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Changes in Atherosclerosis Risk Factors Induced by Hormone Replacement Therapy or Ethanol Consumption

ACADEMIC DISSERTATION
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ABSTRACT

Atherosclerosis is a chronic disease of large and medium sized arteries, characterized by retention of lipoproteins and accumulation of cholesterol in the arterial wall and subsequent narrowing of the arterial lumen. Atherosclerosis is the main cause of death in Western countries. The use of hormone replacement therapy (HRT) to prevent atherosclerosis has been an area of controversy in recent years. However, despite a multitude of earlier studies, there were no previous randomized clinical trials on whether estrogen treatment and estrogen receptor gene variation influence the progression of atherosclerosis in women. Moderate ethanol consumption has been shown to affect the serum lipid profile favorably, and there is a J-shaped association between alcohol consumption and the incidence of coronary heart disease. Nevertheless, associations between the lipid profile and the biomarkers of alcohol consumption carbohydrate-deficient transferrin (CDT) and gamma-glutamyl transferase (GGT) were unresolved.

The aims of this study were to investigate the possible effects of postmenopausal HRT on the oxidation of low density lipoprotein (LDL) particles and progression of cardiovascular diseases and to look for associations between the genotype of estrogen receptor and progression of atherosclerosis. The effects of alcohol consumption on the lipid profile were studied in relation to laboratory biomarkers of alcohol use.

Postmenopausal HRT by estradiol valerate alone, combined estradiol valerate–levonorgestrel, and combined estradiol valerate–medroxyprogesterone acetate was found to be associated with less severe atherosclerotic lesions and diminished LDL oxidation. Genetic variation in the estrogen gene may modulate the effect of postmenopausal estrogen therapy on progression of atherosclerosis. Alcohol consumption, associated with high serum CDT concentration, was associated with a favorable anti-atherogenic lipid profile, whereas alcohol consumption associated with liver induction and elevated serum

GGT activity may favor pro-atherogenic effects on lipid profile. Thus, the biomarkers of alcohol consumption, CDT and GGT seem to detect different populations of subjects in regard to cardiovascular lipid risk factors.

Keywords: atherosclerosis, cholesterol, carbohydrate-deficient transferrin, estrogen receptor genotype, gamma-glutamyl transferase, hormone replacement therapy, alcohol, serum lipids.

TIIVISTELMÄ

Valtimonkovettumatauti eli ateroskleroosi on suurten ja keskikokoisten valtimoiden krooninen tauti, jolle on ominaista kolesterolin kertyminen valtimon seinämään ja valtimon ontelon kaventuminen. Ateroskleroosi on yleisin kuolinsyy länsimaissa. Hormonikorvaushoidon käyttäminen ateroskleroosin ehkäisyyn on ollut kiistanalainen kysymys viime vuosina. Vaikka tutkimuksia on ollut paljon, estrogeenihoidon tai estrogeenireseptorin geneettisen variaation vaikutusta ateroskleroosin etenemiseen ei ole aikaisemmin tutkittu satunnaistetussa kliinisessä tutkimuksessa. Kohtuullisen alkoholin kulutuksen on todettu vaikuttavan suotuisasti seerumin lipideihin. Alkoholin ja sydäntaudin päätetapahtumien välinen yhteys näyttää olevan J-käyrän muotoinen. Seerumista mitattavien alkoholinkulutusta heijastavien biomarkkereiden hiilihydraattiköyhän transferriinin (CDT) ja gamma-glutamyylitransferaasin (GGT) yhteys seerumin lipidiprofiiliin tunnetaan huonommin.

Tutkimuksessa selvitettiin vaihdevuosien jälkeisen hormonikorvaushoidon mahdollisia vaikutuksia low density lipoproteiinien (LDL) hapettumiseen ja sydän- ja verisuonitautien kehittymiseen sekä selvitettiin estrogeenireseptorin genotyypin yhteyttä ateroskleroosin etenemiseen. Alkoholinkäytön vaikutusta seerumin lipideihin tutkittiin suhteessa sen kulutusta heijastaviin seerumin biomarkkereihin.

Vaihdevuosien jälkeinen hormonikorvaushoito estradiolivaleraatilla, yhdistetyllä estradiolivaleraatti-levonorgestreelillä, ja yhdistetyllä estradiolivaleraatti–medroksiprogesteroniasetaatilla oli yhteydessä lievempiasteiseen ateroskleroosiin ja vähentyneeseen LDL:n hapettumiseen. Estrogeenireseptorin geenivaihtelu saattaa vaikuttaa valtimoiden kovettumistaudin kehittymiseen hormonikorvaushoidon aikana. Alkoholinkäyttö, johon liittyy kohonnut seerumin CDT-pitoisuus, oli yhteydessä suotuisaan lipidiprofiiliin, kun taas alkoholinkäyttö johon liittyy maksan induktio ja

kohonnut seerumin GGT-aktiivisuus, näyttää olevan yhteydessä epäsuotuisaan lipidiprofiiliin. Näin ollen alkoholin kulutuksen biomarkkerit CDT ja GGT näyttävät tunnistavan väestöryhmät, joilla on erilainen sydän- ja verisuonitautien riski lipidien osalta.

Avainsanat: ateroskleroosi, hormonikorvaushoito, estrogeenireseptorin genotyyppi, alkoholi, kolesteroli, seerumin lipidit.

ABBREVIATIONS

ANOVA	analysis of variance
AS	atherosclerotic severity sum
BHT	butylated hydroxytoluene
BMI	body mass index
CCA	common carotid artery
CAD	coronary artery disease
CDT	carbohydrate-deficient transferrin
ESR	estrogen receptor
ESR1	estrogen receptor alfa
ESR2	estrogen receptor beta
EV	estradiol valeriate
GGT	gamma-glutamyl transferase
CVD	cardiovascular disease
HDL	high density lipoprotein
HERS	Heart Estrogen/progestin Replacement Study
HRT	hormone replacement therapy
HSA	human serum albumin
IDL	intermediate density lipoprotein
LDL	low density lipoprotein
M	progestin, medroxyprogesterone
MI	myocardial infarction
NAP	number of atherosclerotic plaques
OD	optical density
oxLDL	oxidized low density lipoprotein

P	progestin, levonorgestrel
PBS	phosphate buffered saline
RCT	reverse cholesterol transport
SMC	smooth muscle cell
VLDL	very low density lipoprotein
WHI	Women's Health Initiative

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original publications, referred to as I-IV in the text

I. Koivu TA, Dastidar P, Jokela H, Nikkari ST, Jaakkola O, Koivula T, Punnonen R, Lehtimäki T (2001): The relation of oxidized LDL autoantibodies and long-term hormone replacement therapy to ultrasonographically assessed atherosclerotic plaque quantity and severity in postmenopausal women. *Atherosclerosis* 157:471-479.

II. Koivu TA, Fan YM, Mattila KM, Dastidar P, Jokela H, Nikkari ST, Kunnas T, Punnonen R, Lehtimäki T (2003). The effect of hormone replacement therapy on atherosclerotic severity in relation to ESR1 genotype in postmenopausal women. *Maturitas* 44:29-38.

III. Nikkari ST, Koivu TA, Anttila P, Raunio I, Sillanaukee P (1998): Carbohydrate-deficient transferrin and gamma-glutamyl transferase are inversely associated with lipid markers of cardiovascular risk. *Eur J Clin Invest* 28:793-797.

IV. Nikkari ST, Koivu TA, Kalela A, Strid N, Sundvall J, Poikolainen K, Jousilahti P, Alho H, Sillanaukee P (2001): Association of carbohydrate-deficient transferrin (CDT) and gamma-glutamyl-transferase (GGT) with serum lipid profile in the Finnish population. *Atherosclerosis* 154:485-492.

In addition, some unpublished data are presented. The original articles are reproduced with the kind permission of Elsevier (I, II, IV) and John Wiley and Sons (III).

1. INTRODUCTION

Atherosclerosis is a chronic disease of large and medium sized arteries. It has a multifactorial origin and is characterized by accumulation of intracellular and extracellular cholesterol in the arterial wall with subsequent narrowing of the arterial lumen (Fuster et al. 1992a, Fuster et al. 1992b). Inflammation plays an important role in the progression of atherosclerosis (Ross 1999). There is a chronic inflammatory process in the atherosclerotic plaque, which has a major role in plaque growth and rupture. Plaques that are prone to rupture have often numerous inflammatory cells and a thin fibrous cap (Stoll and Bendszus 2006). Physiological disruption of atheromatous lesions often underlies acute coronary syndromes such as myocardial infarction (MI), angina, or sudden death due to coronary artery occlusion (Falk 1983).

It is well known that increase in serum LDL is a risk factor for atherosclerosis. Oxidative modification of LDL makes it more atherogenic than its native form. Oxidized LDL (oxLDL) is taken up by macrophages via the scavenger receptors leading to foam cell formation and fatty streak development (Steinberg et al. 1989). OxLDL is immunogenic and induces the formation of autoantibodies, which have been detected in human and animal plasma and atherosclerotic plaques (Ylä-Herttuala et al. 1994). OxLDL autoantibodies have been suggested to predict rapid progression of atherosclerosis in humans (Salonen JT et al. 1992, Puurunen M et al. 1994, Wu et al. 1997). On the other hand, oxLDL has also been reported to have no effect on atherosclerosis (van de Vijver et al. 1996), or even a beneficial effect (Fukumoto et al. 2000). Despite the contradictory results concerning the involvement of oxLDL autoantibodies in the pathogenesis of atherosclerosis, autoantibodies may be useful in specifically evaluating the presence of oxLDL in the arterial wall (Tsimikas et al. 2001).

HRT is often given for women as a short-term relief from menopausal symptoms. The use of HRT to prevent heart disease has been an area of controversy in recent years after negative results in randomized trials (Brinton et al. 2008). This thesis studied the effect of HRT on the progression of atherosclerosis and the relation of this therapy to the level of oxLDL autoantibodies – a risk factor for atherosclerosis. We then wanted to find out whether there was a genetic subgroup of women who would benefit more from estrogens than others. In fact, an important hypothesis of this thesis was that some women would receive more atheroprotective benefit than others from HRT depending on their estrogen receptor alfa (ESR1) genotype.

The per capita alcohol consumption in Finland has steadily increased. During the last two decades, the number of alcohol related deaths has doubled, and in 2008 alcohol caused more deaths of men aged 15 to 64 years than coronary artery disease (CAD) (Statistics Finland 2009). Moderate alcohol consumption is not considered a risk factor for atherosclerosis, but excessive alcohol consumption increases the risk for cardiovascular complications. Two cross-sectional studies were carried out to assess the relation between biomarkers of alcohol intake CDT and GGT and serum lipids.

2. REVIEW OF THE LITERATURE

2.1. The structure of arteries

The artery wall consists of three layers, the intima, media and adventitia (Stary et al. 1992). The intima includes a monolayer of endothelial cells or endothelium and a subendothelial space containing extracellular matrix and smooth muscle cells (SMC). Also macrophages, T-lymphocytes and mast cells may be present. The thickness of the intima varies greatly depending on the size and type of artery. Between the intima and media is the fenestrated internal elastic lamina (Stary et al. 1992). The media consists mainly of layers of SMCs that maintain arterial tone. The external elastic lamina separates the media from the adventitia, the outermost layer, which is connective tissue and contains fibroblasts, vasa vasorum and nerves (Geer and Haust 1972, Stary et al. 1992, Kovanen et al. 1995).

The thickness of the intima increases through the activation of a subgroup of native intimal SMC. Intimal thickening begins already during fetal life (Stary 2000). Diffuse and eccentric thickenings are produced. Diffuse intimal thickenings involving the whole circumference of the artery are found in all anatomic locations of medium and large arteries, but eccentric thickenings that cover less than half of the circumference are localized to arterial bifurcations (Geer and Haust 1972, Stary et al. 1992, Stary 1987). Eccentric thickenings may be adaptations to “disturbed” shear stress conditions at arterial bifurcations (Davies 1995, Gimbrone et al. 2000), and it is thought that atherosclerosis progresses more rapidly in these regions than elsewhere (Stary et al. 1992).

2.2. Atherosclerosis

2.2.1. Risk factors of atherosclerosis

The classical risk factors for formation of atherosclerotic lesions include environmental factors, such as smoking, lack of exercise, high fat diet and infectious agents. In addition, also factors with a significant genetic component are strongly associated with atherosclerosis. These include high serum cholesterol, especially LDL cholesterol, low levels of high density lipoprotein (HDL), male gender, diabetes mellitus and arterial hypertension (for review, see Glass and Witztum, 2001).

2.2.2. Metabolism of cholesterol in lipoproteins

Serum cholesterol transport occurs through different lipoprotein particles that are composed of lipids and apolipoproteins (Witztum and Steinberg, 1995). Dietary cholesterol is absorbed in the intestine and packed into triglyceride-rich chylomicrons that contain apolipoproteins B-48 and E. In tissue capillaries the enzyme lipoprotein lipase hydrolyzes a large part of chylomicrons' triacylglycerol to glycerol and free fatty acids. The remaining smaller chylomicron particle is taken up by the liver (for review, see Glass and Witztum, 2001).

The liver synthesises triglyceride-rich very low density lipoprotein (VLDL) particles containing apolipoprotein B-100 and apolipoproteins E and C. Triacylglycerol is removed from VLDL in peripheral tissues by lipoprotein lipase and VLDL remnants are converted to intermediate density lipoprotein (IDL), which are either taken up by the liver or converted to LDL by hepatic lipase. Two thirds of plasma cholesterol is carried by LDL with apolipoprotein apoB-100. LDL is subsequently taken up by peripheral tissues and the

liver by the LDL receptors that recognize apoB-100. Free cholesterol is released into the cell after lysosomal enzymes hydrolyze LDL. Increased LDL cholesterol levels have been shown to be associated with increased risk of atherosclerosis (for review, see Glass and Witztum, 2001).

One third of the plasma cholesterol is carried by HDL, and its main apoprotein is apoA-I (Oram et al. 2006). HDL has a major role in reverse cholesterol transport (RCT), since it accepts free cholesterol from peripheral tissues, esterifies it by the enzyme lecithin:cholesterol acyl transferase, and is taken up by the liver by hepatic scavenger receptor BI. It also exchanges cholesterol with other lipoproteins through cholesteryl ester transfer protein (Acton et al. 1996).

2.2.3. Oxidation of LDL particles

OxLDL has a significant role in the progression of atherosclerosis (Steinbrecher et al. 1989, Parthasarathy et al. 1990, Parthasarathy et al. 1992). OxLDL accumulates in macrophages, accelerates the development of foam cells and stimulates the adhesion of monocytes to endothelium (Jialal and Devaraj 1996, Witztum and Steinberg 1991, Witztum 1991). The small, dense LDL particles are more susceptible to oxidation, which mainly takes place in the arterial wall (Lehtimäki et al. 1999, Krauss 1995, Galeano et al. 1998). The antibody titers of oxLDL have been shown to correlate with the severity of atherosclerosis and it has been thought to predict MI (Salonen et al. 1992, Wu et al. 1997) and restenosis (Eber et al. 1994).

2.2.4. Atherosclerotic lesions

Atherosclerotic lesions are formed when the influx of atherogenic lipoproteins is excessive at susceptible intimal thickenings of large arteries (McGill and McMahan 1998, Williams and Tabas 1995, Williams and Tabas 1998, Kadar and Glasz 2001, Witztum and Steinberg 1991). Trapping of LDL by the extracellular matrix proteoglycans lengthens its residence time in the intima and predisposes it to modifications, such as metal catalyzed oxidation, nonenzymatic glycosylation or lipolysis and proteolysis by enzymes derived from intimal cells (Ross 1993). Modification of LDL is often needed for its enhanced uptake by monocyte-derived macrophages (Witztum and Steinberg 1991). OxLDL as well as modified LDL generally are taken up by macrophages via scavenger receptors, leading to foam cell formation. This is prerequisite for atherosclerotic plaque formation. The Committee on Vascular Lesions of the Council of Atherosclerosis, American Heart Association (Stary et al. 1992, Stary et al. 1994, Stary et al. 1995) has established a specific classification of atherosclerotic lesions, based on histology (Figures 1 and 2). This classification divides the lesions into early (I-III) and advanced (IV-VI) types.

Classification of early lesions include types I, II and III. Type I lesions only contain foam cells without other significant histological changes (Stary et al. 1994). At the next stage, type II lesions form on the inner surfaces of arteries. They are relatively flat, yellow-colored streaks or patches. Type II lesions are called fatty streaks and consist primarily of macrophage foam cells, but SMC-derived foam cells may also be present. The lipid in these cells is primarily cholesterol and its esters. Type III lesions also contain extracellular lipid droplets derived from apoptotic macrophage foam cells in small pools below the macrophage layer. Types I and II and possibly also type III lesions are relatively innocuous and can either regress, or progress depending on favorable or unfavorable changes in risk factors (Stary 2000).

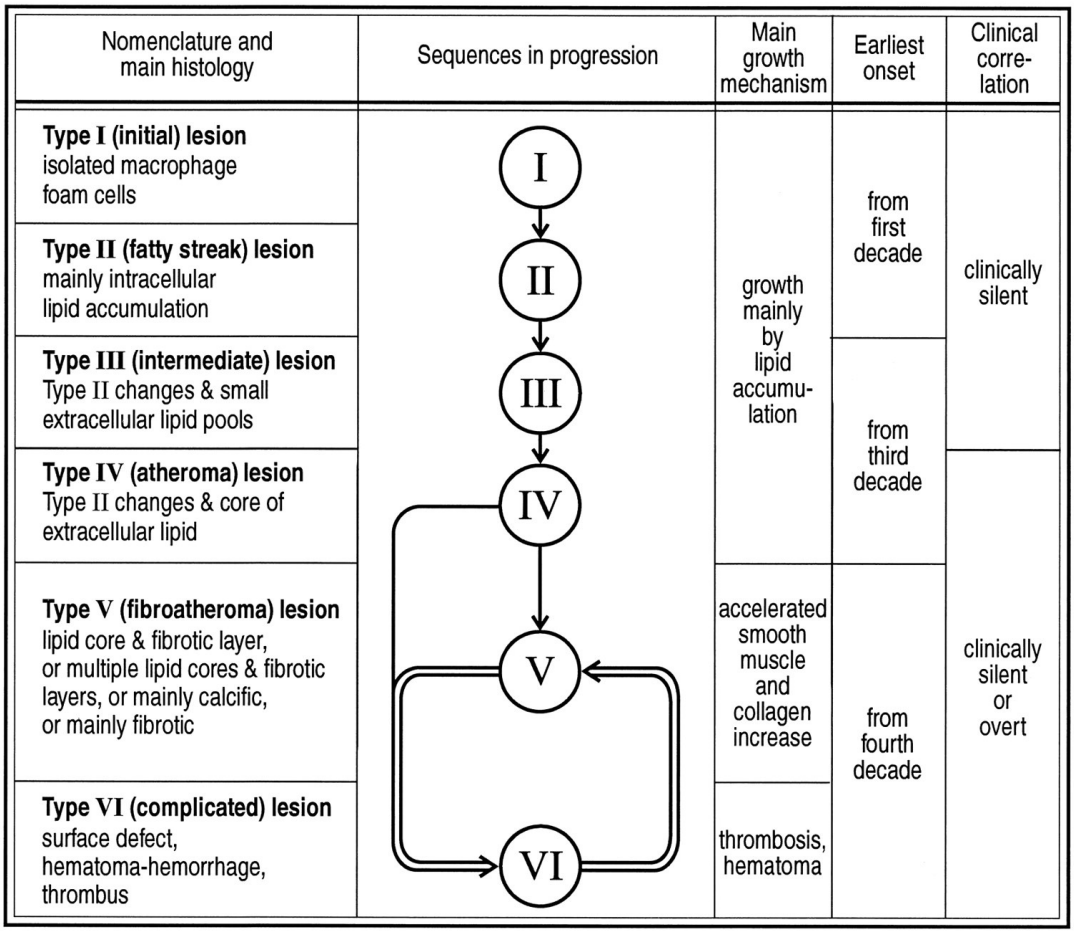


Figure 1. Classification of atherosclerotic lesions (Stary et al. 1995).

Advanced atherosclerotic lesions are classified into type IV – VI. Type IV lesions, also known as the atheromas, evolve from the type II lesions by accumulation and fusion of extracellular lipid pools in the intima, called the lipid core. On top of the lipid core is a thin layer of proteoglycan-rich intercellular matrix containing macrophages, SMCs, mast cells and lymphocytes (Stary et al. 1995, Kovanen et al. 1995, Kaartinen et al. 1998). What makes these lesions dangerous is that they are asymptomatic, not visible by angiography and the thin cap is prone to rupture (MacIsaac et al. 1993, Schroeder and Falk 1995, Davies 1997, Felton et al. 1997). Type V lesions or fibroatheromas, have a prominent fibrous cap of connective tissue between the lipid core and endothelium, and are thus less prone to

rupture. They cause various narrowing of arteries and are often symptomatic due to reduced blood flow of the affected tissue or organ. Fibroatheromas often progress to type VI complicated lesions that show hematoma, hemorrhage or thrombosis. These lesions are unstable and may cause acute complications of atherosclerosis, such as MI and stroke. On the other hand, if the thrombogenic stimulus is relatively limited, it may only lead to local plaque growth. (Stary et al. 1995, Fuster et al. 1992a, Fuster et al. 1992b, Stary 2000).

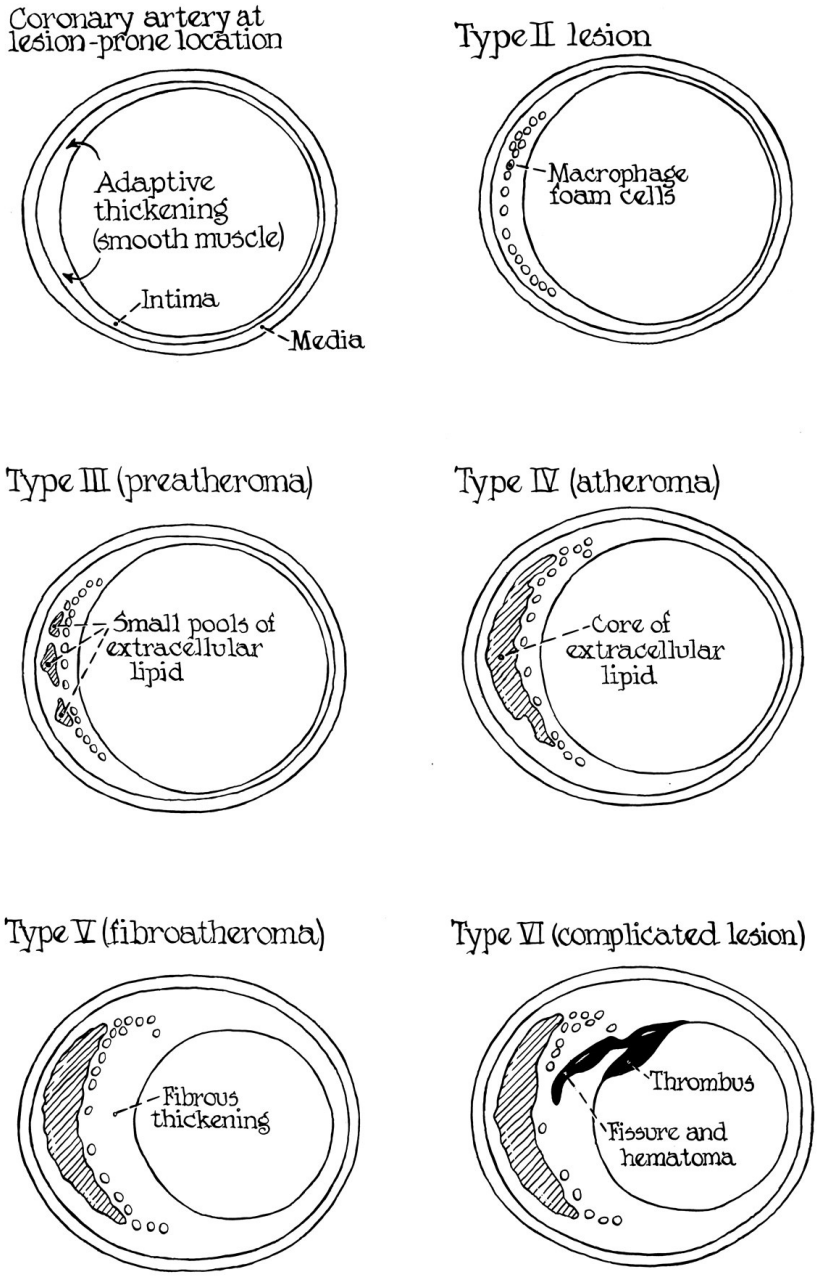


Figure 2. Cross-sections of atherosclerotic lesion types I-VI (Stary et al. 1995).

2.3. Estrogens

2.3.1. Estrogens and the cardiovascular system

The incidence of CHD in premenopausal women is about one-half of that in men of similar age (Ross et al. 1981, Nabulsi et al. 1993, Chae et al. 1997). Since the incidence of CHD rises soon after menopause, estrogens may play a fundamental role as cardioprotective agents (Barret-Connor and Bush 1991, Mendelsohn and Karas 1994, van der Schouw et al. 1996, Hu et al. 1999). Estrogens suppress arterial SMC proliferation and migration (Dai-Do et al. 1996), and they increase the synthesis of collagen and elastin in the arterial intima (Fischer and Swain 1985). Estrogens reduce the accumulation of cholesterol in the arterial wall (Haarbo et al. 1991, Wagner et al. 1991), and protect LDL from oxidation in vitro (Huber et al. 1990).

