Turun yliopisto
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OXIDIZED HDL LIPIDS AND THE RISK FOR ATHEROSCLEROSIS

The Cardiovascular Risk in Young Finns Study

Petri Kresanov

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
Sarja - ser. D osa - tom. 1259 | Medica - Odontologica | Turku 2016

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ISBN 978-951-29-6647-9 (PRINT)
ISBN 978-951-29-6648-6 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Painosalama Oy - Turku, Finland 2016

To My Family

ABSTRACT

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Oxidized HDL lipids and the risk for atherosclerosis.

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Background: Atherosclerosis is a chronic disorder caused by the accumulation of atherogenic lipoproteins in the artery wall leading to cardiovascular diseases (CVDs). Oxidation of lipoproteins may play a role in its etiogenesis. Oxidized low-density lipoproteins (LDL) are associated with the development of atherosclerosis. High-density lipoprotein (HDL) may protect against CVD through its anti-inflammatory, antioxidant and vasoprotective effects, and it may have a role in reverse cholesterol transport. Reverse transport of oxidized lipids by HDL is suggested to be an important mechanism explaining the atheroprotective mechanisms of HDL.

Aims: To study the associations of oxidized HDL lipids (oxHDLlipids) with the risk factors for atherosclerosis including serum fatty acids, developing fatty liver disease and serum paraoxonase-1 (PON1).

Subjects and Methods: The Cardiovascular Risk in Young Finns Study is an ongoing population-based multicenter follow-up study in Finland. The 21-year follow-up was conducted in 2001 with a total of 2,283 participants. Analysis of oxidized lipoprotein lipids was performed from samples collected in 2001 and was based on the determination of conjugated dienes.

Results: OxHDLlipids were directly associated with oxidized LDL lipids (oxLDLlipids) and inversely with age. In women, higher oxHDLlipid levels were associated with a lower polyunsaturated fatty acid (PUFA) proportion and a higher monounsaturated fatty acid (MUFA) proportion. The risk of future fatty liver disease was associated with lower oxHDLlipid levels. Intima-media thickness (IMT) and PON1 activity were not associated with oxHDLlipids.

Conclusion: An elevated cardiovascular risk profile is associated with lower oxHDLlipid levels in a population of young Finnish men and women.

Key Words: Oxidized lipids, atherosclerosis, HDL, LDL

TIIVISTELMÄ

Petri Kresanov

Hapettuneet HDL:n lipidit ja ateroskleroosiriski.

Turun yliopisto, lääketieteellinen tiedekunta, kardiologia ja kardiovaskulaarilääketiede, Turun yliopiston kliininen tohtoriohjelma, sydäntutkimuskeskus, Turku, Suomi

Tausta: Ateroskleroosi eli valtimonkovettumatauti on krooninen etenävä tila, mikä voi johtaa sydän- ja verisuonitauteihin haitallisten lipoproteiinien kerääntyessä valtimon seinämään. Sen on osoitettu olevan yhteydessä useisiin eri riskitekijöihin. Hapettunut LDL on yhteydessä valtimonkovettumataudin kehittymiseen. HDL:n epäillään suojaavan sydän- ja verisuonitaudeilta. Tällä hetkellä on kuitenkin epäselvää, mitkä ovat tärkeimpiä HDL:n sydän- ja verisuonitaudeilta suojaavia ominaisuuksia. Merkittäviä itsenäisiä tekijöitä, jotka voivat selittää HDL:n sydän- ja verisuonitaudeilta suojaavia ominaisuuksia ovat HDL:n kyky ottaa vastaan hapettuneita lipideitä LDL:lta ja kyky kuljettaa hapettuneita lipideitä verenkierrossa kohti maksaa eliminoitavaksi.

Tavoite: Väitöskirjatutkimukseni tavoitteena on selvittää HDL:n sisältämien hapettuneiden lipidien yhteyksiä tunnettujen valtimonkovettumataudin riskitekijöiden kanssa. Tutkittuja riskitekijöitä ovat mm. seerumin rasvahappopitoisuudet, kehittyvä rasvamaksa sekä PON1-aktiivisuus.

Menetelmät: Tutkimusaineistoni perustuu Lasten Sepelvaltimotaudin Riskitekijät (LASERI) –tutkimukseen. Vuonna 2001 seurantatutkimukseen osallistui 2283 henkilöä. Hapettuneiden lipoproteiinien lipidien määritys perustuu lipoproteiinien lipideistä eristettyjen dieeni konjugaattien määritykseen. Näytteet on otettu vuonna 2001.

