7-140.2)	112.6 (99.2-127.8)	114.0 (92.4-140.8)	0.14

Values are means±SD, unless otherwise indicated

* Geometric mean (95% CI), logarithmic transformation used in statistical analyses

In 13-year-old adolescents (IV)

At the age of 13 years, ApoB values (P=0.004), ApoB/ApoA-I ratio (P=0.013) and triglycerides (P=0.037) showed an increasing trend with an increase in longitudinal cotinine level (Table 8).

In the single predictor models, BMI associated with ApoB (ß=0.008, P=0.001), ApoB/ApoA-I ratio (ß=0.012, P<0.001), and triglycerides (ß=0.042, P<0.001).

In the multivariable regression model of lipid variables, longitudinal tobacco smoke exposure remained significant in the final models of ApoB (ß=0.029, P=0.005), ApoB/ApoA-I (ß=0.026, P=0.004), and triglycerides (ß=0.055, P=0.019).

Table 8. Lipid profile of the 13-year-old adolescents in three longitudinal tobacco smoke exposure groups.

	Longitudinal exposure to tobacco smoke*			
	Low	**Intermediate**	**High**	**P for trend**
Number of subject	160	171	163	-
Boys, %	51	59	47	0.47
In STRIP intervention group, %	56	49	47	0.085
Total cholesterol, mmol/l	4.16±0.73	4.22±0.80	4.24±0.65	0.35
HDL cholesterol, mmol/l	1.21±0.24	1.19±0.25	1.21±0.22	0.93
LDL cholesterol, mmol/l	2.60±0.63	2.64±0.69	2.64±0.56	0.52
Triglycerides, mmol/l†	0.73 (0.68-0.78)	0.77 (0.72-0.82)	0.80 (0.76-0.85)	0.037
ApoB, g/l	0.73±0.17	0.77±0.19	0.79±0.16	0.004
ApoA-I, g/l	1.34±0.20	1.35±0.21	1.34±0.17	0.78
ApoB/ApoA-I	0.56±0.15	0.58±0.17	0.60±0.13	0.013
Lp(a), mg/l†	134.8(118.4-153.4)	113.9(101.7-127.5)	118.0(103.8-134.1)	0.14

Values are means±SD, unless otherwise indicated

*Groups are formed by dividing averaged (between ages 8 and 13 years) serum cotinine values into tertiles

†Geometric mean (95% CI), logarithmic transformation used in statistical analyses

5.5. Exposure to tobacco smoke and markers of subclinical atherosclerosis

5.5.1. Arterial reactivity (II, IV)

In 11-year-old children

Regarding baseline ultrasound measures, brachial baseline diameter (P=0.11 for trend) and increase in blood flow after cuff release (P=0.52 for trend) were similar across the cotinine groups.

The peak FMD showed a significant trend across the three cotinine level groups (FMD, mean[SD]: the noncotinine group, 9.10[3.88]%; the low cotinine group, 8.57[3.78]%; and the top decile cotinine group, 7.73[3.85]%; P=0.03 for trend) (Figure 11A). Similarly, AUC was affected by the cotinine level (AUC, mean[SD]: the noncotinine group, 759[491]%·sec; the low cotinine group, 695[488]%·sec; and the top decile cotinine group, 566[434]%·sec; P=0.02 for trend) (Figure 11B). In contrast, NMD was similar across the cotinine groups (NMD, mean[SD]: the noncotinine group, 11.95[4.69]%; the low cotinine group, 12.55[4.89]%; and the top decile cotinine group, 10.55[4.41]%; P=0.57 for trend).

The temporal development of the FMD responses measured between 40 and 180 seconds after cuff release was similar across the cotinine groups, but the magnitude of the response was marginally reduced in children with higher cotinine values (effect of group, P=0.054).

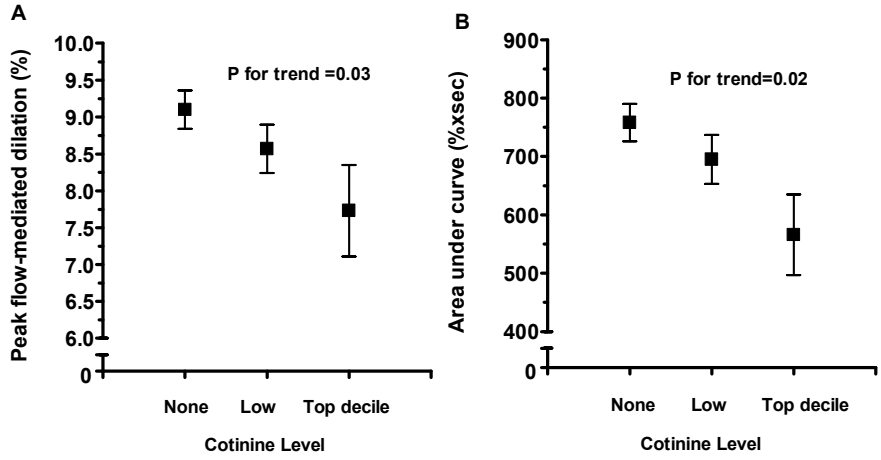

Figure 11. Peak flow-mediated dilation of the brachial artery (A) and area under the dilation response vs. time curve (B) in 11-year-old children according to cotinine group. Values are mean ± SEM. (Study II)

In 11-year-old children with longitudinal cotinine values

As in the cross-sectional analysis in 11-year-old children, FMD and AUC were attenuated in children with several high cotinine concentrations during follow-up (FMD: persistently low cotinine values, 9.14[4.00]%; remittent cotinine values, 8.33[3.61]%; repeatedly high cotinine values, 7.14[3.20]%; P=0.01 for trend), (AUC: persistently low cotinine values, 759[495]%·sec; remittent cotinine values, 638[476]%·sec; repeatedly high cotinine values, 530[379]%·sec; P=0.008 for trend). NMD was similar across the longitudinal cotinine groups (P=0.29 for trend).

The temporal development of the FMD responses measured between 40 and 180 seconds after cuff release differed in cotinine groups (time x group interaction, P<0.001), and the magnitude of the responses was reduced in children with repeatedly high cotinine values (effect of group, P=0.02) (Figure 12).

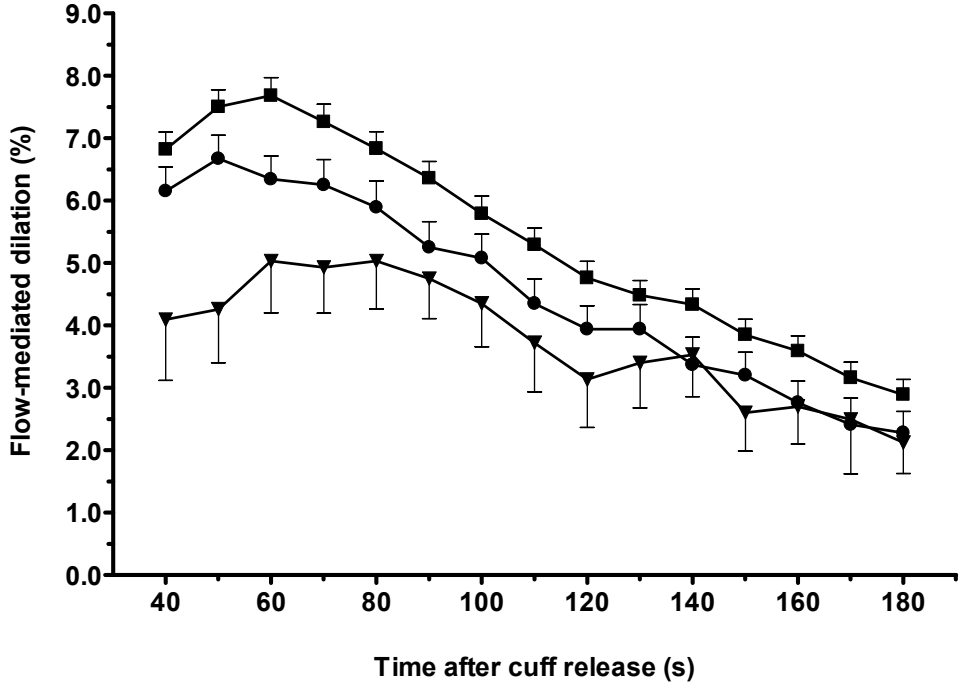

Figure 12. Temporal development of brachial artery flow-mediated dilation responses (mean±SEM) in 11-year-old children with longitudinal cotinine values between the ages 8 to 11 years. The group of children with repeatedly high cotinine values (cotinine concentration in the highest decile at least two times, n=20) is marked with triangles (▼), children with remittent cotinine values (cotinine concentration once in the highest decile, n=96) are marked with circles (●), and children with persistently low cotinine values (cotinine concentration never in the highest decile, n=211) are marked with squares (■). The temporal development of FMD responses differed in cotinine groups (time x group interaction, P<0.001), and the magnitude of the response was reduced in children with repeatedly high cotinine values (effect of group, P=0.02). (Study II)

In the single predictor models, LDL cholesterol was inversely associated with AUC (ß=-74.1, P=0.048), but no other significant associations between the measures of endothelial function and the established risk factors were found (Table 9).