The proposed cardioprotective effects of estrogen are thought to be mediated both by alterations of lipoprotein/lipid profiles and by direct effects on the vessel wall, estrogens i.e. display endothelotherapeutic functions (Mendelsohn 2002). Estrogens are known to increase HDL cholesterol and reduce both total and LDL cholesterol (Walsh et al. 1991, Stevenson et al. 1993, Davis et al. 1994, Tremollieres et al. 1999, Tikkanen et al. 1979), thus generating a favourable lipid profile. On the other hand, it has been argued that the age-related changes in lipids might be due to differences in also other sex hormones, such as testosterone (Rossouw 2002, Smiley and Khalil 2009). Summing up so far, these effects in serum lipids have been estimated to account for only approximately one third of the atheroprotective effects associated to estrogen (Bush et al. 1987, Mendelsohn and Karas 1994).

Direct vascular effects of estrogen on the vessel wall are in part mediated by estrogen–induced changes in gene expression (Vargas et al. 1993, Weiner et al. 1994,

Farhat et al. 1996, Chen et al. 1999). Estrogen has been shown to alter the expression of a number of genes in vascular wall cells, including endothelial nitric oxide synthase, cyclo-oxygenase, prostacyclin synthase, inducible nitric oxide synthase, endothelin-1, collagens, matrix metalloproteinases, vascular cell adhesion molecule, vascular endothelial growth factor, elastin, c-fos and progesterone receptor (reviewed by Mendelsohn and Karas 1999). Furthermore, estrogens have been proposed to act also by rapid "nontranscriptional" signaling actions independent of the synthesis of mRNA. At this level, estrogen triggers rapid vasodilatation, exerts anti-inflammatory effects, stimulates endothelial growth, migration and repair, and protects the vessels from atherosclerotic degeneration (Simoncini et al. 2006). Some proposed nongenomic and genomic vascular effects of estrogens are shown in Figure 3.

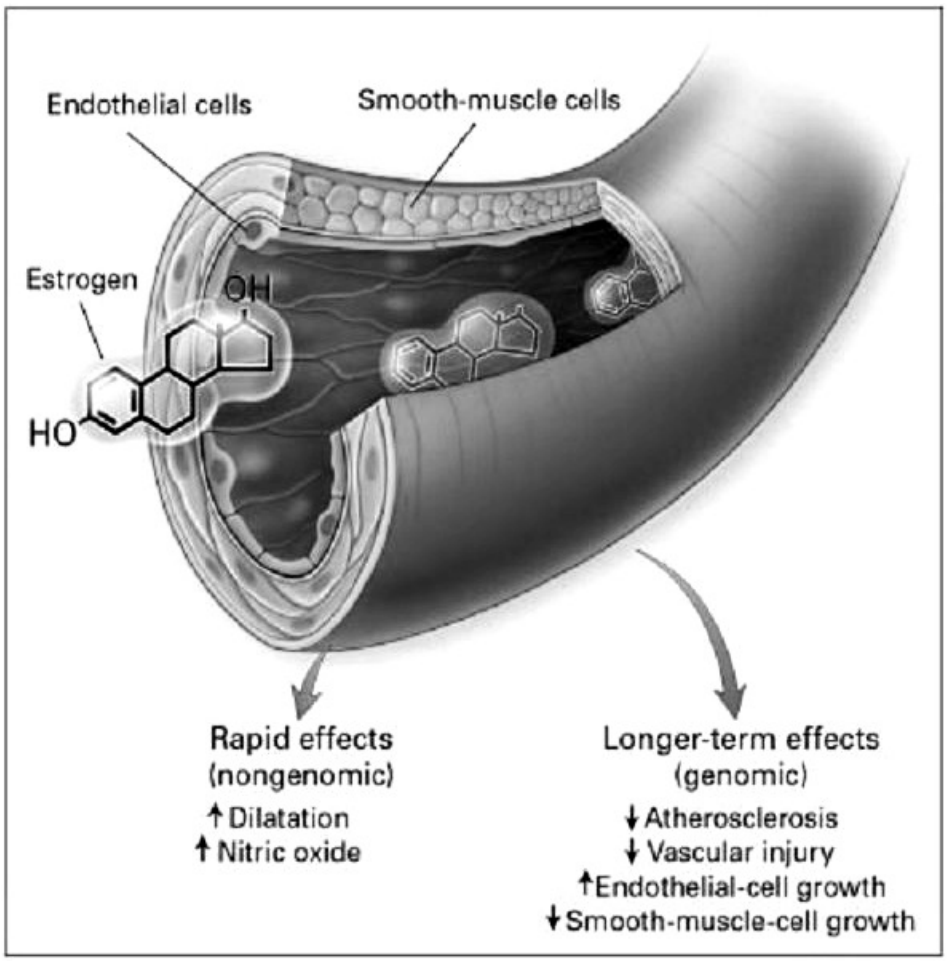


Figure 3. Estrogenic effects on atherosclerosis (Mendelsohn and Karas 1999).

2.3.2. Hormone replacement therapy (HRT) and health

Generally, HRT is used for two primary reasons. Firstly, HRT is used in premenopausal women with certain health conditions, for example in premature ovarian failure (De Vos et al. 2010). HRT helps in maintaining bone health (Pinkerton and Stovall 2010) and reducing the risk of heart disease (Jeanes et al. 2007). Secondly, menopausal and post-menopausal women use HRT to reduce some of the menopausal symptoms, such as hot flashes, night sweats, vaginal dryness and sleep disturbances. It is considered that HRT taken by women with certain health conditions is different than that taken by post-menopausal women. The risks associated with post-menopausal HRT do not apply to pre-menopausal women taking HRT (Writing Group for the Women's Health Initiative Investigators 2002).

Previous studies have suggested that HRT is beneficial with respect to health in postmenopausal women (Ross et al. 1981, Nabulsi et al. 1993, Chae et al. 1997, Stampfer and Colditz 1991, Stampfer et al. 1991, Punnonen et al. 1995, Grady et al. 1992, Grodstein et al. 1997, Mijatovic et al. 1997). However, this concensus is far from clear at present. One of the largest studies of HRT, the Women's Health Initiative (WHI) Hormone Replacement study was designed to explore the usefulness of HRT in preventing disease – particularly heart disease (Writing Group for the Women's Health Initiative Investigators 2002). It was halted in 2002 because women taking the hormones after menopause had a greater risk of breast cancer, heart attack, stroke and blood clots than those who did not take the drugs (Writing Group for the Women's Health Initiative Investigators 2002). However, there were also benefits of estrogen plus progestin compared to those who did not take the hormones, including fewer cases of hip fractures and colon cancer.

Recently Stevenson et al. evaluated the WHI study. They state that the initially published results suggested overall harm from HRT, leading to a dramatic worldwide decrease in its use, and concerns from clinicians and regulatory authorities. Subsequent publications with more detailed analyses appear to have countermanded these initial concerns. Analyses of the studies have not been adherent to those specified in the original published protocol. Initially reported as showing a significant increase in adverse health events with HRT, in a subsequent analysis of the full data the increase was no longer significant. The writers suggest that the raw data should be made available for independent assessment to obtain valid conclusions which may again change clinical practice (Stevenson et al. 2009).

2.3.3. HRT and the development of atherosclerosis

The use of HRT to prevent cardiovascular disease (CVD) has been an area of controversy in recent years. Many animal studies show a clear atheroprotective benefit of estrogen. Estrogen treatment of cholesterol-fed primates and rabbits inhibits the development of atherosclerosis from a 35 % up to an 80 % reduction in lesion size measured in aortic and coronary arteries (Hodgin and Maeda 2002). Animal studies have suggested that estrogen targets atherogenic processes at early stages of lesion development (Hodgin et al. 2001, Hodgin and Maeda 2002). Likewise, other animal studies have also shown that inhibitory effects of estrogen may be lost once atherosclerotic lesions are established (Williams et al. 1995, Hanke et al. 1999). This proposed mechanism, based on animal studies, might be an explanation to why trials of HRT in women have shown no cardioprotection if started late in menopause (Herrington et al 2002, Rossouw et al. 2002).

Most epidemiological studies suggest useful effects of estrogens on the risk of CVD in postmenopausal women (Ross et al. 1981, Stampfer and Colditz 1991, Stampfer

et al. 1991). Although numerous findings from non-randomised experiments show that postmenopausal estrogen therapy slows the development of atherosclerotic disease (Mendelsohn and Karas 1999, Farhat et al. 1996), estrogens have not been shown to slow atherosclerotic progression in women in randomised clinical trials (Hulley et al. 1998, Herrington 1999). It seems that the discrepancies in results between observational and randomized studies from cardioprotective point of view might be explained by differences in selection of participating women, hormone preparations and time elapsed since menopause. Indeed, in a re-analysis of postmenopausal women in the Nurses' Health Study, women beginning HRT near menopause had a significantly reduced risk of CHD, again confirming CHD benefit with HRT in younger women (Grodstein et al. 2006).

2.3.4. Effects of HRT on lipids

Before menopause, serum LDL cholesterol levels are lower and HDL cholesterol levels higher in women compared with men of the same age. After menopause, the levels of LDL cholesterol rise and HDL cholesterol levels decline (Stevenson et al. 1993, Campos et al. 1988, Brown et al. 1993). Postmenopausal estrogen therapy has favorable effects on serum lipoprotein concentrations (Mendelsohn and Karas 1999, Farhat et al. 1996). Orally administered estrogens favorably reduce serum levels of LDL cholesterol and increase levels of HDL cholesterol, each by about 15%, but unfavorably increase levels of triglycerides by 20% to 25% in postmenopausal women (Tikkanen et al. 1978, Granfone et al. 1992, Lobo 1991, Walsh et al. 1991, The Writing Group for the PEPI Trial 1995, Koh and Sakuma 2004). Postmenopausal HRT ordinarily involves estrogen combined with progestin. In Finland, the progestins that are currently combined with estrogen for sequential HRT are norethisterone, medroxyprogesterone and dydrogesterone. It is known that some progestins have unfavorable effects on lipid metabolism, while some do not

(Barnes et al. 1985, Hirvonen et al. 1981, Sonnendecker et al. 1989). Androgenic progestogens of the 19-nortestosterone series (not used in Finland) reverse the beneficial effect of postmenopausal estrogen treatment on HDL cholesterol, whereas the hydroxyprogesterone derivative medroxyprogesterone has no such effect (Hirvonen et al. 1981). Recently it has also been shown that RCT is enhanced by HDL-associated estradiol esters (Badeau et al. 2009).

2.3.5. HRT and oxidized LDL

Modification of LDL in the intima is thought to play an important role in the progression of atherosclerosis (Parthasarathy et al. 1992). Some form of modification of LDL is a prerequisite for rapid accumulation of LDL in macrophages and for the formation of foam cells. LDL isolated from atherosclerotic lesions, but not from normal arteries, resembles oxidized LDL in its physical, chemical, and immunological properties (Ylä-Herttuala et al. 1988, Ylä-Herttuala et al. 1994). Epitopes displaying characteristics of oxidized LDL can be found in atherosclerotic lesions by immunocytochemical techniques (Palinski et al. 1989, Ylä-Herttuala et al. 1989) and furthermore atherosclerotic lesions contain immunoglobulins that recognize oxidized LDL (Ylä-Herttuala et al. 1994, Palinski et al. 1989). In addition, antioxidant therapy reduces atherogenesis in animal models (Carew et al. 1987, Kita et al. 1987). Antibodies against malondialdehyde- or copper modified LDL, detected by radioimmunoassay, have been reported to be predictive of the progression of carotid atherosclerosis (Salonen et al. 1992), CAD (Lehtimäki et al. 1999) and MI (Puurunen et al. 1994). Furthermore, antibodies against copper-oxidized LDL are associated with impaired endothelial function and early atherosclerotic changes (Heitzer et al. 1996, Raitakari et al. 1997, Lehtimäki et al. 1999). However, HRT has not been shown to influence oxLDL antibody titers (Heikkinen et al. 1998).

2.4. Estrogen receptors (ESR)

2.4.1. Types of ESR

Currently, two nuclear estrogen receptors, estrogen receptor alpha (ERα or ESR1) (Green et al. 1986) and estrogen receptor beta (ERβ or ESR2) (Mosselman et al. 1996) are known in humans. In addition, an estrogen membrane receptor coupled to a G protein (GPR30, G-protein-coupled receptor 30) (Thomas et al. 2005, Revankar et al. 2005) has been identified in human arteries and veins (Haas et al. 2007). ESR1 is expressed in vascular endothelial cells (Kim-Schulze et al. 1996, Venkov et al. 1996) and SMCs (Karas et al. 1994). ESR1 activates specific target genes in vascular smooth muscle (Karas et al. 1994), inhibits SMC migration (Kolodgie et al. 1996, Bhalla et al. 1997) and accelerates endothelial cell growth in vitro (Morales et al. 1995) and vivo (Krasinski et al. 1997, Venkov et al. 1996). ESR numbers are higher in females because estrogen production induces their expression (Mendelsohn and Karas 1999). However, atherosclerotic coronary arteries of premenopausal women have fewer ESR1 compared to normal arteries (Losordo et al. 1994).

2.4.2. Genotypes of the ESR1 and coronary artery disease

Allelic variants of the ESR1 gene may have an effect on the amount and function of the expressed receptor. Therefore, it is possible that the effects of estrogens on vascular cells, mediated by ESR1, differ due to the ESR1 variant forms that have different transcriptional effects than the 'wild-type' receptor (Matsubara et al. 1997, Maruyama et al. 2000). The most investigated ESR1 polymorphic sites, associated with risk of CVD, are three tightly

linked polymorphisms, namely c.454-397 T/C (PvuII, rs2234693), c.454-351 A/G in intron 1 (XbaI, rs 9340799), and the $(TA)_n$ VNTR (variable number of tandem repeat) in the promoter region.

The c.454-397T/C genotype (PvuII, rs2234693) consists of a two-allele polymorphism for PvuII restriction enzyme, leading to genotypes P/P, P/p, and p/p (Yaich 1992). The capital letters are used to signify the absence of restriction sites (mutated) and small letters the presence of restriction sites (wild type). These genotypes are referred to by -397(PP), (Pp), and (pp), or (CC), (CT), and (TT), respectively. The PvuII polymorphism is caused by a T-to-C transition in intron 1, and located approximately 0.4 kb upstream of exon 2 (Yaich 1992). This polymorphism is intronic in nature, and its mechanism of action is not clear. It has been speculated that the PvuII polymorphism may alter transcription factor binding and affect expression level of the ESR1 protein (Shearman 2006).

It has been reported that men with the C-allele of the c.454-397T/C polymorphism had more severe CAD compared to men with the TT genotype (Lehtimäki et al. 2002). The Framingham study and another large follow-up study also reported an increased risk of MI among men with the CC genotype (Shearman et al. 2003, Shearman et al. 2006). On the other hand, two large case-control studies from Denmark (Kjaergaard et al. 2007) and Germany (Koch et al. 2005) failed to detect an increased risk of MI with the −397T/C genotype. A weakness of the German study was that the control subjects were not healthy since all had some indication for coronary angiography. Likewise, in sub-analysis of younger men in the Danish case-cohort study, an increased risk of MI in the CC genotype group was found. Even more recently, it has been shown at the population level, using a case-cohort design, that in men, the minor CC genotype of the ESR1 −397T/C polymorphism contributed to a higher risk of CHD, compared to those with the T-allele

(Kunnas et al. 2010). In conclusion, current data supports the view that homozygosity for allele −397C of the ESR1 gene (CC) contributes to the risk of CHD.

2.5. Alcohol

2.5.1. Gamma-glutamyl transferase (GGT) and carbohydrate-deficient transferrin (CDT) as markers of ethanol consumption

Serum CDT and GGT (also known as gamma-glutamyl transpeptidase) are generally considered to be useful laboratory markers for high alcohol consumption (Mihas and Tavassoli 1992, Stibler 1991, Sillanaukee 1996). CDT is currently considered to be the most useful marker of alcohol misuse (Hannuksela et al. 2007). Transferrin is a monomeric, iron-binding glycoprotein, which is synthesized in the liver. Chronic alcohol consumption leads to deficiencies in the carbohydrate content of the protein by a yet unknown mechanism, leading to increases in serum concentrations of CDT (De Jong et al. 1990). The exact mechanisms are not fully understood, but ethanol is thought to affect both protein transport and enzyme activities (Hannuksela et al. 2007). The use of an array of methods for measurement of CDT either in absolute or relative amounts, and possibly covering different transferrin glycoforms, has complicated the comparability of results (Helander et al. 2010).

GGT is known to reflect liver function and its activity in serum may be increased by alcohol and other liver microsomal inducing agents, in most hepatobiliary disorders, obesity, diabetes mellitus and hypertriglyceridemia (Sabesin 1981). Elevation of GGT in serum probably reflects its enhanced hepatic synthesis rate, increased transport to the liver plasma membranes, as well as liver plasma membrane injury (Teschke and Koch 1986,

Nakajima et al. 1994). Release of GGT may be induced by toxic substances (including alcohol), as a result of ischemia, or by damage to hepatocytes due to infection (Hannuksela et al. 2007).

Both CDT and GGT are markers of alcohol consumption, but their serum values do not correlate with each other (Litten et al. 1995, Helander et al. 1996) and may, thus, reflect different drinking patterns. For men, CDT levels respond to number of days drinking, whereas GGT responds to drinks per drinking day. For women, both CDT and GGT were influenced more by drinks per drinking day than by number of days drinking (Anton et al. 1998). However, although widely used, neither GGT nor CDT is sensitive and specific enough to determine the degree of alcohol abuse or its medical complications (Niemelä 2007). GGT and CDT may be combined as the marker gamma-CDT (= 0.8 * ln (GGT) + 1.3 * ln (CDT)), which appears to show better sensitivity, specificity and a stronger correlation with the amount of alcohol intake than other markers (Hannuksela et al. 2007). New biomarkers that may possibly gain foothold in clinical work in the future include phosphatidylethanol, fatty acid ethyl esters, ethyl glucuronide, sialic acid, and acetaldehyde adducts (Hannuksela et al. 2007).

2.5.2. Effects of ethanol on cardiovascular disease

Several studies have shown that moderate consumption of alcohol reduces mortality from vascular diseases (Doll 1997) and reduces the risk of atherosclerosis (Kannel and Ellison 1996). There seems to be a J-shaped association between alcohol consumption and coronary heart disease incidence events (Moore and Pearson 1986, Langer et al. 1992). A recent study suggests that the cardiovascular benefits that may be derived from light-to-moderate alcohol consumption are not mediated through reduced calcium accumulation

(McClelland et al. 2008). Alcohol intake may reduce blood coagulation (Gorinstein et al. 1997). Moderate alcohol consumption is associated with lower levels of several coagulation factors, namely fibrinogen, factor VII and von Willebrand factor (Lee and Lip 2003). On the other hand, excessive alcohol use and alcoholism have detrimental effects on the cardiovascular system and are associated with increased occurrence of stroke, abdominal aneurysms, hypertension, alcoholic cardiomyopathy, arrhythmias, as well as increased CHD (Regan 1990, Ahlawat and Siwach 1994, Klatsky 1987, Knochel 1983, Lip and Beevers 1995).

2.5.3. Effects of ethanol on the metabolism of lipids and lipoproteins

Ethanol has effects on lipoprotein metabolism in several different phases: acetate formed from ethanol acts as a substrate in hepatic triglyceride synthesis, it modulates apolipoprotein synthesis and the activity of the central enzymes of lipoprotein metabolism (ie. lipoprotein lipase, hepatic lipase, cholesteryl ester transfer protein and phopholipid transfer protein) (Hannuksela et al. 2004). Furthermore, ethanol may increase insulin sensitivity (Avogaro et al. 2004). Acetaldehyde, as well as antioxidative reagents found in some alcohol beverages, modify lipoproteins (Frohlich 1996). The unfavorable effects of alcohol on lipoprotein metabolism include hypertriglyceridemia and fatty liver, and in the later phase, hypercholesterolemia and decreased HDL cholesterol (Sabesin 1981).

The beneficial effects of alcohol may partly be mediated by its effects on lipoprotein metabolism, since moderate alcohol consumption has generally been associated with an increase of HDL cholesterol (Glueck 1985, Angelico et al. 1982) and a decrease of LDL cholesterol (Kervinen et al. 1991). Moderate alcohol consumption stimulates apolipoprotein AI secretion by hepatocytes and alters enzymatic activity of several plasma proteins and enzymes involved in lipoprotein metabolism (Hartung et al.

1990, Amarasuriya et al. 1992, Clevidence et al. 1995, Hannuksela et al. 2002). The increase in HDL cholesterol appears to account for approximately half of alcohol's cardioprotective effect (Langer et al. 1992, Hannuksela et al. 2002). Furthermore, environmental and genetic factors may modulate the effects of ethanol on plasma lipids. These include type of alcoholic beverage, lifestyle, drinking pattern, smoking, diet, exercise, liver disease, gender, apoE and cholesteryl ester transfer protein genotype (Hannuksela et al. 2002, Hannuksela and Savolainen 2001).

The alcohol-induced increase in HDL cholesterol has been usually taken as an indicator of a high rate of RCT from peripheral tissues to the liver (Barter et al. 2003). However, HDL are heterogeneous populations of lipoprotein particles (HDL2, HDL3). The increased cholesterol efflux potential of HDL2 may be the anti-atherogenic feature of RCT linked to heavy alcohol consumption (Mäkelä et al. 2008). The effects of alcohol intake on different HDL subclasses are variable (Hannuksela et al. 2002, Sillanaukee et al. 1993a). Chronic alcohol intake appears to have a raising effect on HDL2 cholesterol and lipase activities in both men and women. The protein concentration in HDL2 is increased, while the HDL3 protein concentration is often unchanged in alcoholics. That is why chronic alcohol intake results in a shift towards larger, less dense HDL2 particles. Alcohol withdrawal is associated with a shift to smaller HDL3 particles (Hannuksela et al. 2004). However, there is evidence that larger HDL particles predict the capacity of HDL particles to accept cholesterol from macrophages (Fournier et al. 1997, Matsuura et al. 2006, Vikstedt et al. 2007). Recent advances in lipoprotein research have shown that in addition to its role in RCT, HDL has multiple anti-atherogenic functions such as anti-infectious, anti-thrombotic, anti-oxidative and anti-apoptotic activity. HDL is also important in endothelial repair and increases vasodilatation (Assmann and Gotto 2004, Hannuksela et al. 2004, Ansell et al. 2006).

3. AIMS OF THE STUDY

1) To investigate possible effects of postmenopausal hormone replacement therapy on the oxidation of LDL and progression of cardiovascular diseases [I].

2) To look for associations between the genotype of estrogen receptor 1 and progression of cardiovascular diseases in postmenopausal women [II].

3) To study the effects of alcohol consumption on plasma lipid profile in relation to laboratory biomarkers of alcohol use. These topics were first studied in a pilot study of a group of 70 men [III] and subsequently in a large population study [IV].

4. SUBJECTS, MATERIALS AND METHODS

4.1. Subjects

4.1.1 Women from annual routine gynecological examinations (I, II)

Women attending a private outpatient clinic in Tampere for annual routine gynecological examinations were invited to participate. For the cross-sectional baseline study in 1993 (Punnonen et al. 1995), 141 non-smoking, non-diabetic postmenopausal women aged 45–71 years were enrolled. They had no clinically evident CVD or hypertension and were classified into four groups based on the monthly use of HRT. The HRT-EVP group (n=40) used estradiol valerate (EV) 2 mg per day for 11 days followed by EV continued with progestin (P, levonorgestrel 0.25 mg per day) for 10 days. The HRT-EVM group (n=21) used estradiol valerate (EV) 2 mg per day for 11 days followed by EV continued with progestin (M, medroxyprogesterone acetate 10 mg per day) for 10 days. The HRT-EV group (n=40) used EV alone, and the control group (n=40) had never used HRT. In HRT-EVP, HRT-EVM and HRT-EV groups there was a pause of therapy for 7 days after each 21-day cycle. Of these 141 women 91 (60 in HRT group, 31 controls) participated in 5-year follow up study from 1993 to 1998. HRT, when used, was started at the time of menopause for climacteric symptoms. In the control group, the main reasons not to use HRT were the absence of vasomotor and other climacteric symptoms and dislike of HRT. The mean duration of EVP and EVM was 9.3±3.2 years and of EV treatment 9.9±4.2 years at the beginning of the study. The mean time from menopause in the control group was 11.9±4.1 years (mean 9.9±3.8 years, at baseline). The mean ages in the HRT-EVP, HRT-EVM, HRT-EV, and control groups were 59.6±4.7, 55.9±3.4, 61.0±5.0, and 61.6±5.5 years, respectively (P=0.0002 over all groups in analysis of variance, ANOVA).

The mean body mass indexes were (BMI, mean 25.3±3.2 kg/m2) similar in all studied groups (P=0.8941 over all groups in ANOVA). In the HRT-EV, HRT-EVP, and control groups 24, 4, and 6 women underwent hysterectomy, respectively, due to benign conditions and 4, 2, and 2 women bilateral salpingo-oophorectomy. At baseline, all women were clinically healthy, and used no chronic medication. Nutrient intake analyses were also performed, as described elsewhere (Punnonen et al. 1995), and these analyses did not show any marked differences between the study groups in the amount of used saturated, monounsaturated, and polyunsaturated fats, or dietary cholesterol. Sonography and blood sampling were done in the University Hospital of Tampere. The Ethics Committee of the University Hospital of Tampere approved the study.