Tulokset: Hapettuneet HDL:n lipidit olivat suoraan yhteydessä hapettuneiden LDL:n lipidien pitoisuuteen ja käänteisesti yhteydessä ikään. Korkeammat hapettuneiden HDL:n lipidien pitoisuudet olivat naisilla yhteydessä matalampaan monityydyttymättömien rasvahappojen osuuteen ja korkeampaan kertatyydyttyneiden rasvahappojen osuuteen. Tulevaisuudessa kehittyvän rasvamaksan riski oli yhteydessä matalampiin hapettuneiden HDL:n lipidien pitoisuuksiin. Hapettuneet HDL:n lipidit eivät olleet yhteydessä IMT -arvoon eivätkä PON1-pitoisuuksiin.

Johtopäätökset: Nuorilla suomalaisilla miehillä ja naisilla suurempi sydänsairauksien riskitekijöiden määrä on yhteydessä matalampiin hapettuneiden HDL:n lipidien pitoisuuksiin.

Avainsanat: Hapettuneet lipidit, valtimonkovettumatauti, HDL, LDL

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ABBREVIATIONS

ABCA1 = Adenosine triphosphate -binding cassette transporter A1
ABCG1 = Adenosine triphosphate -binding cassette transporter G1
ALAT = Alanine aminotransferase
Apo-A1 = Apolipoprotein-A1
Apo-B = Apolipoprotein-B
BMI = Body mass index
CETP = Cholesteryl ester transfer protein
CI = Confidence interval
CRP = C-reactive protein
CV = Coefficient of variation
CVD = Cardiovascular disease
FA = Fatty acid
GT = Gamma-glutamyl transferase
HDL = High-density lipoprotein
IDL = Intermediate-density lipoprotein
IMT = Intima-media thickness
LCAT = Lecithin-cholesterol acyltransferase
LDL = Low-density lipoprotein
MUFA = Monounsaturated fatty acid
n3 = omega-3 polyunsaturated fatty acid
n6 = omega-6 polyunsaturated fatty acid
OR = Odds ratio
oxHDLlipids = Oxidized high-density lipoprotein lipids
oxLDLlipids = Oxidized low-density lipoprotein lipids
oxLDLprot = Oxidized low-density lipoprotein proteins
PAI = Physical activity index
PON1 = Paraoxonase-1
PUFA = Polyunsaturated fatty acid
SAS = Statistical Analysis System
SD = Standard deviation
SFA = Saturated fatty acid
SHBG = Sex hormone-binding globulin
SR-B1 = Scavenger receptor -B1
VLDL = Very low-density lipoprotein

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by roman numerals I-IV. Previously unpublished data are additionally presented.

I **Kresanov P**, Ahotupa M, Vasankari T, Kaikkonen J, Kähonen M, Lehtimäki T, Viikari J, Raitakari O.T. The associations of oxidized high-density lipoprotein lipids with risk factors for atherosclerosis: The Cardiovascular Risk in Young Finns Study. *Free radical biology & medicine* 2013*,* vol. 65, pp. 1284-1290.

II Kaikkonen J.E, **Kresanov P**, Ahotupa, M, Jula A, Mikkilä V, Viikari J.S, Kähönen M, Lehtimäki T, Raitakari O.T. High serum n6 fatty acid proportion is associated with lowered LDL oxidation and inflammation: The Cardiovascular Risk in Young Finns Study. *Free radical research* 2014.

III **Kresanov P**, Vasankari T, Ahotupa M, Kaikkonen J, Hutri-Kähönen N, Juonala M, Kähönen M, Lehtimäki T, Viikari J, Raitakari O.T. Paraoxonase-1 and oxidized lipoprotein lipids. The Cardiovascular Risk in Young Finns Study. *Atherosclerosis* 2015*,* vol. 241, no. 2, pp. 502-506.

IV Kaikkonen J.E, **Kresanov P**, Ahotupa M, Jula A, Mikkilä V, Viikari J.S, Juonala M, Hutri-Kähönen N, Kähönen M, Lehtimäki T, Kangas A.J, Soininen P, Ala-Korpela M, Raitakari O.T. Longitudinal study of circulating oxidized LDL and HDL and fatty liver: The Cardiovascular Risk in Young Finns Study. *Free radical research* 2015*,* pp. 1-21.