Regarding 11-year-old children, in the multivariable regression model, controlling for LDL cholesterol had no effect on the association between increase in cotinine concentration and impaired FMD (adjusted P=0.03 for trend) or decreased AUC (adjusted P=0.03 for trend). In the multivariable regression model concerning 11-year-old children with longitudinal cotinine values, the association between decreased FMD and decreased AUC and several high cotinine values was also unchanged after adjustment for LDL cholesterol (FMD: adjusted P=0.01 for trend; AUC: adjusted P=0.007 for trend).

Table 9. **Single predictor regression analysis for determinants of peak FMD and total dilation response (AUC). (Study II)**

		FMD		AUC	
Explanatory variable	**n**	**Regression Coefficient ß**	**P**	**Regression Coefficient ß**	**P**
Male sex	402	0.236	0.54	68.5	0.16
STRIP control group	402	-0.502	0.19	-18.5	0.70
Pubertal	402	0.064	0.87	14.3	0.77
Body mass index, kg/m²	402	0.076	0.28	8.2	0.35
Systolic blood pressure, mmHg	392	0.013	0.60	2.3	0.44
Diastolic blood pressure, mmHg	392	0.025	0.50	2.2	0.64
Total cholesterol, mmol/l	400	0.054	0.84	8.8	0.79
LDL cholesterol, mmol/l	400	-0.447	0.13	-74.1	0.048
HDL cholesterol, mmol/l	400	-0.578	0.42	-74.9	0.41
Triglycerides, mmol/l*	400	0.617	0.16	61.0	0.27
hsCRP, mg/l*	402	0.061	0.68	-0.3	0.99

*Logarithmic transformation

hsCRP indicates high-sensitivity C-reactive protein

In 13-year-old adolescents with longitudinal cotinine values

Endothelial function of the 13-year-old adolescents differed significantly across the three longitudinal tobacco smoke exposure groups (FMD: low exposure group 10.43 [4.34]%; intermediate exposure group 9.78 [4.38]%; and high exposure group 8.82 [4.14]%; P for trend 0.001), (AUC low exposure group, 779[519]%·sec; intermediate exposure group, 741[498]%·sec; and high exposure group, 648[486]%·sec; P=0.021 for trend) (Figure 13).

Regarding 13-year-old adolescents, FMD was not associated with gender, STRIP study group, pubertal status, BMI, lipid profile, blood pressure, hsCRP, or saturated fat intake. The association between longitudinal exposure to tobacco smoke and peak FMD (ß=-0.821, P=0.002) and AUC (ß=-72.7, P=0.018) remained significant after controlling for other risk factors.

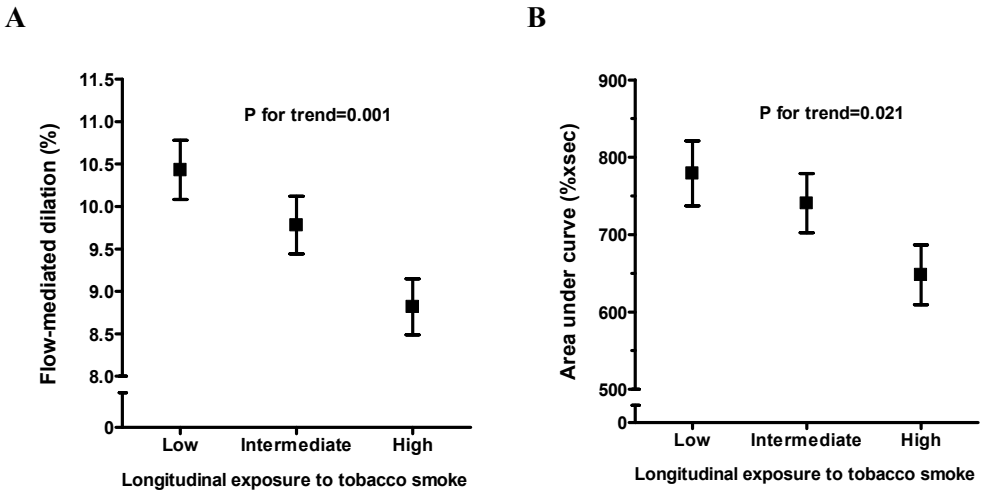

Figure 13. Peak flow-mediated dilation of brachial artery (A) and area under the dilation response vs. time curve (B) in healthy 13-year-old adolescents according to longitudinal tobacco smoke exposure levels. Values are mean±SEM. (Study IV)

5.5.2. Arterial elasticity (III)

In 11-year-old children

In 11-year-old children with arterial elasticity measurements, aortic diastolic diameter was similar across the cotinine groups (P=0.78); however, carotid diastolic diameter increased with increase in cotinine level (P=0.022).

An increase in aSI was observed across the cotinine levels (the noncotinine group, 2.31[0.67]; the low-cotinine group, 2.42[0.78]; and the top decile cotinine group, 2.78[1.35]; P=0.006 for trend; Figure 14A). aYEM also tended to be related to the cotinine level (the noncotinine group, 98[32] mmHg·mm; the low-cotinine group, 102[35] mmHg·mm; and the top decile cotinine group, 111[54] mmHg·mm; P=0.066 for trend; Figure 14B). aD showed a significant inverse trend across the three cotinine groups (the noncotinine group, 5.74[1.69] %/10 mmHg; the low-cotinine group, 5.54[1.79] %/10 mmHg; and the top decile cotinine group, 5.12[1.90] %/10 mmHg; P=0.041 for trend; Figure 14C). In contrast, carotid elasticity indices showed no statistical difference across the cotinine levels (cSI, P=0.10; cYEM, P=0.82; cD, P=0.14) (Figure 15).

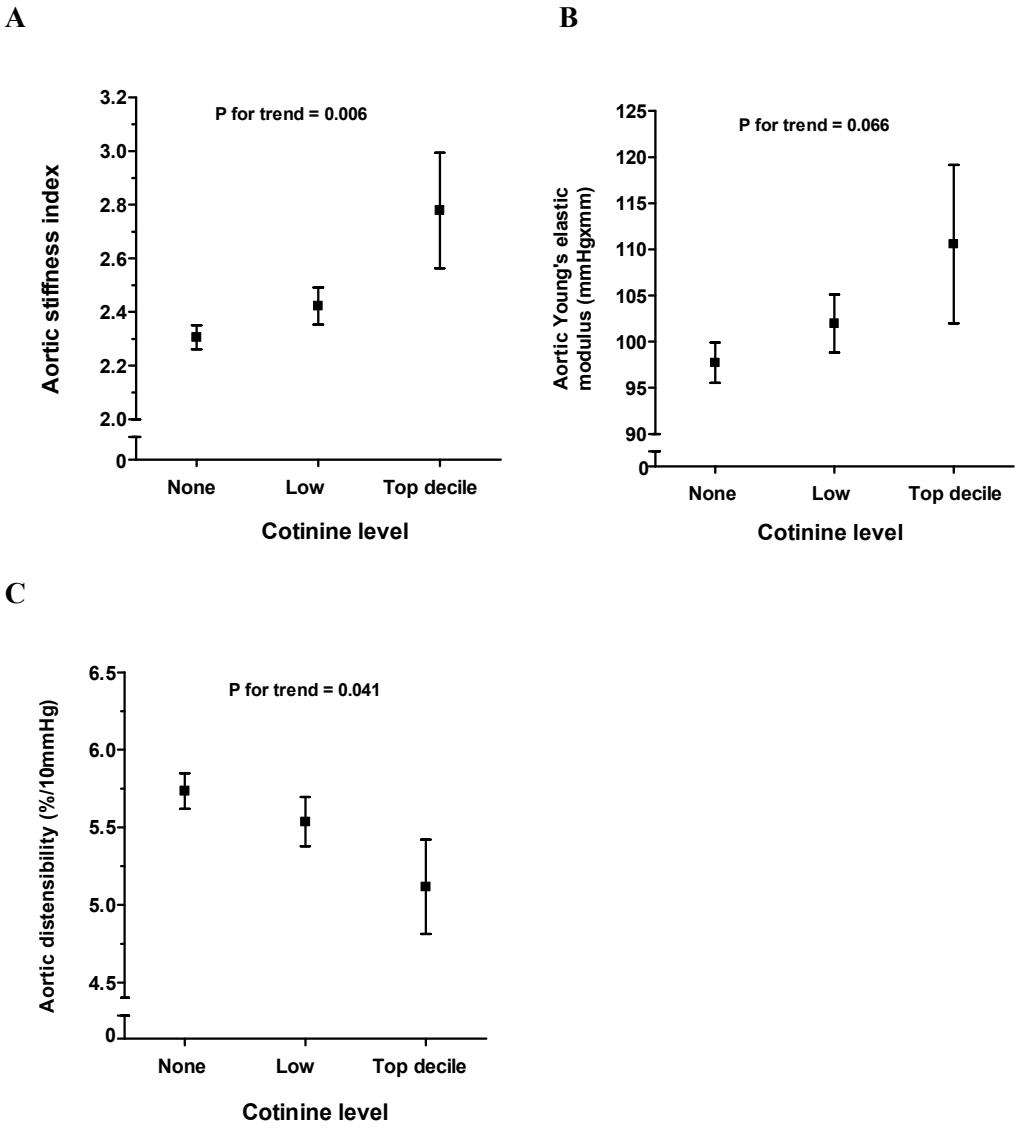

Figure 14. Aortic stiffness index (A), Young's elastic modulus (B), and distensibility (C) in 11-year-old children according to serum cotinine level group. Values are means±SEM. (Study III)