4.1.2. Men from an occupational health survey (III)

Alcohol and lipid profile-related laboratory tests were carried out in 70 consecutive non-alcoholic male employees (mean age 45 years, range 37-58 years, SE 0.7 years) in connection with an occupational health survey in 1996. The employees were both blue-collar and white-collar workers from a board machine manufacturing factory.

4.1.3. Men and women from the 1997 Finrisk study (IV)

The National Public Health Institute of Finland has performed large cross-sectional population surveys, the FINRISK studies, related to the risk factors of CAD every 5 years beginning in 1972 (Puska et al. 1995). The 1997 FINRISK study was conducted in five geographic areas: Helsinki–Vantaa region in Southern Finland, Turku–Loimaa region in Southwestern Finland, Kuopio and North Karelia provinces in Eastern Finland, and Oulu province in Northern Finland. In each study area, an age- and gender-stratified random

sample of 2000 subjects was drawn from the population aged between 25 and 64 years. In addition, a random sample of 500 men and 250 women was drawn in Helsinki–Vantaa region and in North Karelia province from the 65–74 years age-group.

Our study was a sub-study of the 1997 FINRISK project. The total sample size was 11500. The participation rate was 71 % among men and 76 % among women. Subjects who had missing data on serum lipids, GGT or CDT, who had diabetes, who were pregnant or who were using cholesterol lowering medication or hormonal treatment were excluded. Thus, 3097 males and 2578 females were included in the study. The study was conducted according to the Helsinki Declaration of 1975 on Human Experimentation and was approved by the Ethical Committee of Primary Health Clinics in Finland. All participants gave informed consent to scientific use of the data and samples collected in the study.

4.2. Blood samples (I-IV)

Blood samples were taken after the subjects had fasted overnight (I-III), or at least 4 hours (IV). After separation of serum by low-speed centrifugation the sera were divided into aliquots and stored at −70°C until analyzed.

4.2.1. Lipid analyses (I-IV)

In studies I and II serum total cholesterol and triglycerides were determined by commercial methods (Kodak Echtachem 700XR, Eastman Kodak Company, Clinical Products Division, Rochester, USA). Serum HDL cholesterol was separated by a dextran–sulfate–Mg precipitation procedure (Nquven 1989) and the cholesterol content was analyzed with a Monarch 2000 Analyzer (Instrumentation Laboratory, Lexington, USA),

using the CHOD-PAP cholesterol reagent (Cat No. 237574; Boehringer Mannheim, Germany) and a primary cholesterol standard (Cat No. 530; Orion, Finland). The LDL cholesterol concentration was calculated according to the Friedewald formula (Friedewald 1972). Apolipoproteins (apo) A1 and B were determined on a Monarch Analyzer by an immunoturbidimetric method (Riepponen 1987) (Cat No. 67265 and 67249, Orion Diagnostics, Finland). In the 5-year follow-up study the lipid concentrations were determined with Cobas Integra 700 analyzer with reagents and calibrations recommended by the manufacturer (Hoffmann-La Roche Ltd., Basel, Switzerland). In studies III and IV, total and HDL cholesterol were determined from fresh serum samples using CHOD-PAP. Triglycerides were measured by a fully enzymatic method (GPO-PAP, Boehringer-Mannheim).

4.2.2. Determination of autoantibodies against oxLDL (I)

Antigens for this assay included: (A) native LDL prepared from the pooled plasma of ten donors and protected against oxidation by 0.27 mmol/l EDTA and 20 mol/l butylated hydroxytoluene (BHT) in phosphate buffered saline (PBS); and (B) oxidized LDL obtained after 24-h oxidation of the native LDL with 2 mol/l $CuSO_4$. For enzyme-linked immunosorbent assay, half of the wells on a polystyrene plate (Nunc, Roskilde, Denmark) were coated with 50 µl of native and the other half with 50 µl copper-oxidized LDL antigen (both at a concentration of 5 g/ml) in PBS for 16 h at 4°C. After removal of the unbound antigen and washing of the wells, the remaining non-specific binding sites were saturated using 2% human serum albumin (HSA) in PBS and 20 mol/l BHT for 2 h at 4°C. After washing, 50 µl of the serum samples, diluted 1:20, were added to wells coated with native LDL and oxidized LDL and incubated overnight at 4°C. After incubation the wells were aspirated and washed six times before an IgG-peroxidase conjugated rabbit

anti-human monoclonal antibody (Organon, USA No. 55220 Cappel), diluted 1:4000 (v/v) in buffer (0.27 mmol/l PBS, 20 mol/l EDTA, 1% BHT, 0.05% Tween HSA), was added to each well for 4 h at 4°C. After incubation and washing, 50 µl of freshly made substrate (0.4 mg/ml o-phenylenediamine (Sigma) and 0.045% H_2O_2 in 100 mmol/l acetate buffer, pH 5.0) was added and incubated exactly 5 min at room temperature. The enzyme reaction was terminated by adding 50 µl of 2 M H_2SO_4. The optical density (OD) was measured at 492 nm using a microplate reader. All measurements were blinded and done on coded serum samples. The results were expressed as the mean OD values from duplicate determinations, and autoantibody titer against oxidized LDL was calculated by subtracting the binding of antibodies to native LDL from that to copper-oxidized LDL. This approach reduces the possibility of getting false positive values due to cross-reactivity with both LDL epitopes.

4.2.3. DNA extraction and ESR1 genotyping (II)

DNA was isolated from white blood cells using a commercial kit (Qiagen Inc, CA). A region of the ESR1 gene containing a part of intron one and exon two was amplified using primary and secondary (nested) primers designed from those reported by Yaich et al. (Yaich 1992). After digestion of the PCR product with PvuII restriction endonuclease, fragments were separated using agarose gel (1.0%) electrophoresis. Capital (P, mutated) and small (p, wild type) letters denoted the absence and presence of the restriction sites, respectively.

4.2.4. Determination of serum CDT and GGT (III, IV)

CDT concentrations in serum samples were analyzed by a double antibody kit (CDTect™, Pharmacia and Upjohn Diagnostics, Uppsala, Sweden) according to the manufacturer's instructions. The test is based on anion exchange chromatography and radioimmunoassay (Stibler 1991, Stibler et al. 1986) and has a detection limit of 1 U/l and a measuring range of 5–300 U/l. The calibration of the test was assessed from a five-point standard curve derived from the displacement of 125I-CDT from the antibody by known amounts of human transferrin, within the interval 0–100 U/l. CDTect gives the results in units per liter, which is comparable to mg/l in serum (1 U CDT refers to ≈1 mg transferrin). The reference values for CDT (CDTect™) were <20 units/L for men and <26 units/L for women. The activity of serum GGT (reference value <80 IU/L for men and <50 IU/L for women) was determined according to the recommendations of the Scandinavian Society for Clinical Chemistry and Clinical Physiology (1976).

4.3. Sonography (I-II)

Sonography at baseline and follow-up were performed with Toshiba Sonolayer V SSA 100 equipment, as reported elsewhere (Punnonen et al. 1995). Briefly, all the sonographies were done blinded by one experienced sonographer and radiologist. During the examinations women were lying in a supine position. Transverse and longitudinal scans of extracranial carotid arteries were performed bilaterally at four different segments of the carotid: first, at the 10 mm segment of the common carotid artery (CCA) just distal to the origin of carotid bifurcation, second at a 10 mm segment in the area of distal third of the CCA, third at the 10 mm segment between origin of carotid bifurcation and the tip of the flow divider, which separates internal from external carotid arteries, and fourth at a 10

mm segment of the internal carotid artery cranial from flow divider. Only fibrous and calcified atherosclerotic lesions were considered and were defined as plaques when distinct areas of mineralization or/and focal protrusion into the lumen were identified. The thickness and length of such plaques within the artery vessel wall were determined by transverse and longitudinal scans, respectively, and the thickness of a plaque was determined as the distance between the intimal–luminal interface and the medial–adventitial interface. The intimal–medial far-wall thickness equal to or more than 1.3 mm at any segment in carotid arteries was defined as an atherosclerotic plaque (Furberg et al. 1989) and the total number of plaques was calculated. All carotid artery sonographies were done with a 5.0 MHz convex transducer probe.

Longitudinal sonographs of the abdominal aorta were obtained at 1 cm intervals and transverse scans at 2 cm intervals at the area of three aortic segments: (1) supra-pancreatic; (2) pancreatic and infra-pancreatic; and (3) at the area of the aortic bifurcation. As for carotid plaques, significant aortic plaques were defined as an intimal–medial far-wall thickness equal to or more than 3.0 mm (Furberg et al. 1989). All aortic examinations were performed using a 3.75 MHz convex transducer probe. The average duration for the whole examination varied from 25 to 30 min.

The atherosclerotic severity sum (AS) was constructed by dividing the atherosclerotic changes of abdominal aorta and carotid arteries into three severity classes: 1=slight, 2=moderate, and 3=severe, and calculating the sum, i.e. AS. Total number of atherosclerotic plaques (NAP) was subsequently calculated.

The reproducibility of the sonographic protocol for significant aortic and carotid plaques was also examined: 1 month after the first assessment 20 randomly selected subjects were invited to a repeated examination. The reproducibility of the number of plaques (number of plaques initial by repeated sonography) between the first and second

examination was 90% for the carotid artery segment areas and 100% for the aortic segments.

4.4. Estimation of alcohol intake (III)

Estimation of the alcohol intake was carried out by Alcohol Use Disorders Identification Test (AUDIT) by WHO (Saunders et al. 1993, Seppä et al. 1995).

4.5. Quantitative estimation of alcohol intake, smoking and physical activity (IV)

Quantitative estimation of alcohol intake was carried out by using 12 structured questions to determine the amount and frequency of drinking. The measure for average weekly intake included the following drinks: beer, cider, liquor, long drink and wine. The total mean consumption of all alcoholic drinks was expressed as grams of pure ethanol per week. The variable 'number of smokes per day' was computed by adding up the numbers of filter cigarettes, non-filter cigarettes, self-made cigarettes, pipe-fulls and cigars smoked on an average day for all subjects who reported having smoked within 1 month prior to the survey. 'Physical activity' was determined as a session of leisure-time exercise resulting in shortness of breath and sweating (times/week).

4.6. Statistical analyses (I-IV)

All calculations were done with the Statistica for Windows version 5.1 (Statsoft Inc., Tulsa, Oklahoma, USA) software in PC. P-values of less than 0.05 were considered statistically significant.

In study I, two-way analysis of covariance (using age, BMI, and total cholesterol as covariates, when appropriate) was used to assess the interaction between grouping factor, HRT groups and control group, in selected variables of interest (=dependent variables) i.e. AS, total NAP, serum lipids, and apolipoproteins. Two- and one-way ANOVA was used to assess the statistical differences between HRT groups and control group in lipid parameters. Age and BMI were used as covariates when appropriate. Pairwise comparisons of group means were done by using ANOVA, age and BMI as covariates, when appropriate. Multiple regression was used in search for the variables that predict the severity of atherosclerosis. Repeated measures ANOVA was used to study the effect of time and HRT on AS in our study population.

In study II, two-way ANOVA was used to assess the interaction between ESR1 PvuII genotype groups and treatment groups. One-way ANOVA was used to assess the statistical differences between HRT groups and control group and among ESR1 PvuII genotypes in lipid parameters. Repeated measures ANOVA was used to study the effect of time, ESR1 PvuII genotype and HRT on AS in the study population.

In studies III and IV, subjects were divided into quartiles on the basis of CDT concentration or GGT activity. Estimated significance was based on ANOVA.

5. RESULTS

5.1. Effects of postmenopausal hormone replacement therapy on oxidation of LDL and progression of cardiovascular diseases in a 5-year follow up (I)

The purpose of the study was to determine whether HRT has a beneficial attenuating effect on sonographically determined NAP and AS in the CCA and abdominal aorta of 101 postmenopausal women compared to 40 controls without HRT. The interaction of HRT and antibodies against oxidized LDL on AS and NAP progression was also studied.

The HRT-EVP group used estradiol valerate (EV) followed by EV continued with progestin (P, levonorgestrel), the HRT-EVM group used EV followed by EV continued with progestin (M, medroxyprogesterone acetate), the HRT-EV group used EV alone, and the control group had never used HRT. During the 5-year follow-up, the concentration of HDL cholesterol increased and LDL cholesterol decreased significantly in HRT-EV, HRT-EVP and control groups, and total cholesterol decreased significantly in the HRT-EV group and controls, but not in the HRT-EVP group. The triglycerides increased in HRT-EVP group and controls but not in the HRT-EV group. The HRT-EVM group was not included in the lipid follow-up.

HRT-EV, HRT-EVP and HRT-EVM therapies were each associated with lower AS and NAP as compared to controls without HRT. AS was significantly higher in both the HRT groups and control group after follow-up, compared to baseline. In a multiple regression model explaining NAP in the whole study population, the strongest predictors were HRT (P=0.0006) and copper-oxidized LDL autoantibodies (P=0.0491). What comes to oxLDL autoantibodies, it was evident that they seem to predict NAP in postmenopausal women, but do not seem to affect AS at least during the 5-year follow up time (Figure 4 = Figure 2 from I).

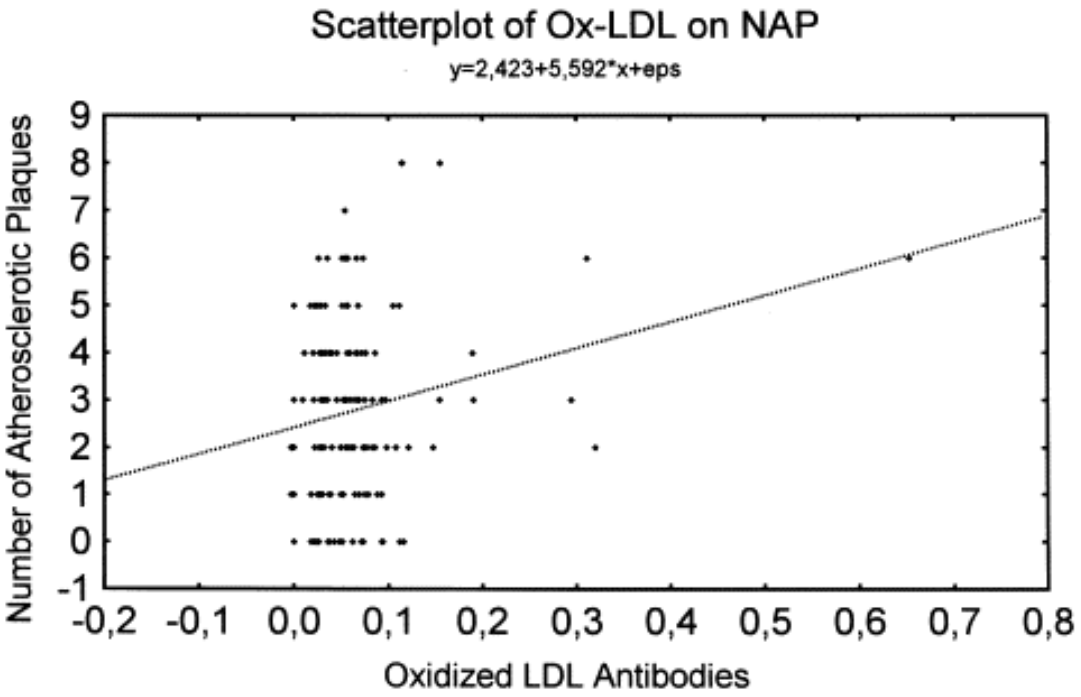


Figure 4. Scatterplot of oxidized LDL antibody titer on X-axis and number of atherosclerotic plaques on Y-axis.

In conclusion, the findings indicated that both estradiol valerate alone, combined estradiol valerate–levonorgestrel, and combined estradiol valerate–medroxyprogesterone acetate therapy are associated with lower total NAP and less severe atherosclerotic lesions, as compared to controls without HRT. This outcome may also be associated with a reducing effect of HRT on LDL oxidation.

5.2. Associations between the genotype of estrogen receptor and progression of cardiovascular diseases in a 5-year follow up (II)

At baseline, the mean age, BMI, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, apoA1 and apoB were not significantly different among the genotypes.

Subjects with the P/P genotype had a higher AS (1.93 ± 0.92) as compared with P/p (1.24 ± 0.85) and p/p (1.43 ± 0.84) genotypes (P=0.012).

*ESR1 Pvu*II genotype, HRT treatment and time had a statistically significant or borderline significant effect on AS during 5-year follow-up (P=0.090, P=0.004 and P<0.001, respectively), when analyzed by repeated measures ANOVA (Figure 5 = Figure 1 from II).

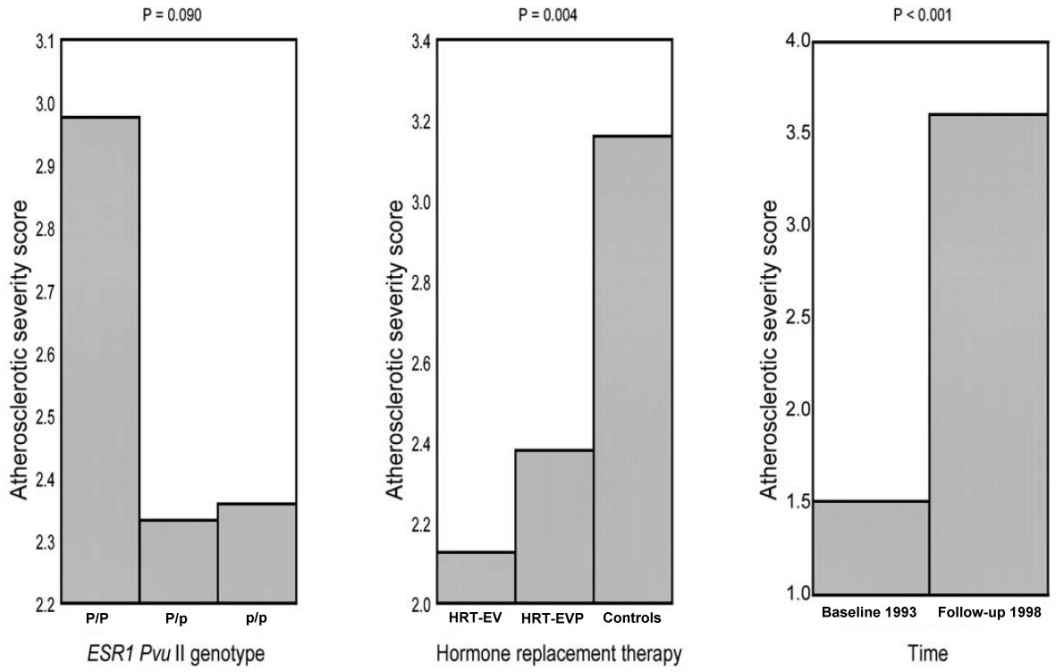


Figure 5. The main effect of *ESR1 Pvu*II genotypes, HRT, and time on atherosclerotic severity score during 5-year follow-up analyzed by repeated measures ANOVA. ESR1 PvuII, estrogen receptor 1 PvuII; HRT, hormone replacement therapy. EV, estradiol valerate, 2.0 mg/day; P, levonorgestrel, 0.25 mg/day. Atherosclerotic severity score is estimated from aorta and carotid artery by sonography. Age was used as covariate.

There was a significant genotype-by-treatment (HRT-EVP and control groups) interaction for AS (P=0.034). In response to HRT-EVP, subjects homozygous for the mutated forms of the receptor P/P, compared with those with P/p and p/p genotypes, had less increase in AS (1.60 ± 1.14 vs. 1.71 ± 1.27 vs. 2.43 ± 1.27). Baseline AS as covariate in similar model did not change the significant interaction effect between HRT-EVP and

control groups (P=0.036). However, this effect was not found between HRT-EV and control groups (Table 1). The results suggest that the effect of HRT-EVP in postmenopausal women on progression of AS may be determined in part by the genotype of *ESR1 Pvu*II.

HRT	**HRT-EVP**			**HRT-EV**			**Controls (no HRT)**		
Genotype (n)	P/P (5)	P/p (14)	p/p (7)	P/P (3)	P/p (19)	p/p (10)	P/P (6)	P/p (13)	p/p (11)
Baseline (SD)	1.80 (0.84)	1.14 (0.95)	1.29 (0.49)	1.33 (1.53)	1.11 (0.66)	1.10 (0.88)	2.33 (0.52)	1.54 (0.97)	1.82 (0.87)
Follow-up (SD)	3.40 (1.67)	2.86 (1.35)	3.71 (1.11)	3.67 (2.08)	3.00 (1.33)	2.50 (1.18)	5.33 (1.75)	4.31 (1.93)	3.73 (1.56)
Change (SD)	1.60 (1.14)	1.71 (1.27)	2.43 (1.27)	2.33 (0.58)	1.89 (1.37)	1.40 (0.84)	3.00 (1.67)	2.77 (1.30)	1.91 (1.14)

Table 1: Baseline and follow-up AS according to *ESR1 Pvu*II genotypes and HRT groups. HRT, hormone replacement therapy; EVP, estradiol valerate and progestin; EV, estradiol valerate alone; P, mutated *ESR1 Pvu*II receptor gene; p, wild type *ERS1 Pvu*II receptor gene.

It is interesting to notice that P/p genotype seemed at baseline to be related to less severe atherosclerosis, and was thus the most beneficial in regard to the development of atherosclerosis. If both alleles were mutated, the severity of atherosclerosis seemed to be increased at baseline. These two findings were trends without statistical significance. Statistically significantly, in the HRT-EVP group, the more mutated alleles the person had, the more she gained benefit of HRT. However, the severity of atherosclerosis increased the most in women with wild-type (p/p) receptor alleles. Interestingly, the trend was just the opposite in HRT-EV group without progestin component, as the wild-type (p/p) receptor carriers seem to benefit the most from estrogen replacement therapy. The

same trend of atherosclerosis progression were seen in both HRT-EV and control groups, as the presence of mutated receptor alleles (P/p and P/P) seemed to be associated with increased AS progression.

In conclusion, one could say, the more mutated *ESR1 Pvu*II alleles a person has, the more she benefits of combined estrogen-progesterone HRT. The same cannot be said about estrogen-only therapy. In HRT-EV and controls, the wild-type receptor gene seemed to be beneficial in regard to atherosclerosis progression. This may have clinical relevance, and it would be possible to evaluate beforehand the benefit of considered HRT based on the *ESR1 Pvu*II genotype.

5.3. Effects of alcohol consumption on the lipid profile (III, IV)

When the men from the occupational health survey (III) were divided into quartiles according to CDT and GGT, the men in the highest CDT quartile had significantly higher serum HDL cholesterol concentration than those in the lowest quartile. The men in the highest GGT quartile had significantly higher serum total cholesterol, lower serum HDL cholesterol, higher serum LDL cholesterol and higher serum triglyceride concentration than the lowest quartile (Figure 6 = Figure 1 from III).

The results obtained from study III were further confirmed in a larger population, in both genders (Table 2; Figure 7, panels A and B). Serum CDT concentrations were positively associated with higher alcohol consumption and smoking and lower BMI in both genders in the FINRISK-study (IV). There was no significant difference in physical activity between the different CDT quartiles. Serum CDT concentrations were negatively associated with age in women, but not in men. Serum HDL cholesterol values were significantly higher and triglycerides lower with increasing serum CDT concentrations for both men and women. These results remained significant even after adjustment for age,

BMI and smoking. Serum total cholesterol decreased with increasing CDT concentrations in women, but not in men. However, this was not significant after adjustment for age, BMI and smoking. Serum CDT concentration and GGT activity were positively associated in men and negatively associated in women, but the negative association in women did not remain significant after adjustment for age, BMI and smoking.

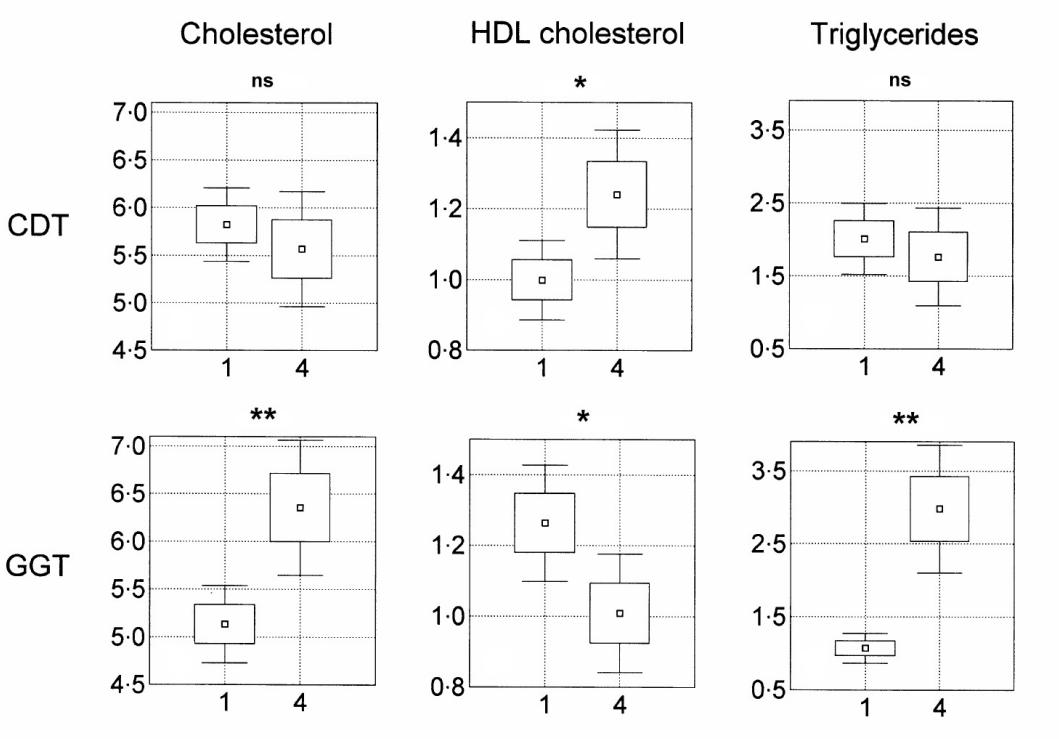


Figure 6. Cholesterol, HDL-cholesterol and triglycerides (mmol/l) in the lowest (1) and highest (4) CDT and GGT quartiles. CDT quartiles: lowest ≤12U/L, highest ≥16U/L. GGT quartiles: lowest < 29U/L, highest ≥75U/L. The larger box, ±1.00 SE; the vertical lines, ±1.96 SE; the small box, mean. Elevated CDT seems to have positive effects on lipid values, whereas elevated GGT seems to have adverse effects on them. Statistical significance *, $p<0.05$; **, $p< 0.01$; ns, not significant.