1. INTRODUCTION

Atherosclerotic cardiovascular diseases (CVDs) are a leading cause of death worldwide (GBD 2013 Mortality and Causes of Death Collaborators 2015). The clinical complications of atherosclerosis, such as strokes and myocardial infarctions, are the severe end-points of this disease (Ross 1999, Usman et al. 2015). Low high-density lipoprotein (HDL) cholesterol and high low-density lipoprotein (LDL) cholesterol are independent risk factors for CVDs (Briel et al. 2009).

The main function of HDL is to transport lipids in circulation. There are additionally various atheroprotective functional properties of HDL. It is hypothesized that an important atheroprotective role of HDL is its ability to promote reverse cholesterol transport (Khera et al. 2011). HDL may also possess anti-inflammatory (Murphy et al. 2009), antioxidant (Navab et al. 1991), antithrombotic (Griffin et al. 1999), vasodilatory (Drew et al. 2004), antidiabetic (Drew et al. 2009), and antiapoptotic activities (Terasaka et al. 2007). HDL may protect endothelial functions (Seetharam et al. 2006). These functional properties of HDL may be more important in the net atheroprotective capacity of HDL than the traditionally measured HDL cholesterol as such (Spillmann et al. 2010). However, the main atheroprotective properties of HDL are not completely understood.

Regardless of therapeutic interventions that lower the levels of atherogenic lipoprotein particles (Robinson, Stone 2015), high-risk patients continue to have cardiovascular events. Findings from studies raising HDL cholesterol levels, however, have not been beneficial in clinical trials (Barter et al. 2007b). It has been found that some genetic mechanisms raising HDL cholesterol do not lower risk of myocardial infarction (Voight et al. 2012). This has shifted interest into the functional properties of HDL for therapeutic gain (Asztalos, Tani & Schaefer 2011). Oxidized LDL has a key role in the pathogenesis of atherosclerosis according to the oxidation hypothesis of atherosclerosis (Ross 1993). Therefore, HDL could alternatively be active in the reverse transport of directly atherogenic lipid peroxides from LDL (Ahotupa et al. 2010). This capacity of HDL may be considered in the future as an important risk-related mechanism in the atherogenesis.

The Cardiovascular Risk in Young Finns Study is an ongoing population-based multicenter follow-up study of 3,596 participants in Finland. It was initiated to assess the biological and lifestyle factors underlying CVDs. In this thesis the main objectives were to study the associations of oxidized high-density lipoprotein lipids with risk factors for atherosclerosis, the relation of serum fatty acid quality with HDL lipid oxidation, the role of paraoxonase-1 (PON1) in HDL lipid oxidation and the relation of oxidized HDL lipids with future fatty liver disease.

2. REVIEW OF LITERATURE

2.1 ATHEROSCLEROSIS

Atherosclerotic CVDs are the leading cause of death in developed countries and the prevalence of CVD continues to rise among these countries (GBD 2013 Mortality and Causes of Death Collaborators 2015). Clinical manifestations of atherosclerosis, including myocardial infarction and stroke, are the severe consequences of this disease and those complications are results of plaque rupture and occlusion of the artery (Muller et al. 1994, Usman et al. 2015). The view is that progression of atherosclerosis is initiated in childhood and that childhood cardiovascular risk factors are associated with the risk of subclinical atherosclerosis in adulthood (Juonala, Viikari & Raitakari 2013). Maintaining a healthy lifestyle already early in life seems therefore to be an important atheroprotective factor.

Atherosclerosis may be considered as a state of chronic inflammation (Ross 1999). The progression of this disease is initiated by the accumulation of cholesteryl esters in the sub-endothelial space and a fatty streak is the first visible lesion of atherosclerosis (Nakashima, Wight & Sueishi 2008). Foam cells, which originate from circulating monocytes, are characteristic of these fatty streaks, and are rich in cholesteryl esters (Nakashima, Wight & Sueishi 2008). These foam cells are a result of an imbalance between the uptake of cholesterol from atherogenic lipoproteins and the cholesterol efflux capacity of HDL (Yvan-Charvet, Wang & Tall 2010). Apolipoprotein-B (Apo-B) containing particles including very low-density lipoprotein (VLDL), LDL and intermediate-density lipoprotein (IDL) particles are significant contributors to plaque cholesterol, and it has been shown that oxidative modification of these particles lead to enhanced uptake by macrophages (Witztum 1993). The development of the plaque is accompanied by intimal inflammation, necrosis, fibrosis and calcification (Usman et al. 2015). The continuous development of atherosclerosis is presented in the Figure 1.