All aortic elasticity indices were associated with BMI and systolic and diastolic blood pressure (Table 10). In the multivariable regression models, the cotinine level was one significant explanatory variable related to all aortic elasticity indices (Table 11). When FMD was also forced into the multivariable model, the associations between cotinine group and aortic elasticity indices remained significant (aSI:ß=0.063, P=0.005; aD:ß=–0.282, P=0.020), except for aYEM (ß=0.042, P=0.071).

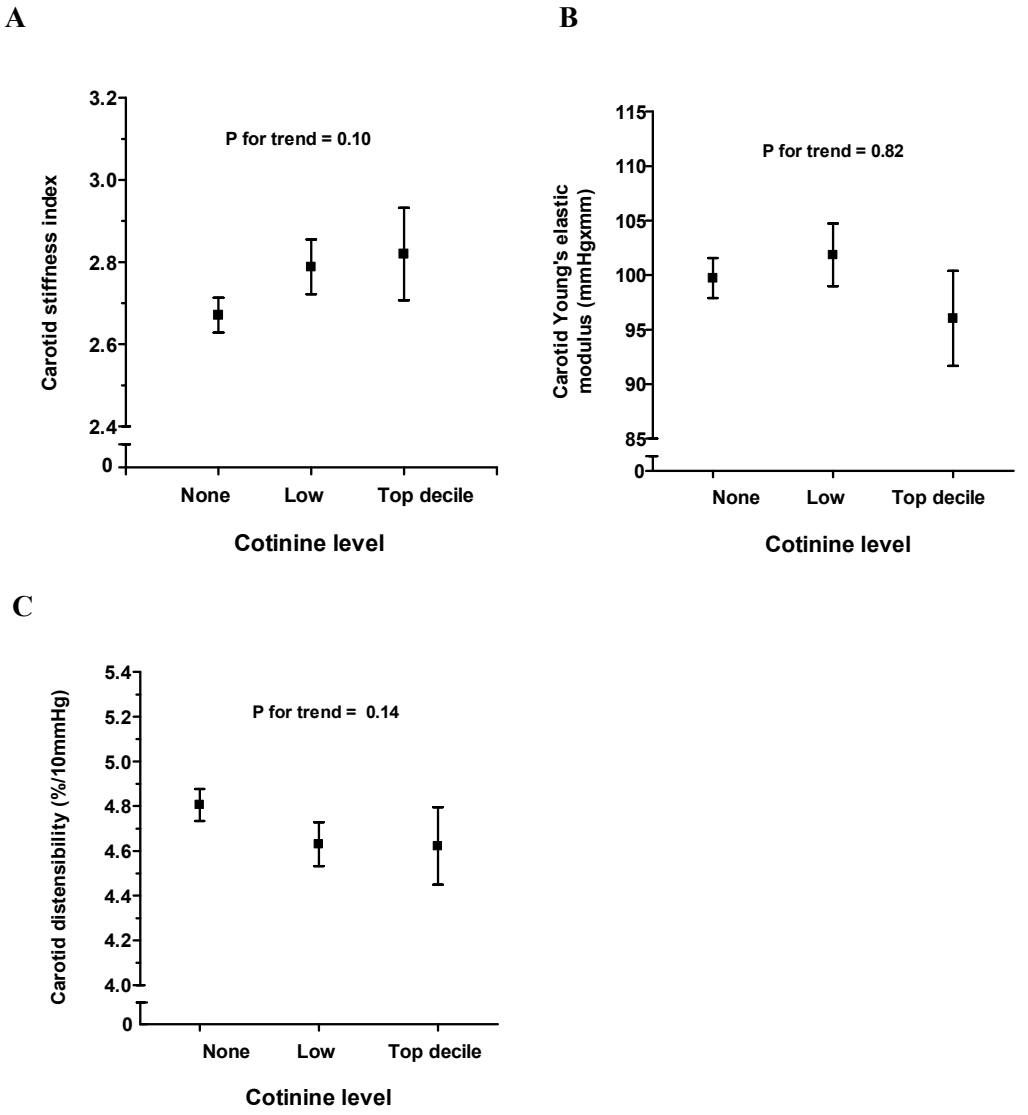

Figure 15. Carotid stiffness index (A), Young's elastic modulus (B), and distensibility (C) in 11-year-old children according to serum cotinine level group. Values are means±SEM.

Independent predictors of cSI were diastolic blood pressure, hsCRP, and male sex (Table 12). Both cYEM and cD were associated with systolic blood pressure and male sex. In the multivariable regression analyses of carotid elasticity indices, cotinine level remained non-significant.

Table 10. Single predictor regression analysis for the determinants of aortic elasticity indices in 11-year-old children. (Study III)

	Aortic stiffness index*		Aortic Young's elastic modulus*		Aortic distensibility	
Explanatory variable	Regression Coefficient ß±SE	P	Regression Coefficient ß±SE	P	Regression Coefficient ß±SE	P
Male sex†	0.030±0.031	0.33	0.020±0.034	0.57	-0.204±0.179	0.26
STRIP control group†	0.014±0.031	0.64	-0.005±0.034	0.90	-0.156±0.178	0.38
Pubertal†	0.021±0.033	0.53	0.060±0.037	0.10	-0.221±0.192	0.25
Body mass index, kg/m²	0.013±0.006	0.023	0.025±0.006	<0.001	-0.089±0.032	0.006
Systolic blood pressure, mmHg	0.007±0.002	<0.001	0.017±0.002	<0.001	-0.089±0.010	<0.001
Diastolic blood pressure, mmHg	-0.006±0.003	0.037	0.007±0.003	0.027	-0.037±0.017	0.025
Total cholesterol, mmol/l	0.011±0.021	0.60	0.023±0.023	0.32	-0.163±0.122	0.18
LDL cholesterol, mmol/l	0.012±0.024	0.61	0.031±0.026	0.25	-0.169±0.138	0.22
HDL cholesterol, mmol/l	0.042±0.057	0.46	0.023±0.064	0.72	-0.298±0.331	0.37
Triglycerides, mmol/l*	-0.029±0.035	0.41	-0.017±0.039	0.67	0.070±0.203	0.73
hsCRP, mg/l*‡	0.016±0.012	0.18	0.021±0.014	0.12	-0.072±0.070	0.30

*Logarithmic transformation

†Regression coefficient indicates difference between group means

‡hsCRP indicates high-sensitivity C-reactive protein

Table 11. Multivariable models of the associations between risk factor variables and aortic elasticity indices. (Study III)

	Aortic stiffness index*		Aortic Young's elastic modulus*		Aortic distensibility	
	Regression Coefficient ß±SE	P	Regression Coefficient ß±SE	P	Regression Coefficient ß±SE	P
Cotinine level group	0.063±0.022	0.005	0.046±0.023	0.044	-0.282±0.120	0.020
Systolic blood pressure, mmHg	0.007±0.002	<0.001	0.016±0.002	<0.001	-0.089±0.010	<0.001
Body mass index, kg/m²†			0.016±0.006	0.007		

*Logarithmic transformation

†Body mass index excluded from final analysis of aortic stiffness index and aortic distensibility with P>0.1

Table 12. Single predictor regression analysis for the determinants of carotid elasticity indices in 11-year-old children.