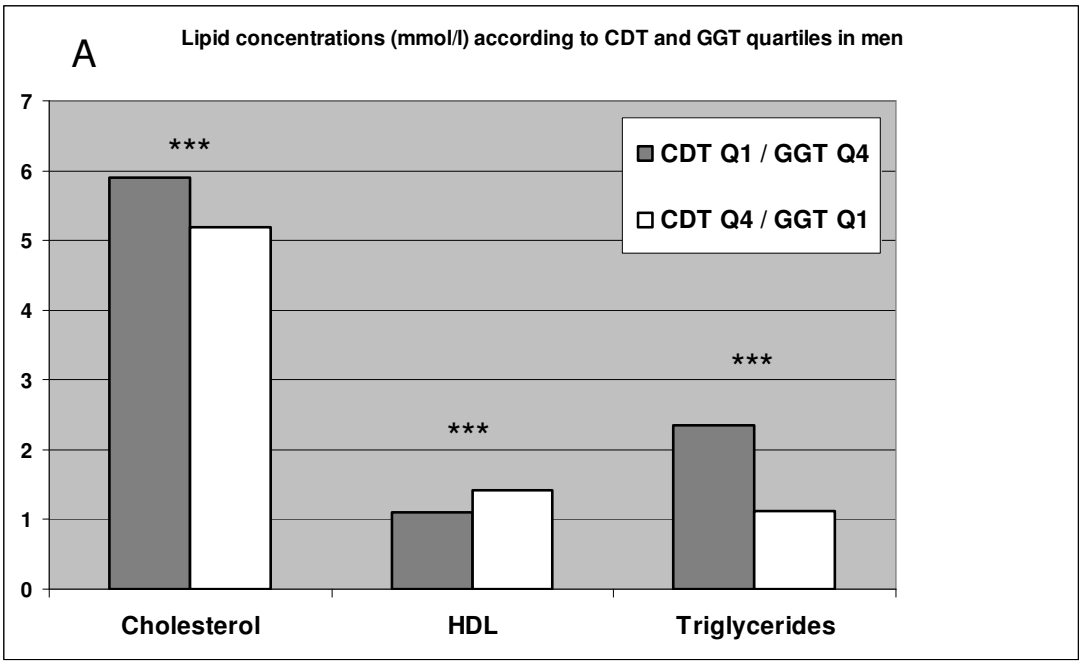
It appears that increased CDT is associated with a favorable lipid profile, including higher HDL cholesterol and lower triglycerides. In contrast, increase in GGT quartiles

appeared to affect the lipid profile unfavorably. There were higher concentrations of total cholesterol and triglycerides for both genders with increasing GGT activity.

Since serum CDT concentration and GGT activity seemed to have contrasting associations with lipid biomarkers for cardiovascular risk, a comparison was made between the lipid values in subjects who had a highest quartile CDT and a lowest quartile GGT (CDT Q4/GGT Q1) or a lowest quartile CDT and a highest quartile GGT (CDT Q1/GGT Q4) (IV). The CDT Q4/GGT Q1 group had significantly lower cholesterol and triglyceride values and higher HDL cholesterol values than the unfavorable CDT Q1/GGT Q4 group in both genders, even after adjustment for age and BMI (Table 2; Figure 7, panels A and B).

Men	**CDT Q1 / GGT Q4 (n=178)**	**CDT Q4 / GGT Q1 (n=180)**	**Significance adjusted to age and BMI**
Total cholesterol	5.90 (1.14)	5.19 (0.98)	p<0.001
HDL cholesterol	1.11 (0.28)	1.42 (0.31)	p<0.001
Triglycerides	2.35 (1.35)	1.12 (0.49)	p<0.001
Alcohol last week	89.9 (109)	80.0 (94.1)	ns
Women	**CDT Q1 / GGT Q4 (n=194)**	**CDT Q4 / GGT Q1 (n=223)**	**Significance adjusted to age and BMI**
Total cholesterol	5.90 (1.09)	5.06 (0.97)	p<0.05
HDL cholesterol	1.36 (0.33)	1.62 (0.31)	p<0.001
Triglycerides	1.84 (1.41)	0.89 (0.37)	p<0.001
Alcohol last week	22.9 (42.2)	28.0 (38.4)	ns

Table 2. Lipid values (mmol/l (S.D.)) and alcohol consumption during last week (g (S.D.)) of subjects belonging to the opposite extreme quartiles of CDT and GGT.
CDT, carbohydrate-deficient transferrin; HDL, high density lipoprotein; GGT, gamma-glutamyl transferase; Q, quartile; ns, not significant.



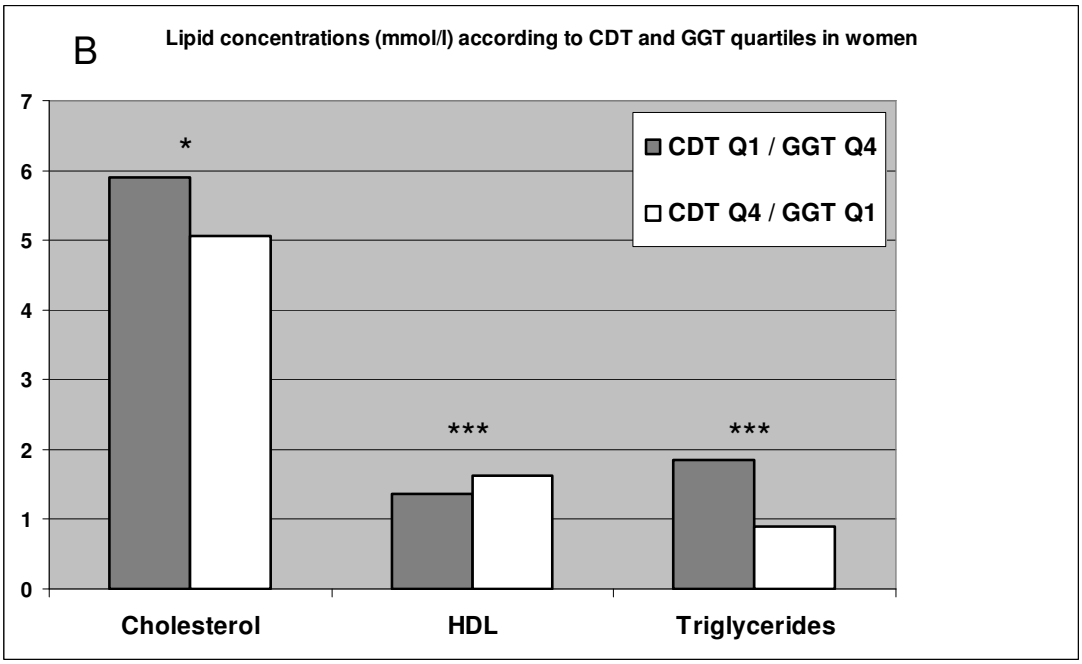


Figure 7. Serum lipid concentrations according to CDT and GGT quartiles in men (panel A) and women (panel B). Significance adjusted by age and BMI is displayed by * symbol. Statistical significance *, $p<0.05$; **, $p< 0.01$; ***, $p<0.001$. Values as mmol/l.

As a curiosity, in our data of the Finrisk study 1997, gamma-CDT seemed to be a more sensitive marker of alcohol use compared to either GGT or CDT (unpublished data from Finrisk study 1997). The correlations of alcohol markers CDT, GGT and gamma-CDT to alcohol use during the previous week are shown in Table 3 below.

MEN (n=3682)	**Mean (S.D.)**	**Correlation**	**Significance**
GGT	45.6 (75.1)	0.28	p<0.05
CDT	14.8 (6.61)	0.31	p<0.05
Gamma-CDT	6.24 (0.71)	0.40	p<0.05
Alcohol last week	82.1 (120)		

WOMEN (n=2864)	**Mean (S.D.)**	**Correlation**	**Significance**
GGT	25.3 (38.0)	0.17	p<0.05
CDT	18.4 (6.71)	0.26	p<0.05
Gamma-CDT	6.13 (0.57)	0.26	p<0.05
Alcohol last week	27.9 (48.1)		

Table 3: Correlation of alcohol markers GGT (IU/L (S.D.)), CDT (U/L (S.D.)) and gamma-CDT (= 0.8 * ln (GGT) + 1.3 * ln (CDT)) to alcohol use (g (S.D.)) during the previous week. CDT, carbohydrate-deficient transferrin; GGT, gamma-glutamyl transferase.

6. DISCUSSION

6.1. Postmenopausal HRT is associated with less severe atherosclerotic lesions and diminished LDL oxidation (I)

Our findings indicated that estradiol valerate alone, combined estradiol valerate–levonorgestrel, and combined estradiol valerate–medroxyprogesterone acetate therapy are associated with lower total NAP and less severe atherosclerotic lesions, as compared to controls without HRT, and that this outcome may be associated with the reducing effect of HRT on LDL oxidation. In the present study, during the 5-year follow-up, the concentration of HDL cholesterol increased and LDL cholesterol decreased in HRT groups, in agreement with previous studies (Koh and Sakuma 2004). Several previous studies have shown that postmenopausal estrogen therapy reduces the risk of CVD (Barrett-Connor and Bush 1991, Kannel et al. 1976, Stampfer and Colditz 1991, Stampfer et al. 1991). In a recent study, postmenopausal women on estrogen therapy had significantly fewer coronary calcifications than those on placebo (Manson et al. 2007).

However, in some studies no beneficial effect (Rosenberg et al. 1976), and even an adverse effect and increase in the incidence of CVD (Gordon et al. 1978), have been reported to be associated with HRT. Furthermore, during recent years, the WHI (Writing Group for the Women's Health Initiative Investigators 2002, Rossouw et al. 2002), Heart Estrogen/progestin Replacement Study (HERS) (Grady et al. 2002) and Million Women's study (Million Women Study Collaborators 2003) have indicated that HRT increases the risk for stroke, breast cancer and possibly also dementia. Even more recent results from the WHI showed that estrogen plus progestin may increase the risk of CVD among postmenopausal women, especially during the first year after the initiation of hormone use

(Manson et al. 2003). However, subsequent publications with more detailed analyses appear to have reversed these initial concerns (Stevenson et al. 2009).

HERS found no overall reduction in risk of CHD events among postmenopausal women with CHD (Grady et al. 2002). Participants were randomly assigned to receive either conjugated estrogens and medroxyprogesterone acetate, or placebo. However, in the hormone group, findings did suggest a higher risk of CHD events during the first year, and a lower risk during years 3 to 5. Lower rates of CHD events among women in the hormone group in the final years of HERS did not persist during additional years of follow-up. After 6.8 years, hormone therapy did not reduce risk of cardiovascular events in women with CHD (Grady et al. 2002). In this study, however, estrogen receptor genotype was not considered, and it would be interesting to reevaluate whether there is a genetic subgroup that benefits more from HRT.

In the present study, the level of oxidized LDL autoantibodies seemed to predict the NAP in postmenopausal women. It is important to note that the subjects had already used HRT for almost 10 years at the beginning of the study. In the study of Heikkinen et al. (1998), a 1-year combined estrogen–progestogen treatment was not effective in changing the oxidized LDL autoantibody titer, regardless of the beneficial effects on serum lipid parameters. It has been suggested that the antioxidative effect of estradiol may be anti-atherogenic by reducing the likelihood of oxidative modification of LDL, and thus preventing lipid accumulation within blood vessel walls and foam-cell formation (Sugioka et al. 1987, Ylä-Herttuala et al. 1996). There is in fact in vitro evidence that estrogens inhibit oxidative modification of LDL (Maziere et al. 1991, Badeau et al. 2005), which is believed to be one of the early events involved in the progression of atherosclerosis. Antioxidant protection provided by estrogens are free radical scavenging, chelating of ions, and importantly stabilizing the conformation of apoB-100, the major apolipoprotein of LDL, to be resistant to oxidation (Brunelli et al. 2000).

6.2. The effect of HRT on progression of atherosclerosis may be determined in part by the genotype of estrogen receptor (II)

Both long-term HRT and ESR1 PvuII genotype have affected the progression of atherosclerosis in postmenopausal women during a 5-year follow-up. Subjects with estradiol-valerate-levonorgestrel therapy and P/P genotype had less increase in AS, compared with those with P/p and p/p genotypes. All subjects in this study were non-smokers, nondiabetics, had normal blood pressure, and were also otherwise clinically healthy. The dietary analysis did not reveal any substantial differences in the use of saturated vs. polyunsaturated fat or dietary cholesterol between the HRT groups (Punnonen et al. 1995). Some other factors that may differ between HRT users and non-users (i.e. socioeconomic status, family history) were not accounted for and may have biased our results. There was also a small number of participants in the study.

In randomized clinical trials, estrogen has not been shown to slow down the progression of atherosclerosis (Hulley et al. 1998, Herrington 1999). In these studies, however, the possible genotypic effects on HRT response were not considered. Therefore, it would be interesting to perform a reanalysis of previous negative trials (Hulley et al. 1998, Herrington 1999) to find out whether there is a genetic subgroup, which would benefit more from HRT than other groups. The mechanism behind the ESR1PvuII polymorphism that affects signalling via ESR1 is not known, but the variant forms are strongly associated with the area of advanced atherosclerotic lesions (Lehtimäki et al. 2002).

The results suggest that women with the PvuII genotype P/P (presently referred to by C/C) of the c.454-397T>C benefit most from estrogen replacement therapy. The same CC genotype has earlier been found to be significantly associated with CAD in the

Helsinki Sudden Death study (Lehtimäki et al. 2002). The Framingham Heart Study has confirmed that the CC genotype is associated with enhanced progression of atherosclerosis. Men with the CC genotype had a substantial increase in risk of MI compared with the TT and CT genotypes (Shearman et al. 2003). Thus the variations in the ESR1 gene may affect the action of estrogen on the artery wall, and the effect of HRT on progression of atherosclerosis.

These results and those of others thus stress the importance of estrogen receptor genotype and suggest that estrogen receptor genotype has potential to explain recent conflicting data on estrogen replacement therapy and CVD susceptibility in women (Rossouw et al. 2002).

6.3. CDT and GGT detect different populations of drinkers with regard to cardiovascular lipid risk factors (III, IV)

The two cross-sectional studies (III, IV) were carried out to assess the relation between the markers of alcohol intake CDT and GGT and serum lipid profile. It was demonstrated that with increasing serum CDT concentrations, serum HDL cholesterol values were significantly higher and triglycerides lower in both men and women. This outcome may partly be caused by alcohol, since CDT, a potent biomarker of alcohol abuse (Sillanaukee 1996, Allen et al. 1994), correlated positively with alcohol consumption. Numerous epidemiological studies have shown with consistency that moderate alcohol intake (variously defined but corresponding to one to four drinks per day) decreases risk of CHD (Doll 1997, Arriola et al. 2010). A part of the protection is thought to be mediated by the effects of alcohol on blood lipid metabolism, particularly increases in HDL cholesterol (Steinberg et al. 1991). Moreover, serum CDT was negatively associated with serum total cholesterol in women, but this effect disappeared after adjusting for age. This might be

due to a decrease of CDT during menopause together with age-related increase of serum total cholesterol (Jousilahti et al. 1999). A negative association of CDT with triglycerides in the present study confirms findings of a previous study, which showed that triglyceride concentrations in diabetic individuals with high CDT values were low rather than high (Fagerberg et al. 1994). A mechanistic view behind this association is not clear.

It is not known why chronic alcohol consumption elevates CDT levels in serum. One explanation might be that the activities of glycosyltransferases involved in transferrin carbohydrate side chain synthesis are diminished in the liver, at least in part, due to enzyme inactivation by acetaldehyde (Salvaggio et al. 1991). Alcohol also influences most metabolic pathways of the liver, and also several endocrine systems in the whole body.

The mechanism behind the association of CDT and lipoprotein metabolism is also not clear at present. In our study BMI was inversely associated with CDT in both genders. However, even after adjusting for BMI, there was a strong independent association of CDT with HDL cholesterol and triglycerides. The baseline values of CDT have been shown to be fairly constant over time within the same individual, but there is some variation between individuals even without any alcohol consumption (Helander et al. 1998). This could suggest that there is a yet unknown mechanism that may influence both (hepatic) CDT formation and lipoprotein metabolism. It is to be noted that the diagnostic value of CDT is not 100% sensitive and specific. For sensitivity and specificity of CDT and GGT as alcohol markers, see table 4 below. A number of confounding factors are known, e.g. pre-analytical issues, mutant transferrins, some metabolic diseases, body composition, arterial hypertension, drug treatment, and exposure to organic solvents (Delanghe and De Buyzere 2009).

TEST	Sensitivity (%)	Specificity (%)
CDT	60-70	80-95
GGT	40-60	80-90
CDT+GGT	60-90	80-95

Table 4. Generalized ranges of laboratory tests of alcohol dependence (Tavakoli et al. 2011).

In contrast to the CDT concentration, increasing serum GGT activity was associated with a more atherogenic lipid profile: higher serum total and LDL cholesterol concentration, and higher triglyceride concentration in both genders. It is possible that the association of GGT with total cholesterol and triglycerides reflects deranged liver lipid metabolism with possible fatty liver formation. This was not accounted for in the present studies, since the study subjects were not imaged for liver damage. Alcohol consumption is known to show a linear dose–response relationship with HDL cholesterol, but again this increase may be modulated by the degree of liver injury (Flegal and Cauley 1985). A positive correlation between GGT activity and HDL cholesterol has also been reported (Pintus and Mascia 1996). In the present study, there was actually a small decrease of HDL cholesterol values in both genders with increasing GGT, but in women this decrease lost its significance after adjustment for age, BMI and smoking. Our observations thus agree with earlier studies showing no positive correlation between GGT activity and HDL cholesterol (Fex et al. 1982, Sillanaukee et al. 1993b). Increases in serum GGT activity were associated with age and BMI in both genders, confirming earlier findings (Kanel et al. 1983, Holder et al. 1995).

With regard to serum CDT concentration and GGT activity, an optimal lipid profile was observed in subjects who belonged to the highest quartile CDT group and simultaneously also to the lowest quartile GGT group, even after adjustment for age and

BMI. It is possible that CDT is an earlier stage or phase biomarker of alcohol consumption than GGT, and GGT might in fact reflect later on more toxic effects of ethanol on hepatic lipid metabolism, leading to hypercholesterolemia, decreased HDL cholesterol and hypertriglyceridemia (Sabesin 1981, Kanel et al. 1983). It has been shown previously that the development of alcoholic liver disease affects plasma lipid levels. HDL cholesterol concentration decreases and LDL cholesterol concentration increases in parallel with the degree of impairment of liver function in alcohol drinkers (Hannuksela et al. 2004).

On the other hand, this hypothesis is not supported by the fact that the average self-reported alcohol consumption was 136 g per week for men and 42 g per week for women in the highest CDT quartile, and 138 g per week and 35 g per week in the highest GGT quartile, respectively. However, this alcohol consumption should be considered in the context that self-reporting of drinking is commonly unreliable and the official per capita mean consumption of absolute ethanol in Finland for inhabitants 15 years and older was 8.4 l per year at the time, corresponding to 162 g per week (Holder et al. 1995). Total alcohol consumption has increased since, being 10.5 l per year at 2007, corresponding to over 200 g per week (Stakes 2008).

In conclusion, the present results indicate that serum CDT concentration is positively associated with alcohol consumption and serum HDL cholesterol, and inversely associated with serum triglycerides. On the other hand, alcohol consumption associated with liver induction and elevated GGT may have adverse effects on lipoprotein metabolism, including hypercholesterolemia and hypertriglyceridemia. CDT and GGT seem to detect different populations of subjects in regard to lipid metabolism. These observations may direct us to a better understanding of the effects of alcohol consumption on serum lipids as well as mechanisms behind favorable and detrimental effects of alcohol on vascular diseases.

7. SUMMARY AND CONCLUSIONS

The present study investigated lipid and other atherosclerosis risk factor changes induced by HRT or ethanol consumption in subsamples representing the Finnish population. The major findings in these studies suggest that HRT and ethanol consumption affect the development of atherosclerosis partly depending on the genotype and phenotype of the individual examined.

The major findings and conclusions are:

1. Postmenopausal HRT with estradiol valerate alone, combined estradiol valerate–levonorgestrel, and combined estradiol valerate–medroxyprogesterone is associated with less severe atherosclerotic lesions and diminished oxidation of LDL particles.
2. The effect of HRT on progression of atherosclerosis in postmenopausal women may be determined in part by the genotype of estrogen receptor.
3. CDT and GGT, when used as biomarkers for alcohol use, detect different populations of drinkers in regard to cardiovascular lipid risk factors. It appears that increased CDT concentration is associated with a favorable serum lipid profile, including higher HDL cholesterol and lower triglycerides. In contrast, increase in serum GGT activity appears to affect the lipid profile unfavorably.

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Kauhava, September 2011

Tommi Koivu

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The relation of oxidized LDL autoantibodies and long-term hormone replacement therapy to ultrasonographically assessed atherosclerotic plaque quantity and severity in postmenopausal women

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Abstract

Background: In epidemiologic studies, the incidence of atherosclerosis rises soon after menopause in women, and hormone replacement therapy (HRT) has proved to be useful in preventing onset of clinical manifestations of the disease. However, it is not known how HRT affects sonographically determined atherosclerotic severity (AS) and number of atherosclerotic plaques (NAP) in large arteries. Furthermore, it is not clear how HRT affects oxidation of low density lipoproteins (LDL), which obviously has an important role in the pathogenesis of atherosclerosis. *Objectives:* The purpose of the study was to determine whether HRT has a beneficial effect on sonographically determined AS and NAP in large arteries of 101 postmenopausal women compared to 40 controls without HRT. We also studied the interaction of HRT and antibodies against oxidized LDL on AS and NAP progression. *Results:* Estradiol valerate alone, combined estradiol valerate–levonorgestrel and combined estradiol valerate–medroxyprogesterone acetate therapy are each associated with lower NAP and AS as compared to controls without HRT. In a multiple regression model explaining NAP in the whole study population, the strongest predictors were HRT ($P = 0.0006$) and copper-oxidized LDL cholesterol autoantibodies ($P = 0.0491$). *Discussion:* Our findings indicate that postmenopausal HRT is associated with a lower total number of atherosclerotic plaques and less severe atherosclerotic lesions, as compared to controls without HRT, and that this outcome may be associated with the effect of HRT on LDL cholesterol oxidation.

Keywords: Hormone replacement therapy; Atherosclerosis; Sonography; LDL oxidation

1. Introduction

Atherosclerotic cardiovascular disease (CVD), causes about one-half of the mortality and morbidity of humans in the western world. The incidence of CVD in premenopausal women is about one-half of that in men of similar age, but rises soon after menopause [1–3]. Most epidemiological studies suggest useful effects of estrogens on the risk of CVD in postmenopausal women [1,4]. Postmenopausal hormone replacement therapy (HRT) ordinarily involves estrogen combined with progestin. However, there is little data in humans about the effects of combined estrogen–progestin therapy on atherosclerosis, or on known risk factors, such as oxidized LDL [5]. To our knowledge, the interaction between long-term estrogen–progestin therapy and oxi-

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dized low-density lipoprotein (LDL) has not previously been studied in terms of prediction of atherosclerosis.

Oxidized LDL is believed to play an important role in the progression of atherosclerosis [6]. Oxidative modification of LDL is a prerequisite for rapid accumulation of LDL in macrophages and for the formation of foam cells. LDL isolated from atherosclerotic lesions, but not from normal arteries, resembles oxidized LDL in its physical, chemical, and immunological properties [7]. Epitopes characteristic of oxidized LDL can be found in atherosclerotic lesions by immunocytochemical techniques [8,9] and atherosclerotic lesions contain immunoglobulins that recognize oxidized LDL [7,8]. In addition, antioxidant therapy reduces atherogenesis in animal models [10,11]. Antibodies against malondialdehyde or copper modified LDL, detected by radioimmunoassay, have been reported to be predictive of the progression of carotid atherosclerosis [12], coronary artery disease (CAD) [13] and myocardial infarction [14]. Furthermore, recent results from Heizer et al. [15] and Raitakari et al. [16] indicate that antibodies against copper-oxidized LDL are associated with impaired endothelial function and early atherosclerotic changes [13]. We hypothesized that there might be an oxidized LDL by HRT interaction, which modulates atherogenesis. Therefore, the purpose of the present study was to assess how long-term HRT affects atherosclerotic changes in large arteries of postmenopausal women, and whether there is an interaction between HRT and oxidized LDL, compared to women who have never used HRT.