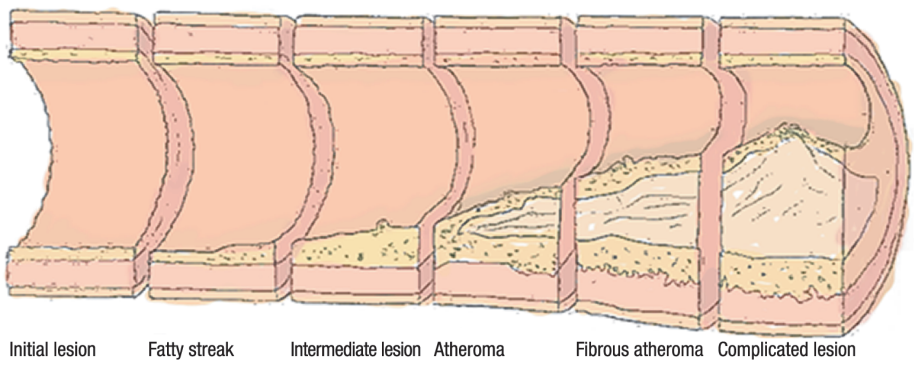

Figure 1. Continuous development of atherosclerosis. The blocks indicate the decades of life. The initial lesion contains atherogenic lipoproteins. The fatty streak is characterized by the accumulation of foam cells in the intima of an artery causing adaptive thickening. Atheromas and fibroatheromas may finally manifest in calcification, hemorrhage, ulceration and thrombosis.

2.2 THE ROLE OF HDL

The role of inflammation in the pathogenesis of atherosclerosis was first discovered by Rudolph Virchow in 1856 (Virchow 1856) and the role of cholesterol in the atherogenesis was suggested by Anitschkow in 1913 who showed that arterial lesions were developed in rabbits after feeding them with a high-cholesterol diet (Anitschkow 1913). The inverse association between HDL cholesterol and CVDs was first found in the 1950s (Barr, Russ & Eder 1951, Nikkilä 1953). It was suggested in 1975 that low HDL levels may accelerate the development of atherosclerosis by impairing the clearance of cholesterol from the artery wall (Miller, Miller 1975) and in 1977, it was shown that HDL cholesterol has an inverse association with the incidence of coronary heart disease (Gordon et al. 1977). The inverse association between HDL cholesterol and CVD risk is a well documented phenomenon (Briel et al. 2009) and this link can be demonstrated at all concentrations of LDL cholesterol (Barter et al. 2007a). It is, however, speculated that the association between HDL and CVD may not be causal as it was shown that genetical mechanisms raising HDL cholesterol did not seem to lower the risk for myocardial infarction (Voight et al. 2012). Elevated LDL cholesterol is shown to be a risk factor for CVDs (Briel et al. 2009, Gordon et al. 1977). In line with this, drugs lowering levels of LDL cholesterol are associated with a decreased number of clinical events (Anonymous 1994, Baigent et al. 2005).

Regardless of these LDL lowering therapeutic interventions mainly with statins (Robinson, Stone 2015), individuals with multiple CVD risk factors still continue to have cardiovascular events. This has fostered interest into HDL metabolism for therapeutic gain (Spillmann et al. 2010). HDL cholesterol level raising strategies have not, however, been necessarily beneficial in clinical trials (Barter et al. 2007b), including cholesteryl ester transfer protein (CETP) inhibition with an increase in the clinical events (Barter et al. 2007b). Clinical studies with nicotinic acid and fibric acid derivatives as therapeutic agents have not shown a clear reduction in the risk for CVDs (Briel et al. 2009). In line with this, it has been shown that risk of myocardial infarction is not lower with genetic mechanisms raising HDL cholesterol levels (Voight et al. 2012). However, there is evidence that intravenous infusions of reconstituted HDL containing a variant of apolipoprotein A-1 (Apo-A1) may promote regression of coronary atherosclerosis (Nissen et al. 2003, Tardif et al. 2007). The atheroprotective role of HDL is therefore controversial.