	Carotid stiffness index*		Carotid Young's elastic modulus*		Carotid distensibility	
Explanatory variable	Regression Coefficient ß±SE	P	Regression Coefficient ß±SE	P	Regression Coefficient ß±SE	P
Male sex†	0.068±0.023	0.004	0.117±0.027	<0.001	-0.316±0.109	0.004
STRIP control group†	0.013±0.024	0.59	0.018±0.028	0.51	-0.103±0.110	0.35
Pubertal†	-0.013±0.025	0.59	0.008±0.030	0.79	-0.003±0.117	0.98
Body mass index, kg/m²	-0.005±0.004	0.30	0.001±0.005	0.81	-0.008±0.020	0.70
Systolic blood pressure, mmHg	0.002±0.001	0.23	0.011±0.002	<0.001	-0.046±0.006	<0.001
Diastolic blood pressure, mmHg	-0.012±0.002	<0.001	0.001±0.003	0.56	-0.003±0.010	0.80
Total cholesterol, mmol/l	-0.011±0.016	0.48	0.008±0.019	0.67	-0.018±0.075	0.81
LDL cholesterol, mmol/l	-0.003±0.018	0.88	0.012±0.022	0.59	-0.054±0.085	0.53
HDL cholesterol, mmol/l	-0.041±0.044	0.35	0.017±0.053	0.76	0.103±0.204	0.62
Triglycerides, mmol/l*	-0.027±0.027	0.32	-0.038±0.033	0.24	0.067±0.125	0.59
hsCRP, mg/l*‡	-0.021±0.009	0.03	-0.018±0.011	0.11	0.074±0.043	0.09

*Logarithmic transformation

†Regression coefficient indicates difference between group means

‡hsCRP indicates high-sensitivity C-reactive protein

5.5.3. Intima-media thickness (IV)

In 13-year-old adolescents

Regarding 13-year-old adolescents, maximum cIMT increased with increase in longitudinal cotinine level (maximum cIMT: low exposure group 0.502 [0.079]; intermediate exposure group 0.525 [0.070]; and high exposure group 0.535 [0.066] mm; P for trend <0.001) (Figure 16). The relationship was similar when mean cIMTs were used instead of maximum values (P for trend <0.001) (Figure 16).

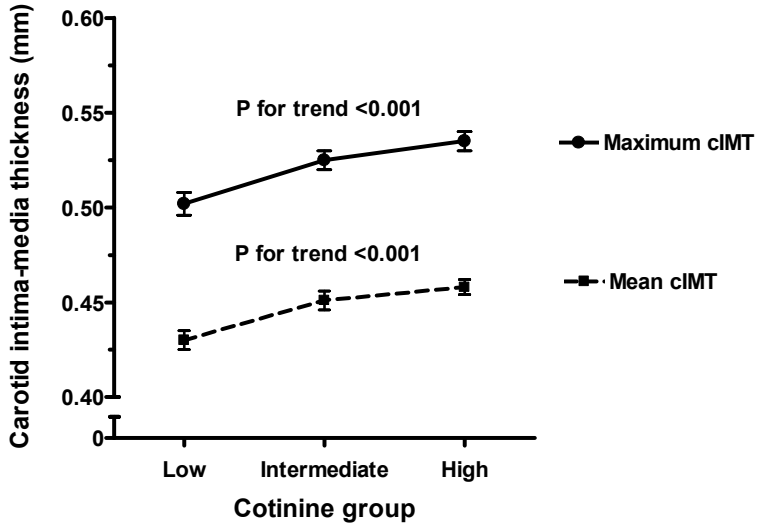

Figure 16. Maximum cIMT (black circles) and mean cIMT (black squares) in healthy 13-year-old adolescents according to longitudinal tobacco smoke exposure levels. Values are means±SEM. (Study IV)

Similarly, maximum aIMT increased across the tobacco smoke exposure groups (maximum aIMT: low exposure group 0.527 [0.113]; intermediate exposure group 0.563 [0.139]; and high exposure group 0.567 [0.126] mm; P for trend 0.004) (Figure 17). Mean aIMT also increased with increase in longitudinal cotinine level (P for trend 0.025) (Figure 17).

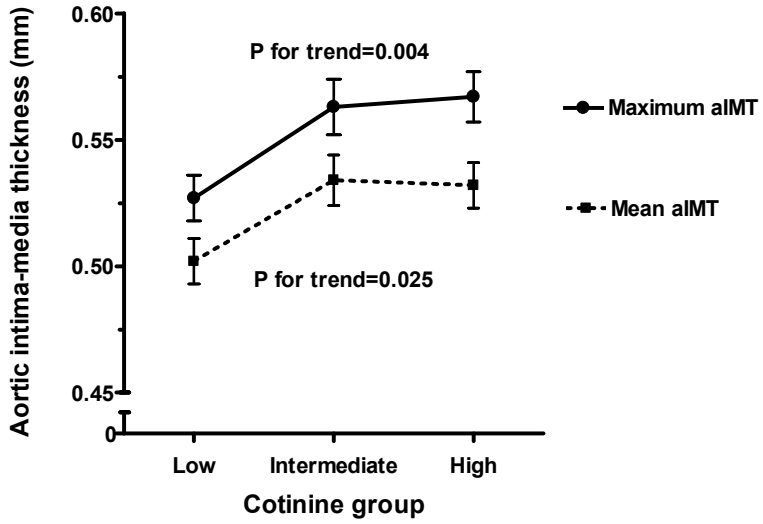

Figure 17. Maximum aIMT (black circles) and mean aIMT (black squares) in healthy 13-year-old adolescents according to longitudinal tobacco smoke exposure levels. Values are means±SEM. (Study IV)

The maximum cIMT was associated with systolic and diastolic blood pressure, total and LDL cholesterol, ApoB, ApoA-I, and gender (greater in boys) (Table 13). The maximum aIMT significantly associated with BMI, diastolic blood pressure, triglycerides, ApoB, ApoB/ApoA-I ratio, and hsCRP.

In the multivariable regression analysis of maximum cIMT, after adjusting for all related covariates, including ApoB and FMD, the cotinine level remained as a significant predictor (regression coefficient ß=0.014, P=0.001) (Table 14). Similarly, regarding maximum aIMT, the final model included the effect of the tobacco smoke exposure group (ß=0.020, P=0.007). Table 14 also shows that regression coefficients in single predictor models and in multivariable models were almost identical, suggesting that the effect of tobacco smoke exposure on vascular changes was not mediated through covariates.

Table 13. Single predictor regression analysis for the determinants of maximum cIMT and aIMT in 13-year-old adolescents. (Study IV)

	Maximum cIMT‡		Maximum aIMT‡	
Explanatory variable	**Regression Coefficient ß±SE**	**P**	**Regression Coefficient ß±SE**	**P**
Male sex*	0.030±0.006	<0.001	0.004±0.012	0.70
STRIP control group*	-0.010±0.007	0.12	-0.002±0.012	0.85
Late Pubertal*	0.003±0.008	0.73	0.013±0.014	0.34
Body mass index, kg/m²	0.001±0.001	0.33	0.016±0.002	<0.001
Total cholesterol, mmol/l	-0.017±0.004	<0.001	-0.002±0.008	0.79
HDL cholesterol, mmol/l	-0.019±0.014	0.17	-0.021±0.025	0.40
LDL cholesterol, mmol/l	-0.019±0.005	<0.001	-0.008±0.009	0.40
Triglycerides, mmol/l†	-0.012±0.008	0.13	0.042±0.014	0.003
ApoB, g/l	-0.032±0.018	0.086	0.058±0.033	0.081
ApoA-I, g/l	-0.053±0.017	0.002	-0.021±0.030	0.48
ApoB/ApoA-I	0.002±0.022	0.93	0.079±0.039	0.044
hsCRP, mg/l†	0.005±0.003	0.12	0.024±0.006	<0.001
Saturated fat intake, E%	-0.0001±0.001	0.90	0.002±0.002	0.45
Systolic blood pressure, mmHg	0.002±0.0004	<0.001	0.001±0.001	0.12
Diastolic blood pressure, mmHg	0.003±0.001	<0.001	0.002±0.001	0.041

*Regression coefficient indicates difference between group means

†Logarithmic transformation

‡All relations were essentially similar when mean IMTs were used instead of maximum IMTs

hsCRP indicates high-sensitivity C-reactive protein

Table 14. Association between tobacco smoke exposure and maximum cIMT, and maximum aIMT in single predictor regression analysis and multivariable regression analysis. (Study IV)

	Longitudinal exposure to tobacco smoke			
	Single predictor model		Multivariable model*	
	ß±SE	P	ß±SE	P
Maximum cIMT, mm	0.016±0.004	<0.001	0.014±0.004	0.001
Maximum aIMT, mm	0.020±0.007	0.004	0.020±0.007	0.007

*Both models include gender, STRIP study group, pubertal status, saturated fat intake, diastolic blood pressure, and triglycerides as covariates. The cIMT analysis also includes ApoB, ApoA-I, LDL cholesterol, and FMD. The aIMT analysis also includes ApoB/ApoA-I, BMI, hsCRP, and FMD.