2. Methods

2.1. Subjects

Women attending a private outpatient clinic in Tampere for annual routine gynecological examinations were invited to participate. For the cross-sectional baseline study in 1993 [5], 141 non-smoking, non-diabetic postmenopausal women aged 45–71 years were enrolled. They had no clinically evident cardiovascular diseases or hypertension and were classified into four groups based on the monthly use of HRT. The HRT-EVP group ($n = 40$) used estradiol valerate (EV) 2 mg per day for 11 days followed by EV continued with progestin (P, levonorgestrel 0.25 mg per day) for 10 days, the HRT-EVM group ($n = 21$) used estradiol valerate (EV) 2 mg per day for 11 days followed by EV continued with progestin (M, medroxyprogesterone acetate 10 mg per day) for 10 days, the HRT-EV group ($n = 40$) used EV alone, and the control group ($n = 40$) had never used HRT. In HRT-EVP, HRT-EVM and HRT-EV groups there was a pause of therapy for 7 days after each 21-day cycle. Of these 141 women 91 (60 in HRT group, 31 controls) participated in 5-year follow up study from 1993 to 1998. HRT, when used, was started at the time of menopause for climacteric symptoms. In the control group, the main reasons not to use HRT were the absence of vasomotor and other climacteric symptoms and dislike of HRT. The mean duration of EVP and EVM was 9.3 ± 3.2 years and of EV treatment 9.9 ± 4.2 years at the beginning of the study. The mean time from menopause in the control group was 11.9 ± 4.1 years (mean 9.9 ± 3.8 years, at baseline). The mean ages in the HRT-EVP, HRT-EVM, HRT-EV, and control groups were 59.6 ± 4.7, 55.9 ± 3.4, 61.0 ± 5.0, and 61.6 ± 5.5 years, respectively ($P = 0.0002$ over all groups in analysis of variance, ANOVA). The mean body mass indexes were (BMI, mean 25.3 ± 3.2 kg/m^2) similar in all studied groups ($P = 0.8941$ over all groups in ANOVA). In the HRT-EV, HRT-EVP, and control groups 24, 4, and 6 women underwent hysterectomy, respectively, due to benign conditions and 4, 2, and 2 women bilateral salpingo-oophorectomy. At baseline, all women were clinically healthy, and used no chronic medication. Nutrient intake analyses were also performed, as described elsewhere [5], and these analyses did not show any marked differences between the study groups in the amount of used saturated, monounsaturated, and polyunsaturated fats, or dietary cholesterol. Sonography and blood sampling were done in the University Hospital of Tampere. The Ethics Committee of the University Hospital of Tampere approved the study.

2.2. Blood samples

Blood samples for serum lipid and genotype analyses were taken after the subjects had fasted overnight. Sampling took place within 3 weeks after the sonography and for HRT users during the third week of the hormone regimen. After separation of serum by low-speed centrifugation the sera were divided into aliquots and stored at -70°C until analyzed.

2.3. Sonography

Sonography at baseline and follow-up were performed with Toshiba Sonolayer V SSA 100 equipment, as reported elsewhere [5]. Briefly, all the sonographies were done blinded by one experienced sonographer and radiologist (P.D.). During the examinations women were lying in a supine position. Transverse and longitudinal scans of extracranial carotid arteries were performed bilaterally at four different segments of the carotid: first, at the 10 mm segment of the common carotid artery (CCA) just distal to the origin of carotid bifurcation, second at a 10 mm segment in the area of distal third of the CCA, third at the 10 mm segment between origin of carotid bifurcation and the tip of the

flow divider, which separates internal from external carotid arteries, and fourth at a 10 mm segment of the internal carotid artery cranial from flow divider. Only fibrous and calcified atherosclerotic lesions were considered and were defined as plaques when distinct areas of mineralization or/and focal protrusion into the lumen were identified. The thickness and length of such plaques within the artery vessel wall were determined by transverse and longitudinal scans, respectively, and the thickness of a plaque was determined as the distance between the intimal–luminal interface and the medial–adventitial interface. The intimal–medial far-wall thickness equal to or more than 1.3 mm at any segment in carotid arteries was defined as an atherosclerotic plaque [17] and the total number of plaques was calculated. All carotid artery examinations were done with a 5.0 MHz convex transducer probe.

Longitudinal sonographs of the abdominal aorta were obtained at 1 cm intervals and transverse scans at 2 cm intervals at the area of three aortic segments: (1) supra-pancreatic; (2) pancreatic and infra-pancreatic; and (3) at the area of the aortic bifurcation. As for carotid plaques, significant aortic plaques were defined as an intimal–medial far-wall thickness equal to or more than 3.0 mm [17]. All aortic examinations were performed using a 3.75 MHz convex transducer probe. The average duration for the whole examination varied from 25 to 30 min.

Predominantly fibrous plaque is moderately to strongly echogenic and the degree of echogenity correlates with the amount of collagen within the plaque architecture [18]. Uniformly fibrous plaque is homogenous in echogenity, but localized hypoechoic regions may be seen when large focal deposits of lipid material or thrombus are present within fibrous plaque [19,20]. Fibrofatty plaque is only faintly echogenic so may be difficult to identify sonografically. It contains a large amount of lipid material. The properties of fibrofatty plaque are very similar to those of blood and thus difficult to decipher. In our study, predominantly fibrous plaques and uniformly fibrous plaques were identified. Calcified plaque shows bright reflections with acoustic shadowing. Tiny areas of calcification on the order of 1 mm in diameter are detected within plaques. Acoustic shadowing from large calcified deposits are troublesome to see on ultrasound [18].

The atherosclerotic severity sum (AS) was constructed by dividing the atherosclerotic changes of abdominal aorta, iliac, and carotid arteries into three severity classes: 1 = slight, 2 = moderate, and 3 = severe, and calculating the sum, i.e. AS. Total number of atherosclerotic plaques (NAP) was calculated according to the criteria given for plaques in Section 2.

The reproducibility of our sonographic protocol for significant aortic and carotid plaques was also examined: 1 month after the first assessment 20 randomly selected subjects were invited to a repeated examination. The repeatability of the number of plaques (number of plaques initial by repeated sonography) between the first and second examination was 90% for the carotid artery segment areas and 100% for the aortic segments.

2.4. Enzyme-linked immunosorbent assay for antibodies against oxidized LDL

Autoantibodies against oxidized LDL were determined as described earlier [13]. In short, antigens for this assay included: (A) native LDL prepared from the pooled plasma of ten donors and protected against oxidation by 0.27 mmol/l EDTA and 20 μmol/l butylated hydroxytoluene (BHT) in phosphate buffered saline (PBS); and (B) oxidized LDL obtained after 24-h oxidation of the native LDL with 2 μmol/l $CuSO_4$. For enzyme-linked immunosorbent assay, half of the wells on a polystyrene plate (Nunc, Roskilde, Denmark) were coated with 50 μl of native and the other half with 50 μl copper-oxidized LDL antigen (both at a concentration of 5 μg/ml) in PBS for 16 h at 4°C. After removal of the unbound antigen and washing of the wells, the remaining non-specific binding sites were saturated using 2% human serum albumin in PBS and 20 μmol/l BHT for 2 h at 4°C. After washing, 50 μl of the serum samples, diluted 1:20, were added to wells coated with native LDL and oxidized LDL and incubated overnight at 4°C. After incubation the wells were aspirated and washed six times before an IgG-peroxidase conjugated rabbit *anti*-human monoclonal antibody (Organon, USA No. 55220 Cappel), diluted 1:4000 (v/v) in buffer (0.27 mmol/l PBS, 20 μmol/l EDTA, 1% BHT, 0.05% Tween HSA), was added to each well for 4 h at 4°C. After incubation and washing, 50 μl of freshly made substrate (0.4 mg/ml *o*-phenylenediamine (Sigma) and 0.045% H_2O_2 in 100 mmol/l acetate buffer, pH 5.0) was added and incubated exactly 5 min at room temperature. The enzyme reaction was terminated by adding 50 μl of 2 M H_2SO_4. The optical density (OD) was measured at 492 nm using a microplate reader (Multiskan MCC/340, Labsystems GmbH, Munich, FRG). All measurements were blinded and done on coded serum samples. The results were expressed as the mean OD values from duplicate determinations, and autoantibody titer against oxidized LDL was calculated by subtracting the binding of antibodies to native LDL from that to copper-oxidized LDL. This approach reduces the possibility of getting false positive values due to cross-reactivity with both LDL epitopes. The intra-assay coefficient of variation was for the antibodies against oxidized LDL 8.5%.

2.5. Other laboratory analyses

For the cross-sectional study the concentrations of serum lipids and apolipoproteins were measured. Serum total cholesterol and triglycerides were determined by a commercial method (Kodak Echtachem 700XR, Eastman Kodak Company, Clinical Products Division, Rochester, and USA). Serum HDL cholesterol and its sub-fractions (HDL_2 and HDL_3) were separated by a dextran–sulfate–Mg precipitation procedure [21] and the cholesterol content was analyzed with a Monarch 2000 Analyzer (Instrumentation Laboratory, Lexington, USA), using the CHOD-PAP cholesterol reagent (Cat No. 237574; Boehringer Mannheim, Germany) and a primary cholesterol standard (Cat No. 530; Orion, Finland). The LDL cholesterol content was calculated according to the Friedewald formula [22]. Apolipoproteins (apo) A1 and B were determined on a Monarch Analyzer by an immunoturbidimetric method [23] (Cat No. 67265 and 67249, Orion Diagnostics, Finland). In the 5-year follow-up study the lipid concentrations were determined with Cobas Integra 700 analyzer with reagents and calibrations recommended by the manufacturer (Hoffmann-La Roche Ltd., Basel, Switzerland).

2.6. Statistical analysis

Two-way analysis of covariance (ANCOVA) using age, BMI, and total cholesterol as covariates, when appropriate) was used to assess the interaction between grouping factor, HRT groups and control group, in selected variables of interest (= dependent variables) i.e. atherosclerotic severity score (AS), total number of atherosclerotic plaques (NAP), and serum lipids, and apolipoproteins. Two- and one-way analysis of variance (ANCOVA) was used to assess the statistical differences between HRT groups and control group in lipid parameters shown in Table 1, age and BMI were used as covariates when appropriate. In Table 1 pairwise comparisons of group means were done by using analysis of variance, age and body mass index as covariates, when appropriate. Multiple regression was used in search for the variables that predict the severity of atherosclerosis. We also used repeated measure analysis of variance to study the effect of time and HRT on atherosclerotic severity in our study population. All calculations were done with the Statistica for Windows version 5.1 (Statsoft Inc., Tulsa, Oklahoma, USA) software in PC. Data are presented as mean $\pm$ SD unless otherwise stated. A P-value less than 0.05 was considered statistically significant.

3. Results

The mean baseline values and differences of serum lipids and apolipoproteins between study groups are presented in Table 1. It is important to notice that the subjects in the HRT groups had already received HRT for 10 years at the beginning of this study. The HRT-EVM group had the lowest baseline LDL/HDL cholesterol ratio (2.22 vs. 3.15 in controls, $P = 0.0018$) and seemed to have the most profitable serum lipid profile (see Table 1).

During the 5-year follow-up (see Table 2), the concentration of HDL cholesterol increased and LDL cholesterol decreased significantly in HRT-EV, HRT-EVP and control groups, and total cholesterol decreased significantly in the HRT-EV group and controls, but not in the HRT-EVP group. The triglycerides increased in HRT-EVP group and controls but not in the HRT-EV group. The HRT-EVM group was not included in the follow-up study.

In a multiple regression model explaining NAP in the whole study population, the strongest predictors were HRT ($P = 0.0006$) and copper-oxidized LDL cholesterol autoantibodies ($P = 0.0491$). Other atherosclerotic risk factors did not reach statistical significance in the model, although there were statistically significant differences in serum lipids between the HRT groups in analysis of variance (Table 1 and Fig. 1). The results and the parameters included in the model are presented in Table 3. In addition, Ox-LDL antibody titer and NAP are visualized in scatterplot (Fig. 2). When HRT was removed from the model, the role of oxidized LDL became even stronger ($P = 0.0306$ for HRT, $P = 0.0128$ for the whole model, data not shown), as the other parameters remained insignificant. In a similar multiple regression model explaining AS in the whole study population, the strongest predictors were HRT ($P = 0.0024$) and HDL cholesterol ($P = 0.0024$ for HRT, $P = 0.0412$ for HDL, $R^2 = 14\%$ and $P = 0.0228$ for the whole model, data not shown). Likewise, when HRT was excluded from the model, no significant parameters remained ($P = 0.25$ for the whole model, data not shown). In otherwise similar regression models performed within different study groups, HDL cholesterol explained AS and NAP in the control group, but not in HRT groups (data not shown).

The effect of HRT on atherosclerosis was also seen in repeated measures analysis of variance performed on the 5-year follow-up study population ($n = 91$). We used the AS of aorta and carotid artery as repeated measure, HRT and control group as independent factor, and age and body mass index as covariates. The HRT group and time had no interaction ($P = 0.1389$), and P-values for the main effects of time and HRT group were 0.0000 and 0.0036, respectively. The result is presented in Fig. 1. The AS after follow-up was significantly higher in HRT-EV ($n = 34$), HRT-EVP ($n = 26$) and control ($n = 31$) groups, compared to baseline (data not shown).

Table 1
Background characteristics in postmenopausal women without or with hormone replacement therapy (HRT)[a]

HRT-GROUP Variable and unit	Controls $n=40$	HRT-EVP $n=40$	HRT-EVM $n=21$	HRT-EV $n=40$	Significance *P*-value	C vs. EVP *P*-value	C vs. EVM *P*-value	C vs. EV *P*-value
Age, year	61.6 (5.5)	59.6 (4.7)	55.9 (3.4)	61.0 (5.0)	**0.0002**	0.0821	**0.0001**	0.6255
Body mass index, kg/m^2	25.4 (3.3)	25.4 (3.0)	24.6 (3.7)	25.4 (2.9)	0.8941	0.9606	0.3914	0.9417
Hypercholesterolemia,% (*n*)	47.5 (19)	27.5 (11)	23.8 (5)	47.5 (19)	0.2280	0.0685	0.0714	1.0000
Triglycerides, mmol/l	1.25 (0.61)	0.79 (0.21)	1.13 (0.46)	1.47 (0.69)	**0.0000**	**0.0000**	0.4522	0.1391
Total cholesterol, mmol/l	6.70 (1.19)	5.92 (0.86)	5.91 (0.89)	6.56 (0.92)	**0.0049**	**0.0013**	**0.0093**	0.5543
LDL cholesterol, mmol/l	4.60 (1.14)	4.10 (0.91)	3.60 (0.92)	4.25 (0.85)	**0.0372**	**0.0328**	**0.0011**	0.1234
Apolipoprotein B, g/l	0.96 (0.24)	0.84 (0.16)	0.82 (0.17)	0.98 (0.18)	**0.0051**	**0.0094**	**0.0182**	0.6874
HDL cholesterol, mmol/l	1.54 (0.33)	1.47 (0.35)	1.79 (0.50)	1.65 (0.37)	**0.0284**	0.3593	**0.0250**	0.1656
HDL_2 cholesterol, mmol/l	0.50 (0.22)	0.45 (0.30)	0.59 (0.24)	0.56 (0.27)	0.1581	0.3370	0.1881	0.3061
Apolipoprotein A1, g/l	1.44 (0.19)	1.34 (0.19)	1.57 (0.19)	1.56 (0.26)	**0.0000**	**0.0325**	**0.0145**	**0.0163**
LDL/HDL cholesterol ratio	3.15 (1.08)	2.96 (1.04)	2.22 (1.01)	2.70 (0.82)	**0.0329**	0.4273	**0.0018**	**0.0369**
Total number of plaques	3.88 (2.09)	2.65 (1.75)	2.10 (1.58)	2.18 (1.69)	**0.0005**	**0.0163**	**0.0107**	**0.0001**
Atherosclerotic severity	2.05 (1.06)	1.48 (0.91)	1.52 (1.03)	1.38 (0.98)	**0.0227**	**0.0240**	0.1922	**0.0048**
Oxidized LDL antibodies	2.20 (0.95)	2.61 (1.92)	2.19 (1.92)	2.08 (0.77)	0.3320	0.1952	0.9420	0.4908

[a] Values are means ($\pm$SD), unless stated otherwise (significant results are in bold). LDL, low density lipoprotein; HDL, high density lipoprotein; C, controls; EV, estradiol valerate, 2.0 mg per day; P, levonorgestrel, 2.5 mg per day; M, medroxyprogesterone acetate. Hypercholesterolemia = total cholesterol > 6.5 mmol/l. Significance is based on analysis of covariance, age and body mass index as covariates, when appropriate.

Table 2
Changes in serum lipid profile during the follow-up[a]

Group	Controls ($n=40$)			HRT-EVP ($n=40$)			HRT-EV ($n=40$)		
Variable and unit	1993	1998	*P*-value	1993	1998	*P*-value	1993	1998	*P*-value
Triglycerides, mmol/l	1.25 (0.61)	1.48 (0.67)	**0.0393**	0.79 (0.21)	1.08 (0.75)	**0.0201**	1.47 (0.69)	1.52 (0.53)	0.6402
Total cholesterol, mmol/l	6.70 (1.19)	6.23 (0.95)	**0.0043**	5.92 (0.86)	5.79 (1.25)	0.5552	6.56 (0.92)	6.10 (0.84)	**0.0002**
LDL cholesterol, mmol/l	4.60 (1.14)	3.89 (0.87)	**0.0000**	4.10 (0.91)	3.56 (1.04)	**0.0005**	4.25 (0.85)	3.65 (0.83)	**0.0000**
HDL cholesterol, mmol/l	1.54 (0.33)	1.67 (0.41)	**0.0023**	1.47 (0.35)	1.74 (0.37)	**0.0000**	1.65 (0.37)	1.76 (0.42)	**0.0442**

[a] Values are means ($\pm$SD), unless stated otherwise (significant results are in bold). LDL, low density lipoprotein; HDL, high density lipoprotein; C, controls; EV, estradiol valerate, 2.0 mg per day; P, levonorgestrel, 2.5 mg per day. Significance is based on repeated measures analysis of variance, age and body mass index as covariates.

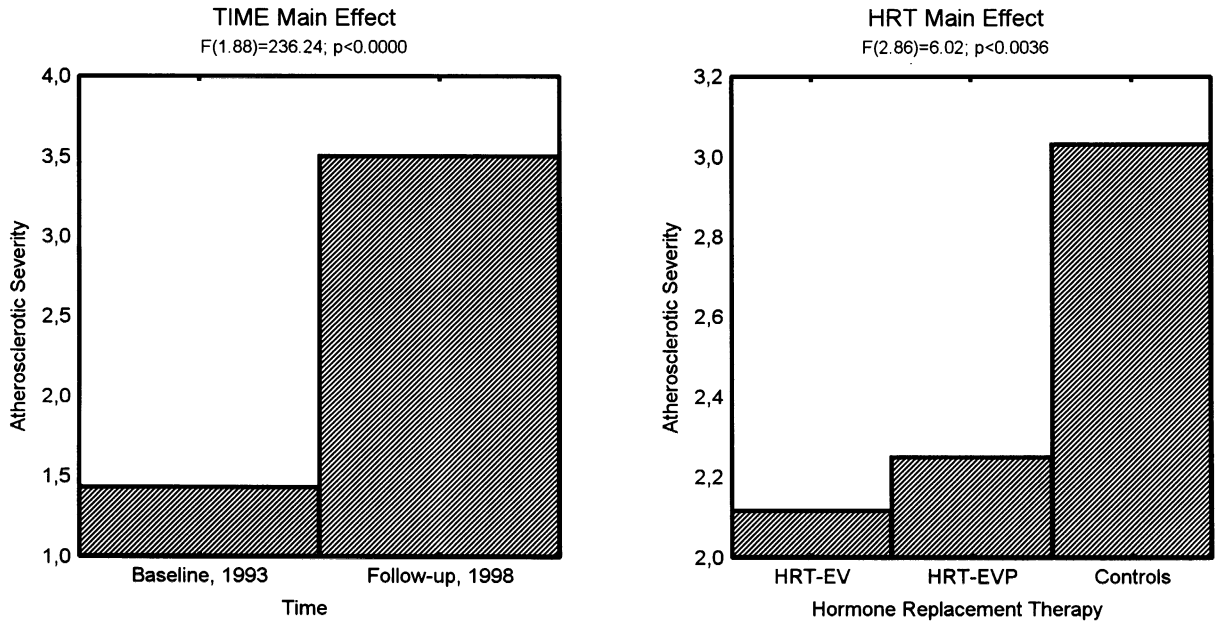

Fig. 1. Repeated measures analysis of variance. Atherosclerotic severity is estimated from aorta and carotid artery by sonography. There was no interaction between the main effects ($P = 0.1389$).

4. Discussion

In this conservative study design, we show that oxidized LDL autoantibodies seem to predict NAP in postmenopausal women, but do not seem to explain AS. To increase our understanding of the effects of HRT on atherosclerosis in postmenopausal women, we tested the relationship of oxidized LDL autoantibodies and three different HRTs in the occurrence of atherosclerosis (as measured by AS and total NAP), after considering the contribution of some other CVD risk factors i.e. age, BMI and serum cholesterol. Further, in this study all subjects were non-smokers, did not have diabetes, had normal blood pressure, and were otherwise clinically healthy. The time elapsed from menopause was similar in HRT groups and controls. In addition, the dietary analysis did not reveal any substantial differences in the use of saturated fat or dietary cholesterol intake between the HRT and control groups. Therefore, our findings are unlikely to be caused by different dietary habits between the groups. Since some other important factors that may differ between HRT and control groups (i.e. socioeconomic status, family history of CVD) were not accounted for, one can say that these unknown factors may have biased our results.

Previous studies have shown that postmenopausal estrogen therapy reduces the risk of CVD [24–26]. The positive effects of postmenopausal HRT on serum lipoproteins have been widely examined, although it is estimated that lipoprotein changes explain only 25–50% of the beneficial effect of estrogen [24]. Recently it has been shown that estrogens also prevent atherosclerosis by directly affecting the function and metabolism of the arterial wall. For example, estrogens reduce the accumulation of cholesterol in the arterial wall [27]. Other reported mechanisms are effects on prostaglandin metabolism [28] and on blood flow [29]. Mendelshon and Karas have written an in-depth review of the protective effects of estrogen on the cardiovascular system [30], in which they list systemic effects and direct effects of estrogen on the cardiovascular system. To our knowledge, estrogen has never been shown to slow atherosclerosis progression in women in randomized

Table 3
Multiple regression model of risk factors predicting total number of atherosclerotic plaques in postmenopausal women receiving hormone replacement therapy and controls[a]

Variable	P-value[b]
Hormone replacement therapy	**0.0006 (0.0007)**
Copper-oxidized LDL autoantibodies	**0.0491 (0.0255)**
HDL cholesterol	0.0568 (0.3609)
Age	0.1108 (0.0646)
Triglycerides	0.1309 (0.2362)
Total cholesterol	0.1333 (0.3873)
Total cholesterol — HDL cholesterol ratio	0.2671 (0.2087)
Body mass index	0.3846 (0.5383)
Dietary saturated fat intake	0.3869 (0.1552)
Dietary cholesterol intake	0.7732 (0.8051)

[a] Total model: $R^2 = 22\%$. $P = 0.00025$ for the whole model.

[b] In parentheses are the P-values when total cholesterol, HDL cholesterol and triglyceride concentration changes are included in the model instead of baseline values; then $R^2 = 21\%$ and $P = 0.00061$ for the whole model.

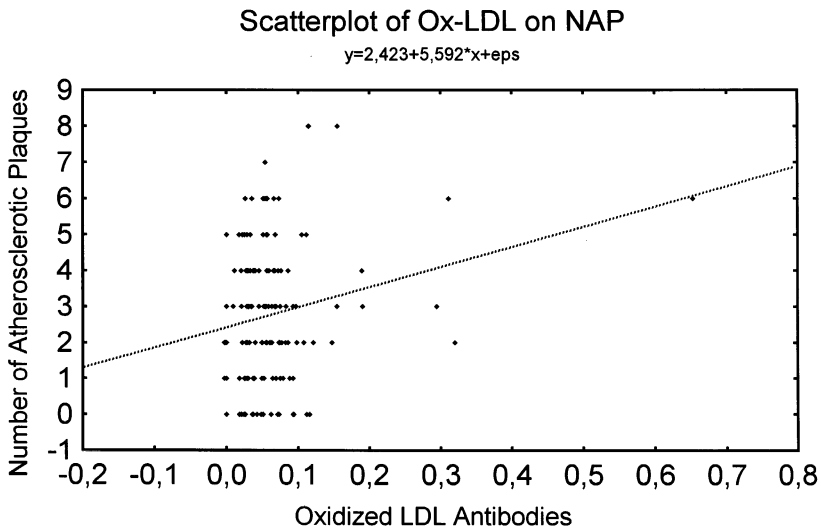

Fig. 2. Scatterplot of oxidized LDL antibody titer on *X*-axis and number of atherosclerotic plaques on *Y*-axis.

clinical trials [31,32], despite numerous findings from non-randomized experiments showing that postmenopausal estrogen therapy has favorable effects on serum lipoprotein concentrations and slows the development of atherosclerotic diseases. Furthermore, in some studies no beneficial effect [33], and even an increase in the incidence of cardiovascular disease [34], have been reported to be associated with HRT. In the present work we show that both time and HRT have an independent effect on AS.