The main function of HDL is to transport the lipids in circulation. HDL particles are heterogeneous in terms of their density, shape, size, surface, apolipoprotein composition and electrophoretic mobility (Rye, Clay & Barter 1999, Rye, Barter 2014), but the clinical importance of these HDL subpopulations is still unknown (Rye,

Barter 2014). For example, the importance of the density of HDL is controversial. Some evidence suggest that the inverse association between HDL cholesterol and CVD is a function of the concentration of the HDL2 (1.063<density<1.125 g/ml) (Miller 1987), while others have presented that CVD is inversely associated with the concentrations of HDL3 (1.125<density<1.21 g/ml) but not HDL2 (Asztalos et al. 2004). Phospholipids, free cholesterol and apolipoproteins coat the surface of HDL and cholesteryl esters and triglycerides form the hydrophobic lipid core. Phospholipids account for 20 to 30 % of total HDL mass. The remaining part of the lipid mass contains cholesteryl esters (15 %), triglycerides (5 %) and free cholesterol (5 %) (Toth et al. 2013). Apolipoproteins, CETP and lecithin –cholesterol acyltransferase (LCAT) are well known proteins in HDL, but there are additionally 85 proteins in the HDL proteome (Toth et al. 2013). Moreover, it is known that apolipoproteins in HDL are exchangeable as they are transferred in the circulation between lipoproteins and therefore the composition of HDL particles varies rather rapidly (Vedhachalam et al. 2010). It was suggested even in 1984 that HDL has many functions in addition to its lipid transporting activity (Eisenberg 1984).

2.2.1 Atheroprotective functional properties of HDL

It is unknown which atheroprotective functions of HDL (Figure 2) are relevant and more clinically important than others. It is therefore suggested that it is significant to focus on these atheroprotective functions of HDL that are independent of cholesterol level (Asztalos, Tani & Schaefer 2011). In the future, therapies focusing on HDL´s functional properties may provide a strategy for addressing the residual cardiovascular risk in these patients. The main atheroprotective properties of HDL are however presently not understood. An important atheroprotective role of HDL may be its ability to promote reverse cholesterol transport (Khera et al. 2011). Other atheroprotective functions of HDL are its anti-inflammatory (Murphy et al. 2009), antioxidant (Navab et al. 1991), antithrombotic (Griffin et al. 1999), vasodilatory (Drew et al. 2004), antidiabetic (Drew et al. 2009) and antiapoptotic activities (Terasaka et al. 2007). HDL may also protect endothelial function (Seetharam et al. 2006). There are possibly several other protective functions of HDL that have not yet been discovered.

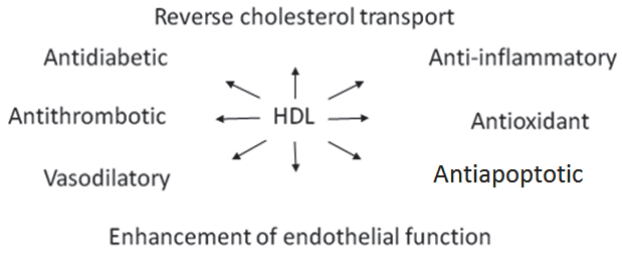

Figure 2. Functional properties of HDL.

Reverse cholesterol transport denotes that HDL accepts cholesterol from the periphery (Yvan-Charvet, Wang & Tall 2010) and delivers it to the liver for excretion as bile acids directly via scavenger receptor-B1 (SR-B1) (Yancey et al. 2003) or indirectly via CETP to LDL (Barter et al. 2007b). It possibly plays a key role in protecting the body from the development of atherosclerosis (Khera et al. 2011). Cholesterol efflux capacity is the first step in the reverse cholesterol transport pathway (Yvan-Charvet, Wang & Tall 2010). This capacity indicates that HDL is able to remove accumulating cholesterol from macrophages, which is mediated by ATP-binding cassette transporters ABCA1 and ABCG1 (Yvan-Charvet, Wang & Tall 2010). It has been shown that the phospholipid content of HDL affects its cholesterol efflux capacity (Gelissen et al. 2006) suggesting that lipid composition of HDL may be essential to its function. Efflux from macrophages is responsible for only a small part of the total flux in the reverse cholesterol transport pathway (Khera et al. 2011), but it is suggested to have an important role in this pathway and in the atheroprotective effect of HDL (Tall 2008). It has additionally been shown to be inversely associated with coronary artery disease and subclinical atherosclerosis, even after the adjustment for HDL cholesterol levels and Apo-A1 (Khera et al. 2011). It has also been shown that cholesterol efflux capacity is inversely associated with incident atherosclerotic CVD after adjustment for traditional CVD risk factors and HDL cholesterol level (Rohatgi et al. 2014). There is evidence that reduced SR-B1 function causes impaired reverse cholesterol transport, which is associated with an increased CVD risk despite elevation in HDL cholesterol levels (Zanoni et al. 2016) suggesting that disturbance in the functional properties of HDL increases the risk for CVDs.