6. DISCUSSION

6.1. Study design and subjects

The study subjects were participants of an ongoing, longitudinal atherosclerosis prevention trial, the aim of which is to influence the atherosclerosis risk factor levels of the study children by individualized dietary and lifestyle intervention. The study families have regularly visited the STRIP team since the child's age of 7 months. In a longitudinal study, drop-out of the study subjects is inevitable. According to dropout analyses made on the project,[272,316] 20% of the families left the study before the 3-year-visit, and 48% discontinued participation at the adolescent's age of 13 years. The main reasons for withdrawal from the project have been changes in the family situation and moving to another area. In addition, the child's fear of blood sampling, the excess effort experienced by the family and school attendance have produced withdrawals. However, the proportion of children who have dropped out the project has been similar among the intervention and control children, as well as among boys and girls. There has been no difference between dropouts and children still participating in the project regarding fat intake, cholesterol concentrations, and growth.

The strengths of the present thesis include its prospective design in determining exposure to tobacco smoke, and using an objective biomarker, serum cotinine concentration, to measure tobacco smoke exposure, as well as the large sample size of children participating in ultrasound studies. Furthermore, healthy subjects of the same age were examined. We excluded children who apparently smoked actively, as the main purpose of our study was to examine exposure to tobacco smoke and its effects on subclinical atherosclerosis development. In addition, children who had a chronic disease that might affect the outcome measures were excluded. However, one possible confounding factor among our study children was ongoing puberty. Pubertal maturation seemed to have a potent and complex confounding effect on the associations between serum lipids and carotid IMT, especially in study IV including 13-year-old adolescents. However, pubertal status was carefully recorded during annual STRIP visits, and it was included in multivariable models whenever warranted.

Due to the fact that we studied healthy children whose overall tobacco smoke exposure levels were only moderate, and the distribution of the serum cotinine concentrations was thus highly skewed, and because the functional sensitivity of the chromatographic method (inter-assay CV <20%) was between 1 and 2 ng/ml, we decided to classify the children according to their cotinine values in exposure groups, which might represent the different exposure levels in this population. We acknowledge that categories of exposure are somewhat arbitrary, and for example, the top decile percentile was not based on any reported cotinine level found dangerous for children's health, but was merely based on the purpose of studying possible differences in extremities of exposure. The cut-off level was decided to be at the 90th percentile because of the suitable number of children still belonging to that group, and because of the level of the functional sensitivity of the chromatographic method. In addition, as half of the children had nondetectable cotinine levels, we could not use the cotinine values as continuous variables in the linear models, but were forced to find a different modeling

technique for the amount of exposure. For the purposes of study II, we also conducted various classifications regarding cotinine level groups. When the group of children with uppermost cotinine values was formed of the 95th percentile, of the 85th percentile, or of the 80th percentile, the associations of tobacco smoke exposure and endothelial function were parallel to the present results with the finding that increased exposure to tobacco smoke decreased endothelial function.

In study IV, we were unable to use similar top decile categories as in studies II and III, because there were only a small number of adolescents with repeatedly high serum cotinine values during the whole follow-up. Thus, we arrived at using averaged cotinine values and formed tertile groups as different tobacco smoke exposure groups. This has produced inconsistency in the categories of exposure to tobacco smoke in studies II-IV, but, possibly, the latest classification (study IV) may provide an even better index for longitudinal passive smoking.

Some children did not volunteer to participate in the ultrasound study at the ages 11 and 13 years. The main reasons given for non-participation were the child's unwillingness to participate, lack of time, fear of the study, and transportation problems. However, serum cotinine concentrations did not differ in 11-year-old children with and without ultrasound mesurements. In addition, we previously showed that those children who had complete ultrasound data did not differ significantly from the other children at baseline of the STRIP project or at the 11-year study with respect to anthropometry, serum lipoproteins, or blood pressure values.[272] Furthermore, participation rates in ultrasound studies were similar in the intervention and control groups.

6.2. Methods and results

6.2.1. Exposure to tobacco smoke

Methodological aspects

As the main purposes of the present study were to gain an insight into the levels of exposure to tobacco smoke among children and adolescents, and analyze the impact of exposure on atherosclerosis risk factors and subclinical atherosclerosis development, the use of a biomarker was important. Cotinine has proved to be valid biomarker in determining passive exposure to ETS,[13] and thus was the biomarker of choice. Interindividual variability exists in all biological measurements, but large numbers of subjects may compensate this variability. Indeed, the use of cotinine as a quantitative marker of smoke exposure is supported by studies showing an association between the biological effects of ETS and cotinine levels.[317,318] Many of the deleterious effects of tobacco smoke on the cardiovascular system, including the endothelium, are caused by other components than nicotine.[5,319] However, intake of nicotine reflects exposure to other constituents of environmental tobacco smoke reasonably well.[13]

Although drawing blood is an invasive action, measurement of cotinine from serum was possible in the STRIP study because the study protocol already included annual blood samples. Serum samples of 8-year-old children were non-fasting and drawn in

the afternoon, whereas serum samples of 9- to 13-year-old children were taken after an overnight fast. This may have influenced on the serum cotinine levels especially at the age of 8 years. The gas chromatographic method with a nitrogen-sensitive detector was used to determine serum cotinine concentrations. The analytical sensitivity of our gas chromatographic method (0.16 ng/ml) was comparable to previous studies.[219] The method has been shown to be feasible, and a more sensitive and specific analytical method[219] than, for example, methods based on radioimmunoassay (sensitivity 1 ng/ml).[221,245] However, more sensitive and specific methods, such as liquid chromatography with atmospheric pressure ionization tandem mass spectrometry (detection limit <0.05 ng/ml), have been used in other laboratories.[222] In our laboratory, gas chromatographic equipment already existed, contributing to the choice of the method.

The other strengths of the present study were that processing of the serum samples for gas chromatographic analysis, the actual analysis phase and the processing of the chromatograms with software was carried out by a single experienced laboratory assistant during the study. The large inter-assay variation at low serum cotinine concentrations was, however, a limitation of the study. The inter-assay CV at a cotinine concentration of 22 ng/ml and at 1 ng/ml was 11.7% and 23.3%, respectively. These variations were more substantial than in a reference laboratory in England.[219] However, cotinine concentrations of the same serum samples measured in our laboratory and at the ABS Laboratories (London, UK) were in good agreement. Moreover, in the present study, children with very low cotinine values formed a group before the analyses of effects of tobacco smoke exposure were conducted.

STRIP study parents' estimation of their child's exposure to tobacco smoke were inaccurate and correlated poorly with the child's cotinine value at the age of 8 years, as almost half of the study children had a detectable serum cotinine concentration, and in 8% parents reported that the child was exposed to tobacco smoke. Poor correlation between reported smoke exposure and cotinine values may result from insufficiencies in the questionnaires, but more likely represents the difficulties in estimating the amounts of exposure. Denial of the child's exposure to tobacco smoke is also possible. Therefore, measurement of exposure with an objective biomarker in further studies was supported by these findings.

The main limitation in using cotinine is that it is not a measure of past exposure, as it reflects the exposure to tobacco smoke during the past few days. However, there are no methods available to objectively quantify long-term exposure to tobacco smoke. Moreover, our longitudinal study design to some extent reflects children's long-term exposure to tobacco smoke. Indeed, it has been stated by Jarvis[320] in the World Health Organization Tobacco Free Initiative Background paper, that frequent measurements of serum cotinine can provide insight into the longitudinal tobacco smoke exposure of children, at least for study purposes. Only few studies have reported the results of repeated cotinine measurements. Henschen et al.[257] found that a single measurement of cotinine is widely stable within an interval of one year (r=0.65). Jarvis et al.[321] also showed a correlation of 0.75 between saliva cotinine concentrations over one year in adolescent girls. To our knowledge there are no previous studies with several cotinine measurements. Thus, uniquely in the STRIP study, an objective biomarker of tobacco

smoke exposure was available in most of the children 5 to 6 times between the ages 8 and 13 years. As the children's cotinine concentrations clearly fluctuated during follow-up, annually measured cotinine values were averaged for the analyses and the children were divided into tertiles. Thus, these three longitudinal exposure groups present different levels of tobacco smoke exposure during follow-up reasonably well.

Levels of tobacco smoke exposure

Our results showed that a notable number of the school aged children and adolescents are exposed to tobacco smoke, as indicated by biomarker serum cotinine concentration. Approximately half of the children aged 8 to 11 years had detectable serum cotinine concentrations, and thereafter until the age 13, less than 20% of the adolescents had nondetectable cotinine. However, the distribution of the STRIP children's serum cotinine concentrations was highly skewed, and a large amount of the children had very low cotinine concentrations indicative of scarce exposure to tobacco smoke.