HRT usually combines estrogen with progestin. However, there is little information on the effects of estrogen–progestin therapy on atherosclerosis. It is known that some progestins have unfavorable effects on lipid metabolism, while some do not [35–37]. Our study is in line with the study of Punnonen et al. [5], who have demonstrated that combined estrogen–progestin replacement therapy prevents the progression of atherosclerosis in large arteries in postmenopausal women.

In recent years, autoantibodies against oxLDL have been widely studied and have been found to predict the progression of carotid atherosclerosis [12] and myocardial infarction [14,38]. There is evidence that estrogens inhibit oxidative modification of LDL [39], which is believed to be one of the early events involved in the progression of atherosclerosis. It has been suggested that the antioxidative effect of estradiol may be *anti*-atherogenic by reducing the likelihood of oxidative modification of LDL, and thus preventing foam-cell formation and lipid accumulation within blood vessel walls [40,41]. In the study of Heikkinen et al. [42], a 1-year combined estrogen–progestogen treatment was not effective in changing the oxidized LDL autoantibody titer, regardless of the beneficial effects on the serum lipid parameters. In the present study, we show that the level of oxidized LDL autoantibodies seems to predict NAP in postmenopausal women. It is important to note that the subjects had already used HRT for almost 10 years at the beginning of the study. This may have resulted in the somewhat unexpected finding that oxidized LDL autoantibodies did not predict AS. One could hypothesize that the progression of early atherosclerotic changes to more advanced forms of atherosclerotic lesions has been slowed by HRT.

In conclusion, our finding indicates that both estradiol valerate alone, combined estradiol valerate–levonorergestrel, and combined estradiol valerate–medroxyprogesterone acetate therapy are associated with lower total number of atherosclerotic plaques and less severe atherosclerotic lesions, as compared to controls without HRT, and that this outcome may be associated with the effect of HRT on LDL oxidation.

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The effect of hormone replacement therapy on atherosclerotic severity in relation to *ESR1* genotype in postmenopausal women

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Abstract

Objective: The atheroprotective action of estrogen is mediated by estrogen receptors (*ESR*) *1* and *2*, expressed in atherosclerotic lesions. The effects of hormone replacement therapy (HRT) and *ESR1 Pvu*II genotypes on atherosclerosis have not previously been studied prospectively in postmenopausal women. *Methods:* We investigated the effect of HRT on the progression of atherosclerosis in a 5-year follow-up study of 88 postmenopausal women aged 45–71 years at baseline allocated into three groups based on the use of HRT. The HRT-EVP group ($n = 26$) used sequential estradiol valerate (EV) plus progestin (P), the HRT-EV group EV alone ($n = 32$), and a control group ($n = 30$) was without HRT. The atherosclerosis severity score (AS) of the abdominal aorta and carotid arteries were determined by sonography and the *ESR1 Pvu*II genotypes (P/P, P/p and p/p) by PCR. *Results:* HRT, time and *ESR1 Pvu*II genotype had a statistically significant or borderline significant main effect on AS during 5-year follow-up ($P = 0.004$, $P < 0.001$ and $P = 0.090$, respectively), when analyzed by repeated measures analysis of variance. There was a significant genotype-by-treatment (HRT-EVP and control groups) interaction for AS ($P = 0.034$). In response to HRT-EVP, subjects with P/P, compared with those with P/p and p/p genotypes, had a less increase in AS (1.61 ± 1.14 vs. 1.71 ± 1.27 vs. 2.43 ± 1.27). Baseline AS as covariate in similar model does not change the significant interaction effect between HRT-EVP and control groups ($P = 0.036$). But this effect was not found between HRT-EV and control groups. *Conclusions:* Our results suggest that the effect of HRT-EVP in postmenopausal women on progression of AS may be determined in part by the genotype of *ESR1 Pvu*II.

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Keywords: Atherosclerosis severity; *ESR1 Pvu*II genotype; Hormone replacement therapy; Ultrasound

1. Introduction

Atherosclerotic diseases cause about one-half of the mortality and morbidity of humans in the western world [1]. Physiological disruption of atheromatous lesions often underlies acute coronary syndromes such as myocardial infarction, angina, or sudden death due to coronary artery occlusion [2]. Numerous findings from non-randomised experiments show that postmenopausal estrogen therapy has favorable effects on serum lipoprotein concentrations and slows the development of atherosclerotic disease [3,4]. However, estrogens have not been shown to slow atherosclerotic progression in women in randomised clinical trials [5,6]. It is estimated that estrogen-induced alterations in serum lipids account for only approximately one third of the possible clinical benefits of estrogens, suggesting that also the direct actions of estrogens on blood vessels contribute substantially to their atheroprotective effects [3,4]. In these previous studies, the possible inherited genotypic effects on estrogen function are not considered. Therefore, it would be interesting to find out if there is a genetic subgroup of women, which would benefit more from estrogens than other.

The atheroprotective action of estrogens may be mediated by estrogen receptors (*ESR*) *1* and *2*, expressed in atherosclerotic lesions [3]. *ESR1* activates specific target genes in vascular smooth muscle [7], inhibits smooth muscle cell migration [8,9] and accelerates endothelial cell growth in vitro [10] and vivo [11,12]. In addition, fewer *ESR1* are found in premenopausal women with atherosclerotic coronary arteries than in those with normal arteries [13]. Therefore, it is possible that the effects of estrogens on vascular cells, mediated by *ESR1*, differ due to the *ESR1* variant forms that have different transcriptional effects than the 'wild-type' receptor [14,15]. *ESR1* has a common two-allele polymorphism for *Pvu*II restriction enzyme, leading to genotypes P/P, P/p, and p/p [16]. The *Pvu*II polymorphism is caused by a T-to-C transition in intron 1 and is located approximately 0.4 kb upstream of exon 2 [16].

Hormone replacement therapy (HRT) may protect against the development of atherosclerosis and several epidemiological studies suggest that estrogens are beneficial with respect to the risk of cardiovascular diseases in postmenopausal women [17–23]. However, the effect of HRT on the development of atherosclerosis in people with different *ESR1 Pvu*II genotypes has not previously been studied prospectively in postmenopausal women. Postmenopausal HRT usually involves estrogens with or without progestin. To our knowledge, the interaction between HRT and genetic factors has not been studied previously in relation to prediction of atherosclerosis progression.

2. Materials and methods

2.1. Subjects

Women attending the private outpatient clinic in Tampere for annual routine gynecological examinations were invited to participate. For the cross-sectional baseline study in 1993 [21], 120 non-smoking, non-diabetic postmenopausal women aged 45–71 years were enrolled. Eighty-eight of 120 women participated in this 5-year follow-up study from 1993 to 1998. They had no clinically evident cardiovascular diseases or hypertension and were classified into three groups based on the use of HRT. The HRT-EVP group ($n = 26$) used estradiol valerate (EV) 2 mg/day for 11 days followed by EV continued with progestin (P, levonorgestrel 0.25 mg/day) for 10 days, the HRT-EV group ($n = 32$) used EV alone, and the control group ($n = 30$) had never used HRT. In HRT-EVP and HRT-EV groups there was a pause of therapy for 7 days after each 21-day cycle. HRT, when used, was started at the time of

menopause for climacteric symptoms. In the control group the main reason not to use HRT was the absence of vasomotor and other climacteric symptoms and dislike of HRT. The mean duration of EVP was 9.3±3.2 years and of EV treatment 9.9±4.2 years. The mean time from menopause in the control group was 11.9±4.1 years (mean 9.9± 3.8 years, at baseline). The mean ages in the HRT-EVP, HRT-EV, and control groups were 59.7± 5.5, 60.4±4.8, and 61.5±5.8 years, respectively ($P = 0.4409$ over all groups in analysis of variance, ANOVA). The mean body mass index was (BMI, mean 25.7±3.0 kg/m^2) similar in all studied groups ($P = 0.9531$ over all groups in ANOVA). At baseline, all women were clinically healthy, and used no chronic medication. Nutrient intake analyzes were also performed as, described elsewhere [21], and these analyzes did not show any marked differences between the study groups in the amount of used saturated, monounsaturated, and polyunsaturated fats, or dietary cholesterol. Sonography and blood sampling were done in the University Hospital of Tampere. The Ethics Committee of the University Hospital of Tampere approved the study. The investigation conforms with the principles outlined in the Declaration of Helsinki [24].

2.2. Blood samples

Blood samples for serum lipid analysis were taken after the subjects had fasted overnight at baseline and after 5-year follow-up. Genotype analysis was performed using blood at baseline. Sampling took place within 3 weeks after the sonography and for HRT users during the third week of the hormone regimen. After separation of serum by low-speed centrifugation the serums was divided into aliquots and stored at −70 °C until analyzed.

2.3. Sonography

Sonography at baseline and follow-up were performed with Toshiba Sonolayer V SSA 100 equipment, as reported elsewhere [21]. Briefly, all the sonographies were done blinded by one experienced sonographer and radiologist. During the examinations women were lying in a supine position. Transverse and longitudinal scans of extracranial carotid arteries were performed bilaterally at four different segments of the carotid: first, at the 10 mm segment of the common carotid artery (CCA) just distal to the origin of carotid bifurcation, second at a 10 mm segment in the area of distal third of the CCA, third at the 10 mm segment between origin of carotid bifurcation and the tip of the flow divider, which separates internal from external carotid arteries, and fourth at a 10 mm segment of the internal carotid artery cranial from flow divider. Only fibrous and calcified atherosclerotic lesions were considered and were defined as plaques when distinct areas of mineralization or/and focal protrusion into the lumen were identified. The thickness and length of such plaques within the artery vessel wall were determined by transverse and longitudinal scans, respectively, and the thickness of a plaque was determined as the distance between the intimal–luminal interface and the medial–adventitial interface. The intimal-media far-wall thickness equal to or more than 1.3 mm at any segment in carotid arteries was defined as an atherosclerotic plaque [25]. All carotid artery examinations were done with a 5.0 MHz convex transducer probe.

Longitudinal sonographs of the abdominal aorta were obtained at 1-cm intervals and transverse scans at 2-cm intervals at the area of three aortic segments: (1) supra-pancreatic, (2) pancreatic and infra-pancreatic, (3) and at the area of the aortic bifurcation. As for carotid plaques, significant aortic plaques were defined as an intima-media far-wall thickness equal to or more than 3.0 mm [25]. All aortic examinations were performed using a 3.75 MHz convex transducer probe. The average duration for whole examination varied from 25 to 30 min.

The atherosclerotic severity score (AS) was constructed by dividing the atherosclerotic changes of abdominal aorta and carotid arteries into three severity classes: 1—slight, 2—moderate, and 3—severe, and calculating the sum, i.e. AS.

The reproducibility of our sonographic protocol for significant aortic and carotid plaques was also examined: 1 month after the first assessment 20 randomly selected subjects were invited to a

repeated examination. The repeatability of the number of plaques (number of plaques initial by repeated sonography) between the first and second examination was 90% for the carotid artery segment areas and 100% for the aortic segments.

2.4. DNA extraction and ESR1 genotyping

DNA was isolated from white blood cells using commercial kit (Qiagen Inc, CA). A region of the *ESR1* gene containing a part of intron one and exon two was amplified using primary and secondary (nested) primers designed from those reported by Yaich et al. [16]. After digestion of the PCR product with *Pvu*II restriction endonuclease, fragments were separated using agarose gel (1.0%) electrophoresis. Capital (P, mutated) and small (p, wild type) letters denoted the absence and presence of the restriction sites, respectively.

2.5. Other laboratory analyzes

For the cross-sectional study the concentrations of serum lipids and apolipoproteins (apo) were measured. Serum total cholesterol and triglycerides were determined by a commercial method (Kodak Echtachem 700XR, Eastman Kodak Company, Clinical Products Division, Rochester, USA). Serum HDL cholesterol were separated by a dextran–sulfate–Mg precipitation procedure [26] and the cholesterol content was analyzed with a Monarch 2000 Analyzer (Instrumentation Laboratory, Lexington, USA), using the CHOD-PAP cholesterol reagent (Cat No. 237574; Boehringer Mannheim, Germany) and a primary cholesterol standard (Cat No. 530; Orion, Finland). The LDL cholesterol content was calculated according to the Friedewald formula [27]. Apo A1 and B were determined on a Monarch Analyzer by an immunoturbidimetric method [28] (Cat No. 67265 and 67249, Orion Diagnostics, Finland). In the 5-year follow-up study the lipid concentrations were determined with Cobas Integra 700 analyzer with reagents and calibrations recommended by the manufacturer (Hoffmann-La Roche Ltd, Basel, Switzerland).

2.6. Statistical analysis

Two-way ANOVA was used to assess the interaction between *ESR1 Pvu*II genotype groups and treatment groups. One-way ANOVA was used to assess the statistical differences between HRT groups and control group and among *ESR1 Pvu*II genotypes in lipid parameters. We also used repeated measures analysis of variance (RANOVA) to study the effect of time, *ESR1 Pvu*II genotype and HRT on AS in our study population. All calculations were done with the *Statistica for Windows* version 5.1 (Statsoft Inc., Tulsa, Oklahoma, USA) software in PC. Data are presented as mean $\pm$SD unless otherwise stated. A P-value less than 0.05 was considered statistically significant.

3. Results

Table 1 presents the baseline characteristics of subjects according to *ESR1 Pvu*II genotype. The mean age, BMI, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, apo A1 and apo B were not significantly different among the genotypes. Subjects with the P/P genotype had a higher AS (1.93 ± 0.92) as compared with P/p (1.24 ± 0.85) and p/p (1.43 ± 0.84) genotypes ($P = 0.012$).

Table 2 shows the baseline and final values and change of lipid levels and AS in postmenopausal women among treatment groups. The changes of total cholesterol, triglycerides, LDL cholesterol, apo A1 and apo B during 5-year follow-up were not significantly different among three treatment groups. Subjects in HRT-EVP group had higher changes of HDL cholesterol and less progression of AS compared with subjects in control group, although this effect was borderline significant ($P = 0.071$ and $P = 0.084$, respectively). Subjects in HRT-EV group had less progression of AS compared with subjects in control group ($P < 0.05$).

Table 3 shows the baseline and final values and change of lipid levels and AS in three treatment groups according to genotype. There was a borderline significant genotype-by-treatment interaction

Table 1
Baseline characteristics of 88 postmenopausal women according to *ESR1 Pvu*II genotypes

Variable and unit	P/P ($n = 14$)	P/p ($n = 46$)	p/p ($n = 28$)	P-value
Age, years	59.4±5.39	60.7±5.08	61.0±5.84	0.670
Body mass index (kg/m^2)	27.0±3.11	25.3±3.05	25.8±2.88	0.218
Total cholesterol (mmol/l)	6.37±1.09	6.31±0.99	6.43±1.14	0.891
Hypercholesterolemia(% (n))	42.9 (6)	39.1 (18)	46.4 (13)	0.868
HDL cholesterol (mmol/l)	1.51±0.23	1.55±0.32	1.60±0.35	0.599
LDL cholesterol (mmol/l)	4.37±0.93	4.21±0.94	4.31±1.05	0.745
Triglycerides (mmol/l)	1.07±0.47	1.23±0.56	1.16±0.76	0.677
Apolipoprotein A1 (g/l)	1.41±0.17	1.46±0.25	1.47±0.21	0.659
Apolipoprotein B (g/l)	0.91±0.19	0.92±0.18	0.92±0.23	0.997
Atherosclerotic severity score	1.93±0.92	1.24±0.85	1.43±0.84	0.028

HDL, high density lipoprotein; LDL, low density lipoprotein. Hypercholesterolemia, total cholesterol > 6.5 mmol/l. Significance is based on analysis of covariance, age as covariate.

for AS ($P = 0.078$, $P = 0.084$ when AS baseline as covariate). There was a significant genotype-by-treatment (HRT-EVP and control groups) interaction for AS ($P = 0.034$). In response to HRT-EVP, subjects with P/P, compared with those with P/p and p/p genotypes, had a less increase in AS (1.61±1.14 vs. 1.71±1.27 vs. 2.43±1.27). When AS at baseline as covariate in above similar model, the significant genotype-by-treatment interaction for changes of AS between HRT-EVP and control groups was still remained ($P = 0.036$). But this effect was not found between HRT-EV and control groups.

The effect of HRT and *ESR1 Pvu*II genotype on AS was also seen in RANOVA. We used AS as repeated measure, treatment groups, and *ESR1 Pvu*II genotype as independent factors, and age as covariate. HRT treatment, time, and *ESR1 Pvu*II genotype had a statistically significant or borderline significant main effect on progression of AS during 5-year follow-up ($P = 0.004$, $P < 0.001$, and $P = 0.090$, respectively). The result is presented in Fig. 1.

4. Discussion

The present study shows that both long-term HRT and *ESR1 Pvu*II genotype have effects on AS in postmenopausal women during 5-year follow-up. All subjects in this study were non-smokers, did not have diabetes, had normal blood pressure, and were also otherwise clinically healthy. The dietary analysis did not reveal any substantial differences in the use of saturated fat or dietary cholesterol between the HRT groups [21]. Therefore, our findings are unlikely to be caused by differences in background characteristics between the HRT and control groups [21]. Since, some other factors that may differ between HRT users and non-users (i.e. socioeconomic status, family history) were not accounted, one can say that these unknown factors may have biased our results.

Estrogen has never been shown to slow atherosclerosis progression in women in randomized clinical trials [5,6], despite numerous findings from non-randomized experiments showing that postmenopausal estrogen therapy has favorable effects on the serum lipoprotein profile and slows the development of atherosclerotic diseases [19,20,23,29]. Our findings might at least partly explain these contradictory results. In these previous studies, the possible genotypic effects on HRT response are not considered. Therefore, it would be interesting to perform a reanalysis of previous negative trials [5,6] to find out if there is a genetic subgroup, which would benefit more from HRT than other groups. Although *ESR1* is expressed in the atherosclerotic plaque [13], no previous study has investigated the association of *ESR1* gene variation with AS. It is possible that the transcriptional effects of *ESR1* variant forms differ from the 'wild-type' receptor and might thus

Table 2
Baseline and follow-up lipid levels and atherosclerosis severity score in postmenopausal women among treatment groups

Variable and unit	HRT-EVP ($n = 26$)	HRT-EV ($n = 32$)	C ($n = 30$)	P-value
Total cholesterol (mmol/l)				
Baseline	5.79 ± 0.87	6.55 ± 0.95	6.64 ± 1.10	
Follow-up	5.64 ± 0.81	6.10 ± 0.86	6.24 ± 0.97	
Δ	−0.16 ± 0.63	−0.44 ± 0.64	−0.40 ± 0.73	0.271
HDL cholesterol (mmol/l)				
Baseline	1.49 ± 0.24	1.66 ± 0.37	1.52 ± 0.30	
Follow-up	1.78 ± 0.38	1.77 ± 0.43	1.68 ± 0.41	
Δ	0.29 ± 0.27*	0.11 ± 0.33	0.16 ± 0.26	0.024
LDL cholesterol (mmol/l)				
Baseline	3.95 ± 0.88	4.24 ± 0.87	4.56 ± 1.06	
Follow-up	3.43 ± 0.78	3.65 ± 0.85	3.91 ± 0.89	
Δ	−0.52 ± 0.62	−0.58 ± 0.73	−0.64 ± 0.67	0.943
Triglycerides (mmol/l)				
Baseline	0.79 ± 0.23	1.44 ± 0.67	1.25 ± 0.62	
Follow-up	0.96 ± 0.41	1.49 ± 0.49	1.44 ± 0.65	
Δ	0.17 ± 0.28	0.06 ± 0.70	0.18 ± 0.52	0.609
Apolipoprotein A1 (g/l)				
Baseline	1.35 ± 0.15	1.57 ± 0.26	1.42 ± 0.17	
Follow-up	1.92 ± 0.31	2.08 ± 0.32	1.92 ± 0.26	
Δ	0.57 ± 0.26	0.51 ± 0.24	0.50 ± 0.19	0.381
Apolipoprotein B (g/l)				
Baseline	0.82 ± 0.16	0.97 ± 0.17	0.95 ± 0.22	
Follow-up	1.21 ± 0.23	1.34 ± 0.22	1.38 ± 0.29	
Δ	0.39 ± 0.14	0.37 ± 0.16	0.43 ± 0.17	0.285
Atherosclerotic severity score				
Baseline	1.31 ± 0.84	1.13 ± 0.79	1.80 ± 0.89	
Follow-up	3.19 ± 1.36	2.91 ± 1.35	4.30 ± 1.80	
Δ	1.88 ± 1.24**	1.78 ± 1.18***	2.50 ± 1.36	0.096

HDL, high density lipoprotein; LDL, low density lipoprotein. C, controls; EV, estradiol valerate, 2.0 mg/day; P, levonorgestrel, 0.25 mg/day. The Δ was calculated as follows:

$$\Delta = \sum_{i=1}^{n} (\text{follow-up}_i - \text{baseline}_i)/n$$

i, number of subgroup subjects. Age was used as covariate.
* $P = 0.071$ EVP vs. C.
** $P = 0.084$ EVP vs. C.
*** $P < 0.05$ EV vs. C.

affect the atheroprotective roles of circulating estrogens [3]. It has been speculated that the *Pvu*II II polymorphism affects the splicing of *ESR1* mRNA, resulting in the alteration of protein expression [14,30] or that the polymorphism is linked to some other polymorphism relevant to protein expression [14]. Since the studied polymorphism is intronic in nature [16], it is most probable that it is in linkage disequilibrium with a mutation elsewhere in the *ESR1* gene. The mechanism how the *ESR1 Pvu*II polymorphism affects signalling via *ESR1* is not known, but the variant forms are strongly associated with the area of advanced atherosclerotic lesions. This supports the hypothesis that at least some variation in *ESR1* gene may affect how the atheroprotective

Table 3
Baseline and follow-up lipid levels and atherosclerosis severity score according to *ESR1 Pvu*II genotypes and treatment groups

	HRT-EVP			HRT-EV			C		
	P/P ($n=5$)	P/p ($n=14$)	p/p ($n=7$)	P/P ($n=3$)	P/p ($n=19$)	p/p ($n=10$)	P/P ($n=6$)	P/p ($n=13$)	p/p ($n=11$)
Total cholesterol (mmol/l)									
Baseline	5.60±1.12	5.82±0.94	5.89±0.61	6.33±0.72	6.60±0.77	6.50±1.35	7.03±0.85	6.40±1.17	6.70±1.16
Follow-up	5.60±1.07	5.76±0.71	5.41±0.88	5.87±0.91	6.18±0.58	6.03±1.28	6.53±0.68	6.04±0.92	6.31±1.16
Δ	0.00±0.39	−0.06±0.72	−0.47±0.51	−0.46±0.43	−0.43±0.65	−0.47±0.73	−0.50±0.31	−0.36±0.72	−0.39±0.9
Triglycerides (mmol/l)									
Baseline	0.81±0.28	0.84±0.23	0.68±0.20	1.08±0.33	1.44±0.40	1.53±1.08	1.28±0.59	1.35±0.78	1.13±0.43
Follow-up	1.00±0.28	0.97±0.36	0.90±0.59	1.39±0.14	1.53±0.45	1.45±0.63	1.51±0.72	1.51±0.74	1.31±0.55
Δ	0.20±0.20	0.13±0.25	0.23±0.40	0.31±0.29	0.09±0.48	−0.08±1.09	0.22±0.65	0.17±0.65	0.18±0.26
HDL cholesterol (mmol/l)									
Baseline	1.37±0.10	1.50±0.26	1.55±0.29	1.86±0.20	1.58±0.38	1.75±0.36	1.47±0.13	1.55±0.32	1.51±0.37
Follow-up	1.68±0.44	1.78±0.42	1.86±0.28	2.04±0.29	1.72±0.48	1.79±0.37	1.59±0.33	1.71±0.44	1.69±0.45
Δ	0.31±0.35	0.27±0.27	0.31±0.24	0.19±0.24	0.14±0.39	0.04±0.25	0.12±0.31	0.15±0.25	0.19±0.26
LDL cholesterol (mmol/l)									
Baseline	3.87±0.99	3.94±1.01	4.03±0.60	3.98±0.67	4.37±0.73	4.07±1.17	4.99±0.71	4.24±1.12	4.69±1.11
Follow-up	3.47±1.01	3.55±0.71	3.15±0.78	3.19±0.65	3.76±0.67	3.58±1.18	4.26±0.64	3.64±0.82	4.02±1.04
Δ	−0.40±0.65	−0.39±0.62	−0.88±0.52	−0.79±0.43	−0.60±0.85	−0.48±0.58	−0.72±0.36	−0.59±0.65	−0.66±0.85
Apolipoprotein A1 (g/l)									
Baseline	1.31±0.13	1.35±0.14	1.38±0.19	1.58±0.23	1.57±0.31	1.59±0.18	1.41±0.12	1.41±0.18	1.43±0.21
Follow-up	1.95±0.36	1.91±0.35	1.92±0.24	2.24±0.19	2.08±0.34	2.03±0.31	1.88±0.21	1.94±0.26	1.92±0.32
Δ	0.64±0.27	0.56±0.29	0.54±0.20	0.66±0.05	0.51±0.27	0.45±0.21	0.46±0.23	0.53±0.18	0.49±0.20
Apolipoprotein B (g/l)									
Baseline	0.80±0.18	0.84±0.18	0.79±0.13	0.86±0.15	0.97±0.13	0.98±0.24	1.02±0.19	0.92±0.21	0.95±0.25
Follow-up	1.25±0.26	1.24±0.23	1.14±0.22	1.20±0.23	1.37±0.17	1.30±0.28	1.50±0.21	1.32±0.28	1.38±0.33
Δ	0.45±0.14	0.39±0.13	0.35±0.16	0.34±0.17	0.40±0.15	0.33±0.18	0.48±0.04	0.40±0.15	0.44±0.22
AS:									
Baseline	1.80±0.84	1.14±0.95	1.29±0.49	1.33±1.53	1.11±0.66	1.10±0.88	2.33±0.52	1.54±0.97	1.82±0.87
Follow-up	3.40±1.67	2.86±1.35	3.71±1.11	3.67±2.08	3.00±1.33	2.50±1.18	5.33±1.75	4.31±1.93	3.73±1.56
Δ[a]	1.60±1.14	1.71±1.27	2.43±1.27	2.33±0.58	1.89±1.37	1.40±0.84	3.00±1.67	2.77±1.30	1.91±1.14

HDL, high density lipoprotein; LDL, low density lipoprotein; AS, atherosclerotic severity score. The Δ was calculated as follows:

$$\Delta = \sum_{i=1}^{n} (\text{follow-up}_i - \text{baseline}_i)/n$$

i, number of subgroup subjects.