Anti-inflammatory activity is another important atheroprotective role of HDL, which may suppress chronic inflammation in the arterial wall due to the actions of LDL (Murphy et al. 2009). This function includes the fact that HDL may inhibit the binding of monocytes to cultured endothelial cells (Navab et al. 1991) and it is capable of reducing cytokine induced expression of vascular cell adhesion molecules, which inhibits the adhesion of mononuclear leukocytes to the endothelium (Cockerill et al. 1995). It has been shown that anti-inflammatory properties of HDL differentiate CVD patients from control subjects better than HDL cholesterol levels (Ansell et al. 2003). The antioxidant activities of HDL are closely linked to the anti-inflammatory activities of HDL and may also inhibit atherogenic properties of oxidized LDL (Navab et al. 1991). An HDL-associated hydrolytic enzyme paraoxonase-1 (PON1) has been suggested to function as a key factor in the antioxidant capacity of HDL (Mackness, Arrol & Durrington 1991). There is also evidence *in vitro* that Apo-A1 could act as an antioxidant (Garner et al. 1998) and that surface phospholipids of HDL may be a factor in the antioxidant function of HDL (Zerrad-Saadi et al. 2009).

Antithrombotic effects of HDL have been suggested to be mediated by inhibition of platelet activation and aggregation (Shah et al. 2001) and by enhancement of the anticoagulant activity of the protein C pathway (Griffin et al. 1999). It has also been shown that high levels of HDL cholesterol are associated with a decreased risk for recurrent venous thromboembolism (Eichinger et al. 2007) and it has been suggested earlier that HDL cholesterol levels should be raised in patients who have an increased risk for atherotrombotic events (Ashen, Blumenthal 2005). The association between HDL and CVD may not however be causal, as it has been shown that genetical mechanisms raising HDL cholesterol do not seem to lower the risk for myocardial infarction (Voight et al. 2012).

There is evidence that HDL may promote endothelial repair by stimulating endothelial cell migration from healthy to damaged endothelium via SR-B1 (Seetharam et al. 2006) and it may enhance progenitor-mediated repair (Tso et al. 2006). HDL is additionally capable of stimulating nitric oxide release by endothelium (Drew et al. 2004) and the production of prostacyclin (Norata et al. 2004), which are considered to be vasodilatory actions of HDL improving endothelial function.

HDL has additionally been shown to have antidiabetic properties (Drew et al. 2009). This effect in glucose metabolism is suggested to take affect via increased insulin levels and reduced glucose levels (Drew et al. 2009). Moreover, HDL has been shown to inhibit apoptosis in pancreatic β-cells (Rutti et al. 2009). HDL may therefore have an influence on the progression of type 2 diabetes. HDL is additionally capable of protecting macrophages from apoptosis induced by oxidized LDL (Terasaka et al. 2007).

It is, however, presently unknown which of these atheroprotective functions of HDL are more clinically significant than others.

2.3 LIPOPROTEIN LIPID OXIDATION

2.3.1 Transport of oxidized lipids

The function of serum lipoproteins such as HDL and LDL is to carry lipids and lipid-soluble material in the circulation. Lipoproteins are able to transport the products of lipid oxidation (Ahotupa et al. 2010). These lipid peroxides are toxic and possess atherogenic potential (Berliner, Watson 2005). According to the oxidation hypothesis of atherosclerosis, oxidized LDL has an important role in the pathogenesis of atherosclerosis (Ross 1993). Oxidized LDL is linked to macrophage accumulation, regulation of macrophage activity, and foam cell formation in vessel walls (Birukov 2006). The knowledge that the transport of oxidized lipids by LDL has been shown to be directed towards peripheral tissues such as arterial wall (Ahotupa et al. 2010) is in line with the oxidation hypothesis of atherosclerosis. HDL contains larger amounts of