In the present study, the geometric mean cotinine level was 0.08 ng/ml in children aged 8-11 years and 0.55 ng/ml in older adolescents. There are no previous studies from Finland measuring cotinine concentrations among children. Compared to studies from other countries, STRIP children's cotinine levels were only moderate. In English adolescents in 1998, the overall saliva cotinine concentration was 0.52 ng/ml,[232] and 11-year-old Scottish children had a salivary cotinine mean of 0.35 ng/ml in 2006.[235] In the NHANES III study, the geometric mean of serum cotinine concentrations among all 4- to 11-year-old children was 0.297 ng/ml,[216] and in the NHANES 1999-2000 study, the 50th percentile points for serum cotinine values were 0.110 ng/ml and 0.107 ng/ml, in children aged 4 to 11 years and for adolescents aged 12-19 years, respectively.[233] The exposure of our study children was thus less severe or equal to that of children in other countries. However, these comparisons between countries are only suggestive, because different age-groups, biological fluids, and analytical methods with various detection limits have been used.

In our study, the cut-off for the top decile percentile was 1.7 ng/ml among 11-year-old children. For comparison, in the NHANES 2001-2002 study, the 90th percentile points for serum cotinine were 2.03 ng/ml and 1.53 ng/ml for 4-11-year-old children and for 12-19-year-old adolescents, respectively,[233] and the cut-off for the top decile percentile was around 3.0 ng/ml in 11-year-old Scottish children.[235]

There are no consistent data on the exposure times and cotinine levels in children, although some reports have presented the correlations between parent's reports of child's exposure to tobacco smoke during the previous 3 days and the cotinine values of the children.[322] In adults, exposure of 0-4 hours within the previous week led to cotinine levels of approximately 0.6 ng/ml in serum, and cotinine values were about 0.9 ng/ml and 2.3 ng/ml in adults with exposure times of 11 hours and 34 hours, respectively.[323] The same study showed that cotinine concentrations increased by an average of 44% for each increase of 10 hours in reported exposure.[323] Compared to these estimates, adolescents in this study were exposed less than 4 hours per week to tobacco smoke. Another way to describe the amounts of exposure has been based on the well established cotinine concentrations of active smokers. If it is assumed that an

average active smoker of 20 cigarettes a day derives a cotinine concentration of 300 ng/ml in blood,[13,240] then the nicotine intake of the 13-year-old STRIP adolescents with longitudinal averaged cotinine values of 0.28, 0.52, and 1.05 ng/ml would be equivalent to the active smoking of roughly 7, 12, and 27 cigarettes a year, respectively.

In the present study, we used a serum cotinine cut-off limit of 15 ng/ml for active smoking, as previously reported.[216] However, several other cut-off points, usually between 10 and 15 ng/ml, have been used.[324] It is possible that cut-off points derived over 20 years ago are no longer optimal, as the prevalence and exposure to passive smoking has declined.[325] According to a recent study by Jarvis and co-workers,[325] a cut-off point of 12 ng/ml in saliva (corresponding cut-off point of about 9.5 ng/ml in plasma) performed best in discriminating smoking status, but some 1.4% of the children had cotinine values slightly raised above this cut-off point, suggesting heavy passive smoking. Indeed, as the number of children in our study with serum cotinine value between 9.5-14.9 ng/ml was 0-3 in different age groups, and none of the STRIP children had a cotinine level between 15-45 ng/ml, it is likely that these cotinine values resulted from heavy passive smoking rather than active smoking.

Determinants of tobacco smoke exposure

Several investigators have examined individual, family, and community factors which may affect cotinine levels of children exposed to tobacco smoke. Maternal smoking has been the primary source of ETS exposure in numerous studies, but also paternal smoking influences the cotinine levels of the children.[231,232,235,239,241,242] In the present study, however, children's cotinine concentrations at the child's age of 8 years were higher if the father smoked than if the mother smoked. In STRIP, the proportion of smokers and the number of cigarettes smoked were clearly lower among the mothers than among the fathers. The detailed interviews also suggested that several STRIP mothers only smoked outside the home, possibly influencing our results. Parent's knowledge of adverse health effects of passive smoking and smoking bans made in households influence the exposure of children to ETS.[246] Finnish parents are known to be more likely than other Nordic parents to protect the child from tobacco smoke exposure at home.[15] Indeed, many cultural issues may affect the amount of ETS exposure. Furthermore, as in previous studies,[232,235] the cotinine levels in our study were highest in families where both parents smoked.

A distinguishable proportion of children from non-smoking families had detectable serum cotinine concentrations, probably because some close adult other than the parents was a smoker, as noted previously.[247] However, children's serum cotinine values varied considerably even in smoking families, suggesting that the proximity of the child to the smoking adult determines the amount of exposure of the child to tobacco smoke. A limitation of the present study was that the information on the smoking of step-parents was inadequate, yet smoking of the step-mother or step-father might be an important source of ETS exposure among children.

We found that as children grow older their serum cotinine values increase. The clear increase in serum cotinine levels in older age groups was somewhat unexpected,

because tobacco smoke exposure levels in many studies have been found to decrease with age.[244,250] However, previous studies usually included younger children than in our study. In addition, Mannino et al.[250] showed that cotinine levels were higher among 12- to 16-year-old adolescents with no reported smoke exposure at home compared to 7- to 11-year-old children, suggesting that these adolescents are being exposed to smoke from friends or other sources outside of the home.

It has been evident that exposure to ETS declines in many populations due to widespread implementation of laws and policies prohibiting smoking in public places.[232,234,235] The serum cotinine concentrations of the STRIP study children were measured during the years 1997-2004, but the current study merely reflects the exposure levels in individuals from prepuberty to puberty rather than secular trends of exposure in the population.

Multiple studies have reported that there is no difference between genders in exposure to ETS.[240,247,250] In line with this, boys and girls in the present study had similar cotinine levels during the whole follow-up. However, contradictory findings have also been reported.[231,245,251] When children were divided into groups according to different tobacco smoke exposure levels, we did not find any differences in body size, pubertal development, systolic blood pressure, hsCRP, energy intake or fat intake across the exposure groups. However, at the age of 13 years, diastolic blood pressure increased and saturated fat intake tended to increase when cotinine levels increased. Thus, these variables were included in multivariable models of ultrasound variables whenever relevant.

One of the aims in the current study was to examine whether lifestyle intervention implemented in the STRIP project has influenced the tobacco smoke exposure of the study children. We did not find any differences in exposure to ETS between the intervention and control groups until the age 13. These findings reflect the observation that active smoking of the study parents has not been significantly different in the intervention and control groups. Our present data suggests that counseling regarding the smoking of the parents and the children's exposure to tobacco smoke has to be specific and intense, it probably needs to be repeated frequently, and attention also has to be paid to the elimination of other sources of tobacco smoke in the environment than that caused by smoking of the parents. It is noteworthy that reducing passive smoking was only one part of the smoking prevention introduced to the intervention children after the child's age of 9. Until the age of 13, only few study children started to smoke actively. Thus, the efficacy of the smoking prevention can not fully be evaluated from this data. According to Finnish postal survey in 2003 (i.e. when the most of the STRIP study children were 13 years old), 7% and 11% of the Finnish 14-year-old boys and girls, respectively, smoked daily, and respective percentages were 1% and 0% in 12-year-old children.[326]

As STRIP parents' smoking has decreased during the study years more than generally in Finnish adult population,[223-225] results indicate that participation of the family in a prospective child-oriented atherosclerosis prevention trial in itself may have had a positive impact on parent's smoking habits in the intervention group as well as in the control group. However, one confusing factor is the recent finding that smoking

parents have discontinued participation in the STRIP project more frequently than other parents.[327] Possibly, this has biased the longitudinal analyses of the parents smoking, but presumably the exposure to tobacco smoke of the study children might thus be underestimated. Nevertheless, the main findings of this study on the association between passive smoking and markers of preclinical atherosclerosis are independent of the family drop-outs at the beginning of the project. Overall, the smoking frequencies of the STRIP parents were lower than in general in Finland, suggesting that the exposure levels found in the STRIP study children may not be totally generalizable.

Impact of tobacco smoke exposure on serum lipid profile

Healthy 13-year-old adolescents who were longitudinally most exposed to tobacco smoke had significantly increased ApoB, ApoB/ApoA-I ratio and triglyceride levels compared to the children with only little exposure. These associations were unchanged even after controlling for gender, STRIP study group, pubertal status, BMI, and saturated fat intake. Previously, there have been no reports on the association between passive smoking and apolipoprotein values among children or adolescents.