[a] Borderline significant genotype-by-treatment interaction for AS ($P=0.078$). Significant genotype-by-treatment interaction (HRT-EVP and control groups) for AS ($P=0.034$). Age was used as covariate.

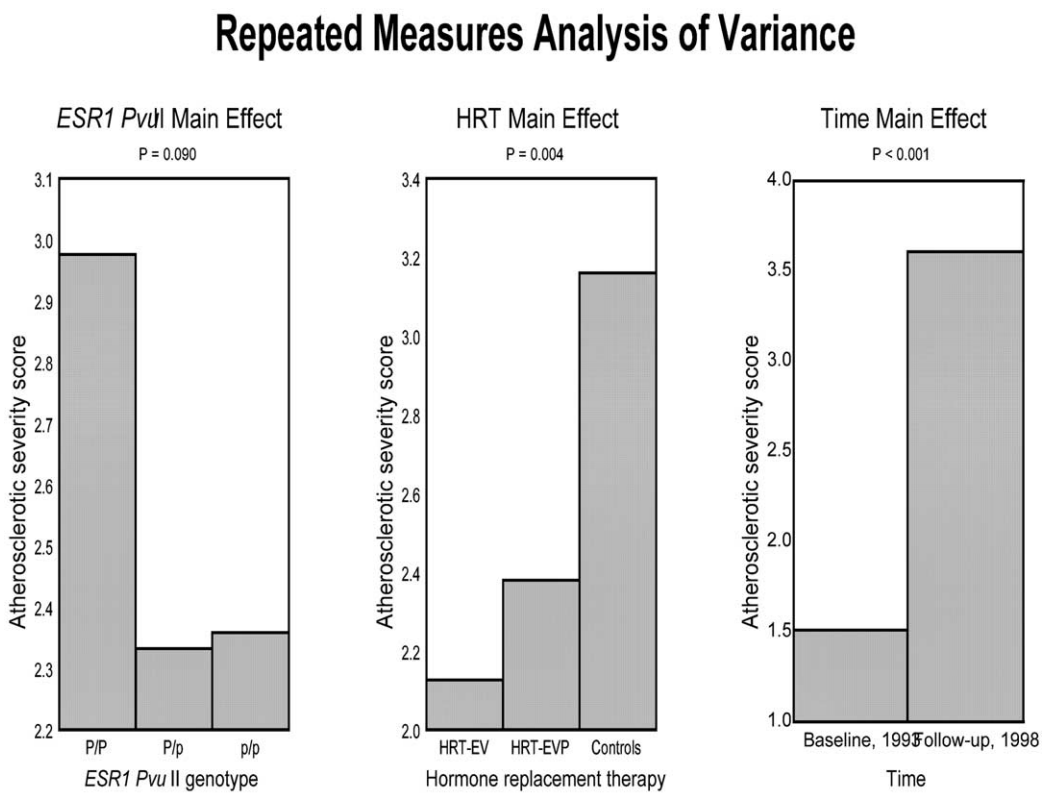

Fig. 1. The main effect of *ESR1 Pvu*II genotypes, HRT, and time on atherosclerotic severity score during 5-year follow-up analyzed by repeated measures ANOVA. *ESR1 Pvu*II, estrogen receptor 1 *Pvu*II; HRT, hormone replacement therapy. EV, estradiol valerate, 2.0 mg/day; *P*, levonorgestrel, 0.25 mg/day. Atherosclerotic severity score is estimated from aorta and carotid artery by sonography. Age was used as covariate.

actions of estrogens are mediated to artery wall cells.

Estrogens suppress arterial smooth muscle cell proliferation and migration [31], and they increase the synthesis of collagen, pro-collagen, and elastin in the arterial intima [32]. Estrogens reduce the accumulation of cholesterol in the arterial wall [33,34], and protect LDL from oxidation [35]. Since estrogens have many anti-atherogenic effects, it is possible that impaired effects of estrogens may lead to progression of atherosclerosis and higher risk for coronary thrombosis. In fact, a recent study suggested an association between diminished estrogen receptor expression and the occurrence of premature coronary atherosclerosis [13].

In the present study we show that a significant genotype-by-treatment (HRT-EVP and control groups) interaction for AS. In response HRT-EVP, subjects with P/P, had a less increase in AS, compared with those with P/p and p/p genotypes. We also found that both HRT and *ESR1 Pvu*II genotype had effect on AS by RANOVA.

In conclusion, our study shows that both long-term HRT-EV alone and combined HRT-EVP are associated with less severe atherosclerotic lesions as compared to controls not taking HRT. Our study also indicated that long-term HRT and *ESR1 Pvu*II genotype have effect on AS in postmenopausal women. *ESR1 Pvu*II polymorphism is associated with the progression of AS to treatment with HRT-EVP. Subjects with P/P genotype had a less progression in AS. The effect of HRT-EVP in postmenopausal women on progression of AS may be determined in part by the genotype of *ESR1 Pvu*II. Our study points out

that the *ESR1* gene is an interesting candidate gene behind the pathogenesis of acute coronary events, although the actual gene variant causing altered function remains to be solved.

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Carbohydrate-deficient transferrin and gamma-glutamyltransferase are inversely associated with lipid markers of cardiovascular risk

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Abstract

Background A variety of epidemiological studies have suggested a U-shaped association between alcohol consumption and atherosclerosis progression and incidence events. Moderate intake of alcohol is considered beneficial, whereas heavy drinking increases cardiovascular disease risk.

Methods Alcohol and cardiovascular risk-related laboratory tests were carried out in 70 consecutive male employees in connection with an occupational health survey in 1996. Carbohydrate-deficient transferrin (CDT) and gamma-glutamyltransferase (GGT) were used as markers for alcohol consumption. The subjects were divided into quartiles on the basis of CDT or GGT value.

Results The highest CDT quartile had significantly higher serum high-density lipoprotein (HDL)-cholesterol ($P < 0{\cdot}05$) than the lowest quartile. The highest GGT quartile had significantly higher serum total cholesterol ($P < 0{\cdot}01$), lower serum HDL-cholesterol ($P < 0{\cdot}05$), higher serum low-density lipoprotein (LDL)-cholesterol ($P < 0{\cdot}01$) and higher serum triglyceride ($P < 0{\cdot}01$) than the lowest quartile.

Conclusions An explanation for the findings is that high alcohol consumption without significant liver induction increases the level of HDL-cholesterol, whereas high alcohol consumption with induction of liver may have adverse effects on lipoprotein metabolism. The results were interpreted to indicate that CDT and GGT detect different populations of drinkers in regard to cardiovascular lipid risk factors.

Keywords Carbohydrate-deficient transferrin, cholesterol, ethanol, gamma-glutamyl-transferase, lipids.
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Introduction

Population studies have suggested that moderate long-lasting intake of alcohol may help to protect the heart from coronary artery disease [1]. This is partly thought to be mediated by the effects of alcohol on lipoprotein metabolism, because alcohol consumption has generally been associated with an increase in high-density lipoprotein (HDL)-cholesterol [2] and/or decrease in low-density lipoprotein (LDL)-cholesterol [3]. In addition, alcohol intake may reduce blood coagulation and enhance the dissolution of blood clots [4,5]. In contrast, heavy drinking and alcoholism can result in cardiomyopathy, arrhythmias and increased blood pressure [6]. The toxic effects of alcohol on lipoprotein metabolism, probably through its action in the liver, may include fatty liver and, in the later phase, hypercholesterolaemia and decreased HDL-cholesterol [7].

Carbohydrate-deficient transferrin (CDT) and gamma-glutamyltransferase (GGT) are generally considered to be the most useful laboratory markers for high alcohol consumption [8,9]. The traditionally used GGT is known to reflect liver status, and the values are increased by alcohol and other liver microsome-inducing agents, by most hepatobiliary disorders and by obesity, diabetes mellitus and hypertriglyceridaemia. As those two markers do not correlate with each other [8–12], they may offer an additional viewpoint for studying the effect of alcohol on lipids. In the present study, differences in total CDT and GGT

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values were compared with the lipid profile of male employees in a routine occupational health survey.

Subjects and methods

Alcohol and lipid profile-related laboratory tests were carried out in 70 consecutive non-alcoholic male employees (mean age 45 years, range 37–58 years, SE 0·7 years) in connection with an occupational health survey in 1996. The employees were both blue-collar and white-collar workers from a board machine manufacturing factory. The sera were stored at −20°C until the first analysis.

The activity of serum GGT was determined according to the recommendations of the Scandinavian Society for Clinical Chemistry and Clinical Physiology [13]. Total cholesterol and HDL-cholesterol were determined by an enzymatic colorimetric method (CHOD-PAP, Labsystems, Finland, or Boehringer Mannheim, Germany) in a standardized laboratory. Triglycerides were determined by enzymatic hydrolysis of triglycerides with subsequent determination of liberated glycerol by colorimetry (Boehringer Mannheim). LDL-cholesterol was calculated according to the Friedewald formula. The CDT in the sera was determined in duplicate determinations using the CDTect RIA kit (Pharmacia Diagnostics, Uppsala, Sweden). Body mass index (BMI) was calculated as weight (kg)/height^2 (m^2). Alcohol consumption was also estimated by the AUDIT test, with a score of 8 points or more indicating high alcohol consumption [14,15]. A current smoker was defined as a person who now smokes daily. In addition, a trained nurse/physician reported drug use, known diseases and relevant clinical symptoms of the employees.

The statistical evaluation was carried out with Statistica software (Statsoft, Tulsa, OK, USA, 1996 version). Estimated significance was based on ANOVA.

Employees were divided into quartiles on the basis of CDT value or GGT value (Table 1). The lowest CDT quartile (Q_1) had serum values of under 12 U L^{-1}, the second quartile (Q_2) was from 12 to under 14 U L^{-1}, the third quartile (Q_3) was from 14 to under 16 U L^{-1} and the highest CDT quartile (Q_4) had values of 16 U L^{-1} or over. The serum GGT values for the GGT quartiles were: Q_1, under 28 U L^{-1}; Q_2, 28–40 U L^{-1}; Q_3, over 40 U L^{-1} but under 69; Q_4, 69 U L^{-1} or over. There was an overlap of four men (6%) between the lowest CDT and lowest GGT quartiles, and an overlap of four men (6%) between the highest quartiles, indicating that the extreme quartiles represent populations distinct from each other.

Table 1 presents background characteristics and lipid values of the CDT and GGT quartiles. There was a statistically significant difference in the AUDIT test scores between the extreme GGT quartiles. There was a trend of increased AUDIT test values between the extreme CDT quartiles, but it did not reach statistical significance (6·2 vs. 7·3 points respectively). The extreme CDT quartiles were similar in regard to BMI, age and smoking. The extreme GGT quartiles differed significantly in BMI values but were similar in regard to age and smoking. None of the participants had diabetes and none was on lipid-lowering medication.

Results

To visualize the relation of markers of alcohol consumption with serum lipid levels, the subjects were divided into quartiles according to CDT and GGT. Figure 1 represents cardiovascular lipid risk factors in the extreme quartiles of CDT and GGT values. The highest CDT quartile had significantly higher HDL-cholesterol ($P<0·05$) (Fig. 1B and Table 1) than the lowest quartile. The highest GGT quartile had significantly higher total cholesterol ($P<0·01$)

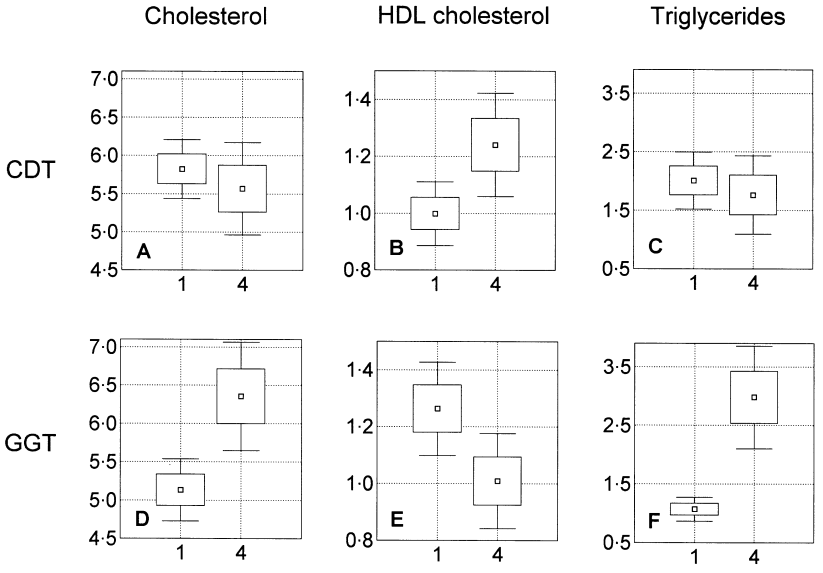

Figure 1 Cholesterol, HDL-cholesterol and triglycerides (mmol L^{-1}) in the lowest (1) and highest (4) CDT and GGT quartiles. CDT quartiles: lowest ≤ 12 U L^{-1}, highest ≥ 16 U L^{-1}. GGT-quartiles: lowest < 29 U L^{-1}, highest ≥ 75 U L^{-1}. The larger box, ± 1·00 SE; the vertical lines, ± 1·96 SE; the small box, mean. Elevated CDT seems to have positive effects on lipid values, whereas elevated GGT seems to have adverse effects on them.

Table 1 Background characteristics and lipid values of the CDT and GGT quartiles among 70 consecutive male employees

	Q_1	Q_2	Q_3	Q_4	Significance
CDT					
n	17	17	18	18	
Age	43·0 ± 1·2	44·9 ± 1·6	44·9 ± 1·4	45·3 ± 1·5	P = NS
BMI	26·1 ± 0·7	27·2 ± 0·9	25·5 ± 0·7	26·4 ± 0·7	P = NS
AUDIT score	6·2 ± 0·8	6·6 ± 1·1	5·9 ± 0·8	7·3 ± 1·0	P = NS
AUDIT score ≥ 8	35%	41%	33%	50%	
Smokers	12%	29%	22%	11%	P = NS
Cholesterol	5·82 ± 0·20	6·12 ± 0·25	5·96 ± 0·28	5·56 ± 0·31	P = NS
HDL-cholesterol	0·99 ± 0·06	1·01 ± 0·07	1·15 ± 0·10	1·24 ± 0·09	P < 0·05
LDL-cholesterol	3·92 ± 0·19	4·10 ± 0·20	3·91 ± 0·32	3·53 ± 0·26	P = NS
Triglycerides	2·01 ± 0·25	2·28 ± 0·46	1·97 ± 0·30	1·76 ± 0·34	P = NS
GGT					
n	16	17	19	18	
Age	42·5 ± 1·3	45·1 ± 1·3	45·0 ± 1·6	45·3 ± 1·5	P = NS
BMI	23·4 ± 0·7	25·5 ± 0·5	27·7 ± 0·6	28·1 ± 0·7	P < 0·01
AUDIT score	5·6 ± 1·0	6·1 ± 0·9	5·9 ± 0·9	8·3 ± 0·8	P < 0·05
AUDIT score ≥ 8	19%	29%	37%	78%	
Smokers	19%	6%	16%	33%	P = NS
Cholesterol	5·13 ± 0·21	6·06 ± 0·20	5·85 ± 0·18	6·36 ± 0·36	P < 0·01
HDL-cholesterol	1·26 ± 0·08	1·23 ± 0·10	0·95 ± 0·05	1·01 ± 0·08	P < 0·05
LDL-cholesterol	3·39 ± 0·22	3·98 ± 0·20	3·99 ± 0·17	3·99 ± 0·36	P < 0·01
Triglycerides	1·07 ± 0·10	1·87 ± 0·24	1·99 ± 0·29	2·98 ± 0·44	P < 0·01

Means and SE of the CDT and GGT quartiles among non-alcoholic male employees. Statistical significance was determined between the lowest (Q_1) and highest (Q_4) quartiles. NS, not significant.

Table 2 Lipid values of subjects belonging to opposite halves of CDT and GGT

	CDT Q_{1-2} /GGT Q_{3-4} (n = 20)	CDT Q_{3-4} /GGT Q_{1-2} (n = 20)	Significance
Age	43·2 ± 1·34	42·7 ± 1·2	P = NS
BMI	27·8 ± 0·5	23·8 ± 0·5	P < 0·01
AUDIT score	7·6 ± 0·8	6·6 ± 0·8	P = NS
AUDIT score ≥ 8	45%	25%	
Smokers	35%	20%	P = NS
Cholesterol	6·25 ± 0·22	5·56 ± 0·25	P < 0·05
HDL-cholesterol	0·96 ± 0·06	1·36 ± 0·08	P < 0·01
LDL-cholesterol	4·22 ± 0·19	3·64 ± 0·23	P = NS
Triglycerides	2·37 ± 0·39	1·23 ± 0·15	P < 0·01

Comparison of means and SE of lipid values in subjects who have a lower half CDT value and a higher half GGT value (CDT Q_{1-2} /GGT Q_{3-4}) or a higher half CDT value and a lower half GGT value (CDT Q_{3-4}/GGT Q_{1-2}). NS, not significant.

(Fig. 1D, Table 1), lower HDL-cholesterol (P < 0·05) (Fig. 1E, Table 1), higher LDL-cholesterol (P < 0·05)(Table 1) and higher triglyceride (P < 0·01) (Fig. 1F and Table 1) than the lowest quartile. There was no statistically significant difference between the extreme CDT quartiles in the total serum cholesterol (Fig. 1A and Table 1) and triglyceride values (Fig. 1C, Table 1). There was no statistically significant difference between the extreme CDT quartiles in LDL-cholesterol values (Table 1).

The highest CDT quartile and the lowest GGT quartile seemed to be associated with a favourable lipid profile, and the lowest CDT quartile and the highest GGT quartile were associated with an unfavourable lipid profile. We therefore additionally compared the lipid values in subjects who had a higher half CDT value and a lower half GGT value (CDT Q_{3-4}/GGT Q_{1-2}) or a lower half CDT value and a higher half GGT value (CDT Q_{1-2}/GGT Q_{3-4}) (Table 2). The favourable CDT Q_{3-4}/GGT Q_{1-2} group had significantly lower cholesterol values, higher HDL-cholesterol values and lower triglyceride values than the unfavourable CDT Q_{1-2}/GGT Q_{3-4} group (Table 2).

Discussion

CDT has proved to be a useful marker of high continuous alcohol consumption [8,9,16]. We show that the subjects in the highest CDT quartile had an elevated HDL value

compared with the lowest quartile. This is consistent with the hypothesis that the ingestion of alcohol has a protective effect against coronary heart disease, presumed to be mediated mainly through elevation of HDL values [1,6,17]. An impact of alcohol on sialylated lipoproteins, such as LDL, very low-density lipoprotein (VLDL) and HDL [18], may be one of the mechanisms leading to increased HDL values in the highest CDT quartile. It appears that CDT is also associated with factors related to insulin/glucose metabolism, because a previous study has shown that triglyceride concentrations in diabetic individuals with high CDT values are low rather than high [19].

In contrast to the beneficial effects of moderate ingestion of alcohol, excessive alcohol consumption has been shown to lead to derangements of lipid and lipoprotein metabolism. This is due to the toxic effects of ethanol on peripheral and hepatic lipid metabolism, leading to hypercholesterolaemia, decreased HDL-cholesterol and hypertriglyceridaemia [7]. Ethanol-induced changes in hepatic metabolism are hypothesized to be the basis for delayed lipoprotein clearance [20]. Elevation of GGT in serum probably reflects its induced hepatic synthesis and increased transport to the liver plasma membranes [21,22]. Thus, alcohol-induced GGT [9,16] may offer a tool to detect non-beneficial effects of alcohol on cardiovascular lipid risk factors. GGT has previously been shown to have a positive association with total serum cholesterol, HDL-cholesterol and triglycerides [23]. However, we report here decreased HDL-cholesterol, which is more in line with the chronic effects of high alcohol consumption on lipoprotein metabolism [7]. There is a known relationship between GGT values and BMI [23]. This was also apparent in the present study, with subjects in the highest GGT quartile having statistically higher BMI values than those in the lowest quartile. Nevertheless, this quartile had a considerably high proportion of alcohol abusers, as supported by the high mean AUDIT-score and the high percentage of subjects with an AUDIT score of ≥ 8.

In conclusion, the present results indicate that high alcohol consumption with normal serum level of GGT is associated with increased serum HDL-cholesterol, whereas high alcohol consumption with liver induction leading to elevated GGT may have adverse effects on lipoprotein metabolism, including hypercholesterolaemia, decreased HDL-cholesterol, increased LDL-cholesterol and hypertriglyceridaemia. CDT and GGT are independently correlated with heavy drinking [8–12], and they seem to detect different populations of drinkers in regard to lipid metabolism. The observation may help to understand different biochemical effects of alcohol consumption on lipids.

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Association of carbohydrate-deficient transferrin (CDT) and gamma-glutamyl-transferase (GGT) with serum lipid profile in the Finnish population

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Abstract

Background: Moderate consumption of alcohol may reduce mortality from vascular diseases. The beneficial effects of alcohol may partly be mediated by its effects on lipoprotein metabolism. We studied the connection between alcohol consumption and the serum lipid profile from a well-documented national health program study. *Methods and results:* Carbohydrate-deficient transferrin (CDT) and gamma-glutamyl-transferase (GGT) were used as biochemical markers for alcohol consumption. The laboratory analyses were carried out on 5675 subjects (3097 males and 2578 females). The subjects were divided into quartiles on the basis of CDT or GGT value. The highest CDT quartile and the lowest GGT quartile seemed to be associated with a favorable lipid profile and the lowest CDT quartile and the highest GGT quartile were associated with an unfavorable lipid profile. Serum high density lipoprotein (HDL) cholesterol values were significantly higher and triglycerides lower with increasing serum CDT concentrations for both men and women. Increasing serum GGT was associated with higher serum total cholesterol and higher triglycerides in both men and women and lower HDL cholesterol in men. *Conclusions:* CDT and GGT seem to detect different populations of subjects in regard to lipid metabolism. These observations may lead to a better understanding of the effects of alcohol consumption on lipids as well as mechanisms behind favorable and detrimental effects of alcohol on vascular diseases. *Condensed abstract:* Carbohydrate-deficient transferrin (CDT) and gamma-glutamyl-transferase (GGT) were used as biochemical markers for alcohol consumption. A total of 3097 males and 2578 females were divided into quartiles on the basis of their CDT or GGT values. The highest CDT quartiles had higher HDL and lower triglycerides, whereas the highest GGT quartiles appeared to be associated with higher total cholesterol and triglycerides in both genders and lower HDL in men. CDT and GGT seem to detect different populations of subjects in regard to lipid metabolism. These observations may have important clinical and public health implications.

Keywords: CDT; GGT; Ethanol; Cholesterol; Lipids

1. Introduction

Several studies have shown that moderate consumption of alcohol reduces mortality from vascular diseases [1]. There seems to be a U-shaped association between alcohol consumption and coronary heart disease (CHD) incidence events [2]. The beneficial effect of

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alcohol may partly be mediated by its effects on lipoprotein metabolism, since alcohol consumption has generally been associated with an increase of high density lipoprotein (HDL) cholesterol [3,4] and occasionally, a decrease of low density lipoprotein (LDL) cholesterol [5]. Alcohol intake may also reduce blood coagulation [6]. Excessive alcohol use and alcoholism have detrimental effects on the cardiovascular system and are associated with increased occurrence of stroke, abdominal aneurysms, hypertension, alcoholic cardiomyopathy, arrhythmias, as well as increased CHD [7]. The unfavorable effects of alcohol on lipoprotein metabolism include hypertriglyceridemia and fatty liver, and in the later phase, hypercholesterolemia and decreased HDL cholesterol [8].