oxidized lipids than LDL (Proudfoot et al. 2009). There is evidence suggesting that HDL could be active in the reverse transport of these directly atherogenic oxidized lipids (Ahotupa et al. 2010) as it has been shown that HDL and LDL are able to response to food-derived and endogenous oxidized lipids by increasing their transport (Ahotupa et al. 2010). Therefore, the levels of oxidized lipoprotein lipids could be indicative of lipoproteins oxidized lipid transport functions. This oxidized-lipid transport function of HDL may protect macrophages and endothelium from toxic lipid peroxides (Shao, Heinecke 2009) and this function of HDL may be an important risk-related mechanism in the atherogenesis, as it logically combines both oxidation and reverse cholesterol transport theories. A schematic view of reverse oxidized lipid transport is presented in the Figure 3.

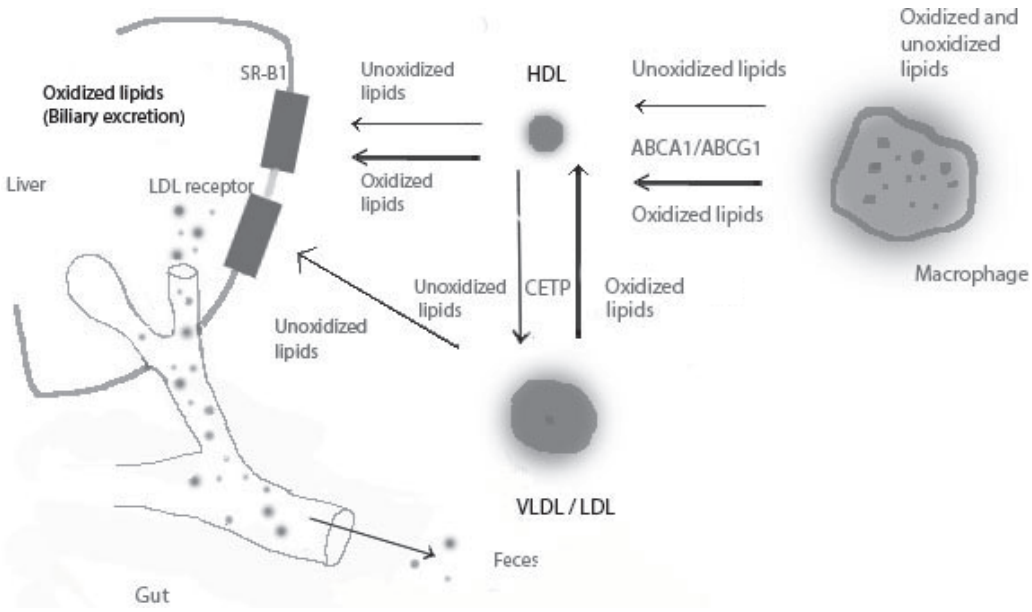

Figure 3. Schematic view of reverse oxidized lipid transport. HDL removes oxidized and unoxidized lipids from macrophages, which is mediated by ABCA1 (adenosine triphosphate -binding cassette transporter A1) and ABCG1 (adenosine triphosphate -binding cassette transporter G1). Transfer of oxidized and unoxidized lipids between LDL and HDL, facilitated by CETP (cholesteryl ester transfer protein), proceeds in opposite directions and oxidized lipids are removed to the liver from HDL but not from LDL. Finally, HDL delivers oxidized and unoxidized lipids to the liver for excretion as bile acids via SR-B1 (scavenger receptor-B1) through the undirect pathway of reverse cholesterol transport. Unoxidized lipids are also removed to the liver through the direct pathway of reverse cholesterol transport.

2.3.2 The analysis of lipid oxidation

Reliable determination of lipoprotein oxidation is demanding due to the complexities concerning the nature of the chemistry of lipoprotein oxidation, and presently, no single method exists that could enable objective determination of oxidative stress

(Grune 2014). It is known that oxidation of LDL may involve several constituents of LDL, such as cholesterol, protein, and polyunsaturated fatty acids, and these may result in several other products of oxidation. Several methods have therefore been used to measure oxidized lipoproteins.