These findings are partly in line with studies made among active smokers, as active adult smokers show dose-dependent elevations of serum total cholesterol, VLDL cholesterol, LDL cholesterol, triglycerides, and ApoB, as well as decreases of HDL cholesterol, HDL_2, and ApoA-I as compared with non-smokers.[101,103,104] The ApoA-I/ApoB ratio has also been reported to be lower in smokers than in non-smokers.[106] The mechanisms of the effects of smoke exposure on lipid profiles have not been fully explored. However, cigarette smoking has been shown to increase hepatic lipase activity,[167] inhibit lecithin cholesterol acyl-transferase activity,[168,169] and decrease lipoprotein lipase activity.[169] Exposure to tobacco smoke may also be associated with the unfavorable quality of diet.[171]

We did not find any differences in HDL cholesterol values in children or adolescents variously exposed to tobacco smoke. In contrast, previous studies in healthy children[126,162] and adolescents,[163,164] as well as in dyslipidemic children[165] have shown an association between decreased HDL cholesterol and passive smoking. The discrepancy with previous studies may probably be due to the generally low tobacco smoke exposure levels found in the current study. The finding that neither HDL cholesterol nor ApoA-I were associated with tobacco smoke exposure increases the concordance of our results, as HDL cholesterol and ApoA-I are highly correlated.[65] We have previously shown that lipid values dramatically change during pubertal development,[328] and that might provide one explanation for the inconsistency with previous studies.[126,162-164] However, pubertal status was taken into account as a covariate in multivariable analyses examining the association between exposure to tobacco smoke and serum lipid profile.

Although an association between passive smoking and ApoB was found in adolescents, we did not observe any differences in apolipoprotein values in tobacco smoke exposure groups at the age of 11 years. Possible reasons for this might be the smaller study groups examined at younger age, or the fact that the pubertal development of the children was different at that time. In addition, the exposure to tobacco smoke

increased with increasing age. Thus, the discrepancy between different age groups in the association between passive smoking and apolipoprotein values may reflect the more substantial tobacco smoke exposure confronted by adolescents.

6.2.2. Markers of subclinical atherosclerosis

Methodological aspects

All ultrasound studies were performed using high-resolution ultrasound, and according to predetermined, standardized protocols for brachial arteries, abdominal aorta and carotid arteries. Other strengths of the present study were standardized study conditions, and that a single experienced vascular sonographer blinded to the subjects' details performed the ultrasound scanning and the offline analysis of the images at certain age points. Moreover, studies were performed among healthy children with the same age.

Arterial reactivity

In the present study, brachial artery FMD was determined ultrasonically according to the method originally described by Celermajer et al.[261] As the between-visit variation was 9.3% and inter-observer variation 8.6%, the reproducibility of the FMD measurements was high, and comparable to other reports.[329] An additional strength of our study was that brachial artery diameter was measured at several time points between 40 and 180 seconds after cuff release to detect the true maximal dilatory response, as a pediatric study has shown that the time needed to achieve the peak vasodilation response may vary considerably between the subjects.[76] Moreover, we could include the measurement of the total dilation response (AUC) i.e. the area under the dilation response vs. time curve during the first 3 minutes of hyperemia.

Brachial artery FMD is a functional measure of endothelial function. Dilation of the artery after the increased blood flow is mainly mediated through endothelial release of NO.[262] To ascertain that decreased FMD is a consequence of endothelial dysfunction and not resulting from smooth muscle dysfunction, measurement of endothelium-independent NMD has usually added to the study protocol of FMD testing.[261] However, impaired NMD has been related with low FMD and higher IMT in children.[330] In this study, the NMD capacity was tested by administration of a 250 micrograms sublingual dose of glyceryl nitrate in 11-year-old children, but the NMD test was not applied at the age of 13 years due to practical reasons.

Despite the widespread use of this non-invasive ultrasound technique for study purposes, successful measurement of brachial reactivity needs a skilled sonographer, and problems with reproducibility are still possible, limiting the use of the FMD method in clinical use. Moreover, there is lack of data of the cut-off point values for normal FMD. Brachial measurements of FMD correlate with coronary endothelial function,[265] the status of systemic endothelial function may modify the association between risk factors and atherosclerosis,[331] and FMD predicts future cardiovascular events.[332,333] However, no data yet exists on the predictive value of FMD measured in childhood on cardiovascular outcomes.

Arterial elasticity

Arterial elasticity in the present study was assessed using ultrasonically measured indices, which are based on the measurement of blood pressure and arterial diameter changes during systole and diastole. The stiffness index, Young's elastic modulus, and distensibility reflect different aspects of the mechanical properties of arteries.[279,280] The stiffness index characterizes the elastic properties of the arterial wall relatively independently of blood pressure. Young's elastic modulus is a measure of arterial stiffness that is independent of arterial wall thickness. Distensibility measures the ability of the arteries to expand as a response to pulse pressure caused by cardiac contraction and relaxation.

To calculate the elasticity indices, we measured arterial dilation in the aorta and in the carotid artery and pulse pressure in the brachial artery. Thus, an important challenge of the present study was the method of blood pressure measurement. It would have been more ideal to study the pulse pressure from the artery in question, but obviously, in this pediatric study, that was not a possible choice. The use of brachial artery blood pressure may overestimate pulse pressure in central arteries.[334] Although it has been shown that aortic elastic modulus calculations using brachial or aortic pressure are highly correlated,[335] and invasively measured aortic and noninvasively measured brachial artery blood pressure values significantly correlate,[336] there are no studies in children to evaluate the accuracy of arterial elastic indices measurement derived from peripheral pressure measurements. However, the difference between central and peripheral pulse pressure is likely to be rather similar between different cotinine level groups and between study subjects of a similar age. In the high cotinine group amplification may decrease due to increased pulse wave velocity and increased wave reflections, and consequently, the actual distensibility could be even smaller in the high cotinine group than the one measured. Therefore, the findings of the present elasticity study may be an underestimation.

Another limitation of the present study on arterial elasticity was the quite large intra-individual variation of 15 to 27% in the elasticity indices. These between-visit variation values were in line with previous reports in adults.[283,337] As variation in diameter measurements was clearly lower (between 6 and 8%), and the two ultrasound studies were performed several months apart, the large variation in elasticity indices is rather due to physiological fluctuation than to a measurement error. Nevertheless, the noise in the data is likely to weaken the observed association.

Intima-media thickness

In the current study, cIMT was measured both from the right and left common carotid arteries approximately 10 mm distally below the carotid bulb. At least four measurements from the posterior (far) wall from both sides were acquired to assess mean and maximum cIMT. The interobserver and between-visit variability of cIMT measurements were 3.0% and 3.9%, respectively. Thus the reproducibility of the method was good and in agreement with those from previous reports.[300]

In pediatric studies, the common carotid artery for cIMT measurement has usually been the artery of choice.[55,196] In adults, a more complex carotid IMT score involving

both internal and common carotid measurements may have a better predictive value than either IMT measure alone.[295] However, another study has shown that common carotid artery IMT may provide a better surrogate marker for myocardial infarction than carotid IMT score.[338] In addition, the relationship between carotid and coronary atherosclerosis is enhanced only marginally when IMT data from the internal carotid artery and carotid bulb are added to the common carotid IMT.[339] Furthermore, as IMT of the common carotid artery has been shown to be easily visualized and the method is highly repeatable, the above data support the use of that location alone, at least for studies of risk factor associations.

cIMT measurement using a non-invasive ultrasound technique has, indeed, proved to be useful in assessing the extent of preclinical atherosclerosis. cIMT is related to atherosclerosis risk factors,[44] coronary atherosclerosis,[340] and is an independent predictor of cardiovascular events.[295,297] On the other hand, the use of IMT of the abdominal aorta as a marker for preclinical atherosclerosis has not yet gained wide acceptance, as it is still a rather new marker, prospective data have not yet accumulated, and the visualization of the aIMT is more demanding that that of the cIMT.

aIMT was measured in the present study from the most distal 15 mm of the abdominal aorta. At least 4 to 6 IMT measurements covering the entire far wall segment of interest were taken for analyses, and these data were used for maximum and mean aIMT determinations. The reproducibility of the aIMT measurements was good and in agreement with cIMT measurements. A possible limitation of this method in children is that it provides data only in the dorsal arterial wall of the most distal abdominal aorta. Fatty streaks in the aortas of children may be located in the distal part of the abdominal aorta or in the arterial branches, and there may be a considerable variation in the distribution of these lesions. However, as autopsy studies have shown that the earliest structural alterations in the vascular tree appear in the dorsolateral abdominal aorta,[17] the measurement of aIMT may provide an even better index of subclinical atherosclerosis than cIMT, particularly among children and adolescents. Indeed, aIMT has identified subjects with increased atherosclerosis risk factor load even more efficiently than cIMT.[58,308]

Impact of tobacco smoke exposure on markers of subclinical atherosclerosis

Healthy 11- and 13-year-old children and adolescents with exposure to tobacco smoke had significantly decreased endothelial function as measured with FMD compared to children with no exposure. In addition, recent exposure to tobacco smoke was associated with decreased elasticity of the aorta in 11-year-old children. Moreover, 13-year-old adolescents with longitudinal tobacco smoke exposure had significantly higher IMT both in the carotid arteries and the aorta compared with adolescents with exiguous exposure. All these associations were evident even after adjustments with traditional atherosclerosis risk factors.