Serum carbohydrate-deficient transferrin (CDT) and gamma-glutamyl-transferase (GGT) are generally considered to be useful laboratory markers for high alcohol consumption [9,10]. Transferrin is a monomeric, iron-binding glycoprotein, which is synthesized in the liver. Chronic alcohol consumption leads to deficiencies in the carbohydrate content of the protein by a yet unknown mechanism [11]. GGT is known to reflect liver function and its activity in serum is increased by alcohol and other liver microsomal inducing agents, in most hepatobiliary disorders, obesity, diabetes mellitus and hypertriglyceridemia. Elevation of GGT in serum probably reflects its enhanced hepatic synthesis rate, increased transport to the liver plasma membranes, as well as liver injury [12].

Both CDT and GGT are markers of alcohol consumption, but their serum values do not correlate with each other [13,14] and may, thus, reflect different drinking patterns [15]. We have previously reported, in a pilot study of 70 men, that high alcohol consumption with an elevated serum CDT concentration was associated with increased serum HDL cholesterol, whereas high alcohol consumption with an elevated serum GGT activity had adverse effects on lipoprotein metabolism, including hypercholesterolemia, decreased HDL cholesterol, increased LDL cholesterol and hypertriglyceridemia [16]. In the present study, we wished to determine whether this finding could be substantiated in a large population and in both genders. In the effort to reduce population cholesterol levels, it is of great interest to study the effect of alcohol consumption on the serum lipid profile from a well-documented national health program study population [17].

2. Methods

2.1. Subjects

The National Public Health Institute of Finland has performed large cross-sectional population surveys, the FINRISK studies, related to the risk factors of coronary artery disease every 5 years beginning in 1972 [17]. The 1997 FINRISK study was conducted in five geographic areas: Helsinki–Vantaa region in southern Finland, Turku–Loimaa region in southwestern Finland, Kuopio and North Karelia provinces in eastern Finland, and Oulu province in northern Finland. In each study area, an age- and gender-stratified random sample of 2000 subjects was drawn from the population aged between 25 and 64 years. In addition, a random sample of 500 men and 250 women was drawn in Helsinki–Vantaa region and in North Karelia province from the 65–74 years age-group. The present study is a sub-study of the 1997 FINRISK project. The total sample size was 11 500. The participation rate was 71% among men and 76% among women. Subjects who had missing data on serum lipids, GGT or CDT, who had diabetes, who were pregnant or who were using cholesterol lowering medication or hormonal treatment were excluded. Thus, 3097 males and 2578 females were included in the study. The present study was conducted according to the Helsinki Declaration of 1975 on Human Experimentation and was approved by the Ethical Committee of Primary Health Clinics in Finland. All participants gave informed consent to scientific use of the data and samples collected in the study.

2.2. Measurements

The survey included a self-administered questionnaire, which was sent to the subjects in advance, including 165 questions about previous and existing diseases, socioeconomic background, smoking habits, occupational status and alcohol intake. The participants returned the questionnaires to the survey site. Subsequently, the participants' height, weight and blood pressure were measured using standard procedures and a venous blood specimen was taken. Body mass index (BMI) was calculated as the ratio of weight (kg) to height squared (m^2).

Quantitative estimation of the alcohol intake was carried out by using 12 structured questions to determine the amount and frequency of drinking. The measure for average weekly intake included the following drinks: beer, cider, liquor, long drink and wine. The total mean consumption of all alcoholic drinks was used, expressed as grams of pure ethanol per week. The variable 'number of smokes per day' was computed by adding up the numbers of filter cigarettes, non-filter cigarettes, self-made cigarettes, pipe-fulls and cigars smoked on an average day for all subjects who reported having smoked within 1 month prior to the survey. 'Physical activity' was determined as a session of leisure-time exercise resulting in shortness of breath and sweating (times/week).

CDT concentrations in serum samples were analyzed by a double antibody kit (CDTect™, Pharmacia and Upjohn Diagnostics, Uppsala, Sweden) according to the manufacturer's instructions. The test is based on anion exchange chromatography and radioimmunoassay [9,18] and has a detection limit of 1 U/l and a measuring range of 5–300 U/l. The calibration of the test was assessed from a five-point standard curve derived from the displacement of ^{125}I-CDT from the antibody by known amounts of human transferrin, within the interval 0–100 U/l. CDTect gives the results in units per liter, which is comparable to mg/l in serum (1 U CDT refers to $\approx$1 mg transferrin). The activity of serum GGT was determined according to the recommendations of the Scandinavian Society for Clinical Chemistry and Clinical Physiology [19]. Total and HDL cholesterol were determined from fresh serum samples by an enzymatic method (CHOD-PAP, Boehringer Mannheim, Mannheim, Germany). Triglycerides were measured by a fully enzymatic method (GPO-PAP, Boehringer-Mannheim) using the Olli-C analyzer (Konelab, Espoo, Finland). The blood samples were analyzed in the laboratory of the National Public Health Institute in Helsinki, Finland and in the Pharmacia and Upjohn laboratory in Uppsala, Sweden. External quality assessments were made in collaboration with the World Health Organization Lipid Research Center in Prague.

Because the data was not normally distributed, subjects of both genders were divided into quartiles on the basis of CDT concentration or GGT activity (Tables 1 and 2). The statistical evaluation was carried out with Statistica software (Statsoft, Inc., Tulsa, OK, 1996 version). Estimated significance was based on analysis of variance (ANOVA).

3. Results

The serum concentrations of lipids in the CDT quartiles together with alcohol consumption during the previous week and confounding variables (age, BMI and smoking) are presented in Table 1. Serum CDT concentrations were positively associated with higher alcohol consumption and smoking and lower BMI in both genders. There was no significant difference in physical activity between the different CDT quartiles. Serum CDT concentrations were negatively associated with age in women, but not in men. Serum HDL cholesterol values was significantly higher and triglycerides lower with increasing serum CDT concentrations for both men and women. These results remained significant even after adjustment for age, BMI and smoking. Serum total cholesterol decreased with increasing CDT concentrations in women, but not in men. The decrease of total cholesterol in women disappeared, however, after adjustment for age, BMI and smoking. Serum CDT concentration and GGT activity were positively associated in men and negatively associated in women, but the negative association in women did not remain significant after adjustment for age, BMI and smoking (Table 1).

The data for the GGT quartiles are presented in Table 2. Increases in serum GGT activity were positively associated with higher alcohol consumption, smoking, age, and BMI in both genders. There was a significant difference in physical activity between the different GGT quartiles in both genders, but it disappeared after adjustment for age, BMI and smoking. Increasing serum GGT was associated with higher serum total cholesterol, slightly lower HDL cholesterol, and higher triglycerides. The small decrease of HDL cholesterol in women with increasing GGT lost its significance after adjustment for age, BMI and smoking. The corresponding decrease of HDL cholesterol in men and the other lipid results remained statistically significant even after adjustment for age, BMI and smoking. After this adjustment, serum GGT activity and CDT concentration were positively associated in men but not in women.

The unadjusted data for lipids in the CDT quartiles are shown in Fig. 1. It appears that increased CDT is associated with a favorable lipid profile, including higher HDL cholesterol and lower triglycerides. However, the decrease of total cholesterol with increased CDT in women was not significant after adjustment for age, BMI and smoking in the statistical analysis. In contrast to the favorable association of increased CDT with lipid risk factors, increase in GGT quartiles appeared to affect the lipid profile unfavorably. There were higher concentrations of total cholesterol and triglycerides for both genders with increasing GGT activity. The differences in HDL cholesterol between GGT quartiles were small in both men and women, but still there was a statistically significant decrease of HDL cholesterol in men even after adjustment for age, BMI and smoking. We did not find any differential effects of alcohol from various sources or frequency and quantity per one occasion of drinking on CDT concentrations or GGT activity (data not shown).

Since serum CDT concentration and GGT activity seemed to be inversely associated with lipid markers of cardiovascular risk, a comparison was made between the lipid values in subjects who had a highest quartile CDT and a lowest quartile GGT (CDT Q_4/GGT Q_1) or a lowest quartile CDT and a highest quartile GGT (CDT Q_1/GGT Q_4) (Table 3). The favorable CDT Q_4/GGT Q_1 group had significantly lower cholesterol and triglyceride values and higher HDL cholesterol values than the unfavorable CDT Q_1/GGT Q_4 group in both genders, even after adjustment for age and BMI.

4. Discussion

This cross-sectional study was carried out to assess the relation between the alcohol markers CDT and GGT and the lipid profile. We show that with increasing serum CDT concentrations, serum HDL cholesterol values were significantly higher and triglycerides lower in both men and women. The effect may partly be caused by alcohol, since CDT correlated positively with alcohol consumption. CDT has been extensively investigated as a potent marker of alcohol abuse [10,20]. The association of CDT with a favorable lipid profile is in agreement with our earlier pilot study, conducted in connection with a routine occupational health examination [16]. Numerous epidemiological studies have shown with consistency that moderate alcohol intake (variously defined but corresponding to one to four drinks per day) decreases risk of CHD [1]. A part of the protection is thought to be mediated by the effects of alcohol on blood lipid metabolism, particularly increases in HDL cholesterol [21]. A negative association of CDT with triglycerides in the present study confirms findings of a previous study, which showed that triglyceride concentrations in diabetic individuals with high CDT values were low rather than high [22].

Serum CDT was negatively associated with serum total cholesterol in women, but this effect disappeared after adjusting for age. This might be due to a decrease

Table 1
Background characteristics and serum lipid values in CDT quartiles[a]

Men ($n=3097$)	Q_1 ($n=779$) under 11.0 U/l (mean (S.D.))	Q_2 ($n=790$) 11.0–13.3 U/l (mean (S.D.))	Q_3 ($n=758$) 13.4–16.6 u/l (mean (S.D.))	Q_4 ($n=770$) 16.7 U/l or over (mean (S.D.))	Significance	
					P	P_a
Cholesterol (mmol/l)	5.48 (1.01)	5.57 (1.02)	5.51 (1.07)	5.57 (1.04)	ns	ns
HDL-cholesterol (mmol/l)	1.16 (0.27)	1.22 (0.28)	1.26 (0.29)	1.39 (0.35)	***	***
Triglycerides (mmol/l)	1.79 (1.05)	1.72 (1.17)	1.66 (1.12)	1.47 (0.92)	***	***
Alcohol last week (g)	54.9 (84.8)	61.7 (94.1)	82.9 (104.2)	136 (171)	***	***
Physical activity (times/week)	2.27 (1.95)	2.37 (3.40)	2.39 (2.16)	2.48 (2.47)	ns	ns
GGT (IU/l)	40.7 (39.5)	41.8 (82.1)	43.0 (52.6)	49.9 (72.9)	*	**
Age	48.7 (13.5)	48.0 (13.6)	48.1 (13.7)	49.8 (13.5)	*	
Body mass index (kg/m^2)	27.3 (3.71)	27.0 (3.87)	26.9 (3.88)	26.1 (3.92)	***	
Smoking (smokes/day)	2.99 (7.62)	3.39 (7.84)	4.10 (8.70)	6.85 (10.2)	***	
Women ($n=2578$)	Q_1 ($n=618$) under 13.9 U/l (mean (S.D.))	Q_2 ($n=634$) 13.9–17.3 U/l (mean (S.D.))	Q_3 ($n=667$) 17.4–21.9 U/l (mean (S.D.))	Q_4 ($n=659$) 22.0 U/l or over (mean (S.D.))		
Cholesterol (mmol/l)	5.65 (1.12)	5.58 (1.14)	5.42 (1.05)	5.27 (0.99)	***	ns
HDL-cholesterol (mmol/l)	1.41 (0.32)	1.47 (0.32)	1.57 (0.33)	1.67 (0.34)	***	***
Triglycerides (mmol/l)	1.47 (1.00)	1.31 (0.68)	1.15 (0.70)	0.98 (0.49)	***	***
Alcohol last week (g)	19.0 (35.0)	26.3 (49.8)	25.9 (41.7)	42.3 (60.1)	***	***
Physical activity (times/week)	2.37 (2.27)	2.33 (2.24)	2.40 (2.04)	2.38 (2.05)	ns	ns
GGT (IU/l)	26.9 (21.3)	27.6 (66.4)	23.4 (29.0)	21.0 (15.5)	**	ns
Age	49.3 (13.7)	46.8 (13.6)	44.2 (12.6)	42.1 (11.3)	***	
Body mass index (kg/m^2)	28.0 (5.45)	26.7 (5.24)	25.2 (4.49)	24.2 (4.03)	***	
Smoking (smokes/day)	1.73 (5.01)	1.80 (5.00)	1.89 (5.03)	2.89 (6.53)	***	

[a] P_a, P-value adjusted to age, body mass index and smoking; ns, nonsignificant. Significance is based on ANOVA.
* $P<0.05$.
** $P<0.01$.
*** $P<0.001$.

Table 2
Background characteristics and serum lipid values in GGT quartiles[a]

Men ($n=3097$)	Q_1 ($n=758$) under 22 IU/l (mean (S.D.))	Q_2 ($n=815$) 22–30 IU/l (mean (S.D.))	Q_3 ($n=790$) 31–46 IU/l (mean (S.D.))	Q_4 ($n=734$) 47 IU/l or over (mean (S.D.))	Significance	
					P	P_a
Cholesterol (mmol/l)	5.13 (0.96)	5.41 (0.93)	5.69 (0.99)	5.91 (1.10)	***	***
HDL-cholesterol (mmol/l)	1.30 (0.29)	1.25 (0.29)	1.23 (0.31)	1.25 (0.35)	***	***
Triglycerides (mmol/l)	1.26 (0.70)	1.48 (0.84)	1.77 (1.09)	2.17 (1.36)	***	***
Alcohol last week (g)	51.1 (74.3)	63.6 (84.7)	84.4 (115)	138 (176)	***	***
Physical activity (times/week)	2.46 (2.21)	2.53 (2.28)	2.31 (2.07)	2.19 (3.50)	*	ns
CDT (U/l)	14.5 (5.59)	14.4 (5.72)	14.9 (6.36)	15.6 (8.58)	**	***
Age	45.5 (14.5)	49.1 (14.0)	49.6 (13.3)	50.3 (11.9)	***	
Body mass index (kg/m^2)	24.9 (3.06)	26.3 (3.46)	27.4 (3.71)	28.8 (4.17)	***	
Smoking (smokes/day)	3.14 (7.05)	3.85 (8.20)	4.78 (9.00)	5.58 (10.4)	***	
Women ($n=2578$)	Q_1 ($n=703$) under 14 IU/l (mean (S.D.))	Q_2 ($n=655$) 15–18 IU/l (mean (S.D.))	Q_3 ($n=632$) 19–25 IU/l (mean (S.D.))	Q_4 ($n=588$) 26 IU/l (mean (S.D.))		
Cholesterol (mmol/l)	5.14 (1.00)	5.33 (1.02)	5.66 (1.10)	5.85 (1.10)	***	***
HDL-cholesterol (mmol/l)	1.55 (0.31)	1.55 (0.32)	1.52 (0.36)	1.50 (0.38)	*	ns
Triglycerides (mmol/l)	1.00 (0.50)	1.07 (0.56)	1.30 (0.67)	1.57 (1.07)	***	***
Alcohol last week (g)	21.6 (33.3)	28.0 (43.7)	30.8 (48.7)	34.9 (64.7)	***	***
Physical activity (times/week)	2.42 (2.00)	2.52 (2.32)	2.39 (2.07)	2.12 (2.18)	*	ns
CDT (U/l)	19.5 (6.32)	18.8 (6.37)	18.3 (7.08)	17.4 (7.09)	***	ns
Age	40.9 (11.9)	43.6 (12.7)	47.5 (13.4)	51.2 (12.0)	***	
Body mass index (kg/m^2)	24.0 (3.46)	25.0 (4.28)	26.6 (5.22)	28.8 (5.75)	***	
Smoking (smokes/day)	1.54 (4.46)	1.92 (5.00)	2.30 (5.69)	2.70 (6.58)	**	

[a] P_a, P-value adjusted to age, body mass index and smoking; ns, nonsignificant. Significance is based on ANOVA.
* $P<0.05$.
** $P<0.01$.
*** $P<0.001$.

of CDT during menopause together with age-related increase of serum total cholesterol [23]. Although the subjects on hormone therapy were excluded from the study, the effect of decrease of estrogen with menopause could also affect the results.

The mechanism by which chronic alcohol consumption causes an elevation of CDT levels in serum is still not understood. It may be that the activities of glycosyltransferases involved in transferrin carbohydrate side chain synthesis are diminished in the liver, at least in part, due to enzyme inactivation by acetaldehyde [24]. Most metabolic pathways of the liver are affected by alcohol, as are several endocrine systems in the whole body. The mechanism behind the association of CDT and lipoprotein metabolism is also not clear at present. We report that a decrease of BMI is associated with increased CDT in both genders, which is known to have a favorable effect on the lipid profile. However, even after adjusting for BMI, there is a strong independent association of CDT with HDL cholesterol and triglycerides. The baseline values of CDT have been shown to be fairly constant over time within the same individual, but there is some variation between individuals even without any alcohol consumption [25]. This could suggest that there is a yet unknown mechanism that may influence both (hepatic) CDT formation and lipoprotein metabolism.

In contrast to the beneficial effects of moderate alcohol consuption on CHD risk, excessive alcohol intake and alcoholism are associated with increased CHD risk. Alcohol use is associated with lipoprotein metabolism by hypertriglyceridemia and, once hepatic damage occurs, serum HDL cholesterol levels may actually be lower than normal [8]. Heavy alcohol consumption is known to elevate both CDT concentrations and GGT activity, but the contribution of drinking pattern to these effects is not completely understood. Alcohol consumption associated almost identically in the present study with CDT and GGT and we did not find any differential effects of alcohol from various sources (beer, wines or spirits) or frequency and quantity per one occasion of drinking on these markers. On the other hand, it has previously been proposed that in men, CDT levels may respond primarily to frequency of drinking, whereas GGT may be influenced primarily by drinking quantity per one occasion [15].

Unlike the CDT concentration, increasing serum GGT activity was associated with higher serum total cholesterol, and higher triglycerides in both genders. The association of GGT with total cholesterol and triglycerides may reflect deranged liver lipid metabolism possibly associated with fatty liver formation. Alcohol consumption is known to show a linear dose–response relationship with HDL cholesterol, but this increase may be modulated by the degree of liver injury [26]. Our observations agree with earlier studies showing no positive correlation between GGT activity and HDL cholesterol [27,28], although positive correlations have been reported [29]. With increasing GGT, there was actually a small decrease of HDL cholesterol values in both genders, but in women this decrease lost its significance after adjustment for age, BMI and smoking. Increases in serum GGT activity were associated with age and BMI in both genders, confirming earlier findings [29,30].

With regard to serum CDT concentration and GGT activity, an optimal lipid profile was observed in subjects who belonged to the highest quartile CDT group and also to the lowest quartile GGT group, even after adjustment for age and BMI. It is possible that CDT is an earlier phase marker of alcohol consumption than GGT, and GGT might in fact reflect toxic effects of ethanol on hepatic lipid metabolism, leading to hypercholesterolemia, decreased HDL cholesterol and hypertriglyceridemia [8,30]. On the other hand, this hypothesis is not supported by the fact that the average self-reported alcohol consuption was 136 g per week for

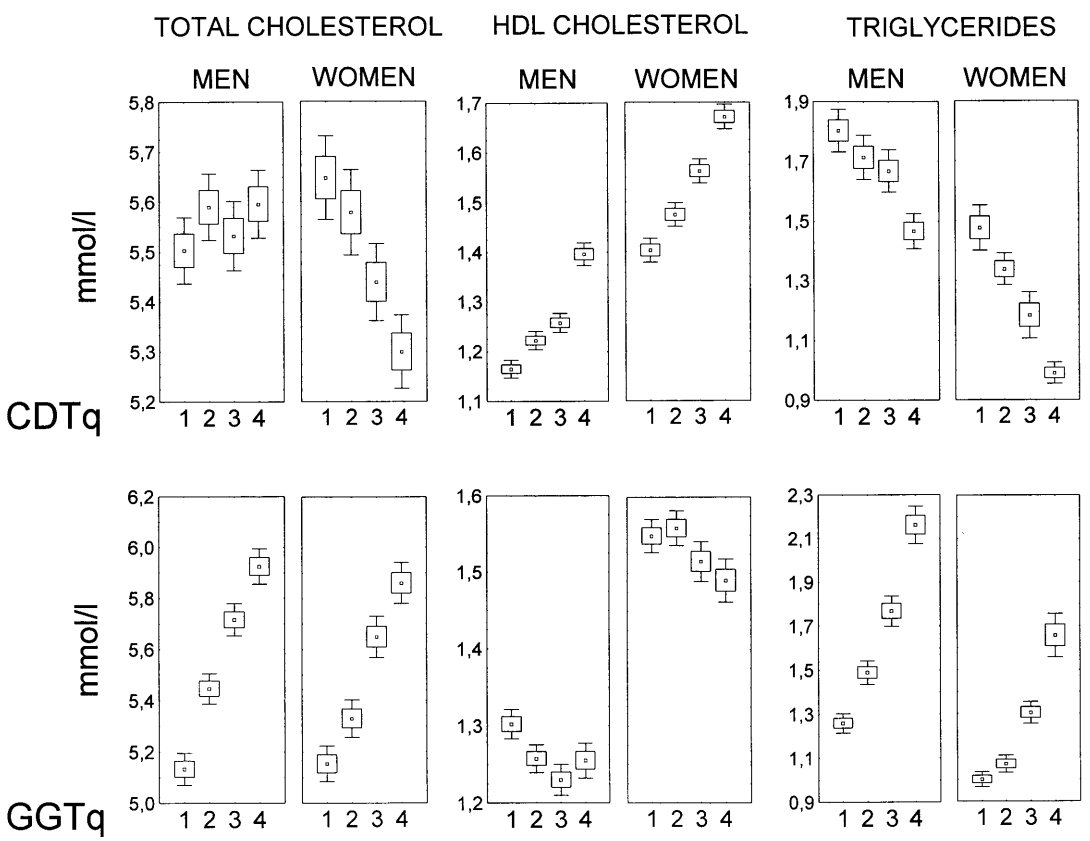

Fig. 1. Cholesterol, HDL cholesterol and triglycerides (mmol/l) in the CDT- and GGT-quartiles in men and women. The larger box, ±1.00 S.E.; the vertical lines, ±1.96 S.E.; the small box, mean.

Table 3
Lipid values of subjects belonging to opposite extreme quartiles of CDT and GGT[a]

Men	CDT Q_1/GGT Q_4 (S.D.) ($n=178$)	CDT Q_4/GGT Q_1 (S.D.) ($n=180$)	Significance	
			P	P_a
Cholesterol (mmol/l)	5.90 (1.14)	5.19 (0.98)	***	***
HDL-cholesterol (mmol/l)	1.11 (0.28)	1.42 (0.31)	***	***
Triglycerides (mmol/l)	2.35 (1.35)	1.12 (0.49)	**	***
Alcohol last week (g)	89.9 (109)	80.0 (94.1)	ns	ns
Physical activity (times/week)	2.11 (1.87)	2.56 (2.54)	ns	*
Age	49.9 (12.4)	47.3 (14.8)	ns	
Body mass index (kg/m^2)	29.2 (3.71)	24.3 (2.76)	***	
Smoking (smokes/day)	4.53 (9.57)	5.79 (8.83)	ns	
Women	CDT Q_1/GGT Q_4 (S.D.) ($n=194$)	CDT Q_4/GGT Q_1 (S.D.) ($n=223$)		
Cholesterol (mmol/l)	5.90 (1.09)	5.06 (0.97)	***	*
HDL-cholesterol (mmol/l)	1.36 (0.33)	1.62 (0.31)	***	***
Triglycerides (mmol/l)	1.84 (1.41)	0.89 (0.37)	***	***
Alcohol last week (g)	22.9 (42.2)	28.0 (38.4)	ns	ns
Physical activity (times/week)	1.89 (1.99)	2.19 (1.81)	ns	ns
Age	53.3 (12.0)	39.1 (10.0)	***	
Body mass index (kg/m^2)	30.4 (5.59)	23.1 (2.98)	***	
Smoking (smokes/day)	2.08 (5.58)	1.80 (4.70)	ns	

[a] P_a, P-value adjusted to age and body mass index; ns, nonsignificant. Significance is based on ANOVA.
* $P<0.05$.
** $P<0.01$.
*** $P<0.001$.

men and 42 g per week for women in the highest CDT quartile, and 138 g per week and 35 g per week in the highest GGT quartile, respectively. However, this alcohol consumption should be considered in the context that self-reporting of drinking is commonly unreliable, and the official per capita mean consumption of absolute ethanol in Finland for inhabitants 15 years and older is 8.4 l per year, corresponding to 162 g per week [31].

Concerning alcohol induced changes of lipoprotein metabolism, CDT appears to be associated with an increased serum HDL cholesterol concentration. In turn, GGT is known to be related to increased very low density lipoprotein (VLDL) cholesterol (reflected by increased triglycerides) [29]. GGT may also reflect increased low density lipoprotein (LDL) cholesterol, since elevation of VLDL was not large enough to explain the observed elevation of total cholesterol in the higher GGT quartiles.

In conclusion, the present results indicate that alcohol consumption, as determined by serum CDT concentration, is associated with increased serum HDL cholesterol and decreased triglycerides. On the other hand, alcohol consumption associated with liver induction and elevated GGT may have adverse effects on lipoprotein metabolism, including hypercholesterolemia, and hypertriglyceridemia. CDT and GGT seem to detect different populations of subjects in regard to lipid metabolism. These observations may lead to a better understanding of the effects of alcohol consumption on lipids as well as mechanisms behind favorable and detrimental effects of alcohol on vascular diseases.

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