Although several cardiovascular risk factors have been associated with early functional and structural vascular wall changes in childhood,[55,57,58,77,289,300,301,308] there are no previous studies in healthy children or adolescents of the association between FMD,

IMT or elasticity and objectively measured tobacco smoke exposure. Only one previous study has found that 15-year-old adolescents and young adults have attenuated FMD in connection with passive smoking measured by questionnaires.[179] In children and adolescents with type 1 diabetes, exposure to tobacco smoke assessed through questionnaire was associated with decreased carotid distensibility, but not with cIMT.[196]

Regarding FMD, our results are in line with previous studies in adults showing an association with long-term tobacco smoke exposure and impaired endothelial function in passive smokers,[177,179] and showing that only 30 minutes of acute passive smoke exposure in non-smokers led to impairment of coronary endothelial function.[128] In addition, our findings are partly in line with previous studies on passive smoking and abnormalities in arterial elasticity. Decreased carotid arterial elasticity has been previously reported following chronic exposure to tobacco smoke in non-smokers,[193] but only in one study. Experimental studies, however, support this finding, as CHD patients have had impaired aortic elastic properties after acute exposure to tobacco smoke,[194] and acute exposure has had a deleterious effect on the arterial pressure waveform in healthy young males.[195] Finally, regarding IMT, our findings are in agreement with results from the ARIC study, as they reported that both past and current passive smoking in adults is associated with increased cIMT,[93,94] and also longitudinal association between exposure to tobacco smoke and the progression of cIMT was found.[199] Taken together, these results from adult studies suggest that cigarette smoke is a potent risk for arterial function and structure.

Carotid elasticity was not significantly associated with passive smoking, although one previous study has reported the association between reported tobacco smoke exposure and decreased carotid distensibility in diabetic children.[196] However, in our study, elasticity of the abdominal aorta was decreased in connection with the exposure to tobacco smoke, a finding in line with previous post-mortem analysis showing that atherosclerotic alterations are first seen in the abdominal aorta.[17] There is also evidence that in children affected by cardiovascular risk factors, intima-media thickening is first present in the abdominal aorta.[58,308] Thus, the positive association between exposure to tobacco smoke and aortic stiffness at the age of 11 years may reflect the very early manifestation of vascular changes at this site.

In the present study, conventional risk factors for atherosclerosis were not related to FMD. Regarding aortic elasticity indices, we found that BMI and blood pressure were associated with aortic stiffness, in line with previous studies.[289,290] Diastolic blood pressure associated with both IMT measurements, and aIMT also related with BMI and triglycerides, and cIMT with gender and total and LDL cholesterol. As cholesterol values decrease as puberty progress,[328] the unexpected inverse association between total and LDL cholesterol and cIMT found in the current and our previous study[341] is probably due to ongoing puberty. Nevertheless, pubertal status and relevant risk factors were taken into account as covariates in multivariable analyses examining the association between exposure to tobacco smoke and markers of subclinical atherosclerosis.

Other known causes of vascular damage presumably did not affect the main findings, as the tobacco smoke exposure groups did not differ in terms of atherosclerotic risk factors. It is noteworthy that hsCRP, the inflammatory marker related to atherosclerosis, was similar across the cotinine groups, indicating that mechanisms other than the induction of systemic inflammation probably contribute to the association between exposure to tobacco smoke and vascular changes in children. At the age of 13 years, ApoB and triglyceride values increased with the increase in cotinine level, but this did not confound the association between exposure to tobacco smoke and the IMT of the carotid arteries or the abdominal aorta. Thus, although exposure to tobacco smoke can promote atherosclerosis in part by the effects on the ApoB metabolism, this did not offer a mechanistic explanation for the relationship between smoke exposure and the IMT of the carotid arteries or the abdominal aorta among healthy adolescents.

6.3. Clinical implications

Environmental tobacco smoke is a known risk to health and a preventable cause of morbidity.[10] Children and young people are particularly vulnerable to the health effects of passive smoking.[10] Exposure to ETS causes multiple damage to the cardiovascular system, many of which are also reported among children,[12] and even exposure to tobacco smoke in utero may have direct consequences on the cardiovascular physiology.[342] Multifactorial atherosclerosis begins in childhood, although clinical manifestations of the disease occur decades later. This enables realization of early detection and prevention strategies. The development of ultrasound technology has provided an opportunity to detect early vascular changes related to atherosclerosis in healthy populations.

The present study showed that even moderate exposure to tobacco smoke may be harmful to the function and structure of the arteries, and to some part of the lipid metabolism already in childhood. Thus, exposure to tobacco smoke appears to be an important risk factor for atherosclerosis in children and adolescents, which should be taken into account both in clinical studies, in the primary prevention of the atherosclerosis, and in clinical practice.

Our results confirm that school-aged children and adolescents are potentially at risk for the adverse cardiovascular effects of passive smoking, even in a developed country where the smoking frequency of the adults is moderate, and smoking bans in public places have been implemented. Thus, these data emphasize the importance of endorsing smoke-free environments for children and adolescents both at home and in public places.

Exposure to ETS is best measured through the biomarker cotinine, as cotinine level provides valid quantitative measure of average ETS exposure.[13] Although cotinine measurements are commonly used in research studies, such measurements are not currently feasible in routine pediatric health care, because of the cost and time constraints. Thus, simple screening methods are still needed. In order to reduce passive smoking in children, the health education of parents is fundamental. Both reducing

active smoking among parents and diminishing smoking near children, as well as counseling on the adverse health effects of passive smoking in children are important. Results from the present study, showing the adverse effects of the tobacco smoke exposure on arterial structure and function, could provide one concrete theme to add to the counseling of smoking parents. Our data further suggest that counseling regarding the smoking of the parents and the children's exposure to tobacco smoke is challenging, has to be well targeted, probably needs to be repeated frequently, and attention also has to be paid to the elimination of other sources of tobacco smoke in the environment than that caused by smoking of parents.

6.4. Future research needs

This research suggests that frequent exposure to tobacco smoke may have a role in the development of early atherosclerotic changes. Although exposure to tobacco smoke was related to ApoB and triglyceride levels, the effect of smoke exposure on preclinical vascular changes did not seem to be mediated through these changes according to multivariable analyses. In addition, ETS exposure independently associated with each of the markers of subclinical atherosclerosis, suggesting that multiple mechanisms may be responsible for the vascular effects of cigarette smoke. Thus, more studies on the possible underlying mechanisms and their interactions are needed. Furthermore, it is not known whether the vascular changes related to tobacco smoke exposure are reversible, and what their predictive value is for future disease development.

The present study was the first one to show the association between passive smoking and ApoB in adolescents. Thus, these results should be reproduced in different settings. In addition, the longitudinal stability and implications of this association remain to be resolved.

Additional research is needed to better determine the level of tobacco smoke exposure that is biologically important in children and adolescents. Preventive action to diminish exposure to tobacco smoke among children definitely deserves further study.

Little is known about the tracking of the markers of subclinical atherosclerosis among children and adolescents. In the future, the STRIP study will produce data of several IMT, FMD and elasticity measurements among young healthy subjects, allowing study of the longitudinal patterns of these measurements, as well as the associations of childhood risk factors and changes in ultrasound markers. Although these ultrasound measures are currently widely used for study purposes, it is essential to gain more prospective data until these methods are acceptable as a tool for clinical practice in children and adolescents.

7. CONCLUSIONS

1. The prevalence of active smoking of parents has been reduced among all participants in the atherosclerosis prevention trial STRIP. Repeated and individualized child-oriented lifestyle intervention has not influenced the active smoking of the fathers, and has had only a marginal effect on the active smoking of mothers belonging to the intervention group. Intervention did not have any effect on the study children's exposure to tobacco smoke. Serum cotinine values of the study children were highest in the families where either the father or both parents were smokers. However, objectively measured exposure varied both in smoking and non-smoking families, suggesting that the smoking of other people than the parents also influenced the passive smoking of the school-aged children.

2. Exposure to tobacco smoke confirmed by serum cotinine concentration was related to decreased FMD of the brachial artery and decreased elasticity of the aorta. These findings suggest that exposure to tobacco smoke alters endothelial function and the mechanical properties of the elastic arteries in children, and thus may have a role in the development of early functional arterial changes associated with subclinical atherosclerosis.

3. Exposure to tobacco smoke was not associated with total, HDL, or LDL cholesterol, but exposed adolescents showed elevations in ApoB, ApoB/ApoA-I ratio and triglyceride values, suggesting that tobacco smoke exposure may play a role in early atherosclerosis by interfering with some parts of the lipid metabolism.

4. Frequent exposure to tobacco smoke was related to increased IMT both in the abdominal aorta and the carotid arteries in healthy adolescents, suggesting that passive smoking is a potent risk factor for early structural atherosclerotic changes. In addition, FMD was impaired among exposed adolescents. Thus, exposure to cigarette smoke may play a significant role in the development of early atherosclerotic changes.

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