Often the methods measuring oxidized lipoproteins are based on the determination of autoantibodies to oxidized lipoproteins (Ahotupa et al. 1998, Holvoet et al. 2006). These methods are based on the antibodies generated *in vivo* or raised against oxidatively damaged LDL *in vitro*. Oxidized LDL was measured in this study also with a method based on antibodies against oxidized Apo-B (Holvoet et al. 2006). A considerable limitation of those methods is that preparation of identical antigens between laboratories is not possible. Therefore, various assays may not give similar results. It is suggested that antibodies against oxidized LDL would not be reliable indicators of oxidative damage as it was shown that a healthy diet rich in fruits and vegetables and reduced in saturated fat increased the levels of antibodies against oxidized LDL (Miller et al. 2005).

Another method frequently used for detecting oxidized LDL is plasma and urinary levels of F2-isoprostanes, which are indicators of free radical-induced oxidative stress (Patrono, FitzGerald 1997). Production of F2-isoprostanes occurs during cell- or copper- induced oxidation of LDL *in vitro* (Lynch et al. 1994). F2-isoprostanes are associated with high LDL cholesterol levels (Davies, Roberts 2011). The lack of specifity is, however, a problem concerning this method measuring F2-isoprostanes as it represents lipid oxidation occurring in whole body and it is therefore not possible to detect how the measured F2-isoprostanes reflect LDL oxidation (Ahotupa, Vasankari 1999). Because F2-isoprostanes are formed, at least in part, enzymatically, they are therefore not absolutely associated with oxidative stress (Tsikas et al. 2012). Using F2-isoprostanes as a method to assess the risk for atherosclerosis related to lipoprotein oxidation should therefore be considered with caution.

The levels of oxidized HDL and LDL lipids were analyzed in this study by the determination of the baseline level of conjugated dienes in lipoprotein lipids (Ahotupa et al. 1998). The formation of conjugated dienes indicates a rearrangement of double bonds in polyunsaturated fatty acids (Ahotupa et al. 1998). It is an early event of lipid oxidation in a cascade leading to more advanced oxidative damage of lipids, such as the formation of 4-hydroxy-2-nonenal or malondialdehyde (Ayala, Munoz & Arguelles 2014). Therefore this method is considered to be an indicator of mildly oxidized lipoproteins (Ahotupa, Vasankari 1999). A significant advantage of this method compared to other methods measuring oxidatively modified lipids is that it measures oxidative modifications of lipids in lipoprotein particles *in vivo* (Ahotupa et al. 1998).

In addition to this method, the baseline diene conjugation measurement is fast and simple to perform in a standard laboratory (Ahotupa, Vasankari 1999).

2.4 OXIDIZED LIPOPROTEIN LIPIDS AND RISK FACTORS FOR ATHEROSCLEROSIS

2.4.1 Oxidized lipoprotein lipids and traditional risk factors for atherosclerosis

Knowledge regarding the associations of oxidized HDL lipids (oxHDLlipids) with risk factors for atherosclerosis at the population level is limited. There are, however, results showing that oxidized LDL lipids (oxLDLlipids) are associated with increased risk for atherosclerosis.

It is known that oxLDLlipids are associated with several known risk factors for atherosclerosis. It has been shown that men with borderline hypertension had increased arterial intima-media thickness (IMT) as a marker for subclinical atherosclerosis and higher levels of oxLDLlipids (Toikka et al. 2000). Cholesterol levels are shown to be associated with oxidized lipoproteins as higher levels of LDL cholesterol and lower levels of HDL cholesterol are associated with elevated oxLDLlipid levels (Vasankari et al. 2000, Toikka et al. 1999). It has also been shown that higher postprandial levels of oxLDLlipids are associated with lower HDL cholesterol levels (Tiainen et al. 2014). Waist circumference and BMI are shown to be higher in subjects with increased levels of oxLDLlipids (Vasankari et al. 2000) and a 10-month exercise program resulting in weight reduction is associated with lower levels of oxLDLlipids (Vasankari et al. 1998). In addition, postpartum weight retention associates with higher oxidized LDL/ HDL cholesterol ratio compared to subjects who lost weight (Puhkala et al. 2013). Studies have shown that weight reduction is associated with the reduced oxLDLlipid levels (Vasankari et al. 2001). It was additionally shown that higher levels of oxLDLlipids were associated with impaired insulin sensitivity in a 32-month life-style intervention study (Linna et al. 2015). In addition, intensive physical training for several years is associated with reduced levels of oxLDLlipids (Kujala et al. 1996) and physical inactivity is associated with higher levels of oxLDLlipids (Vasankari et al. 2000). In line with this, lower levels of oxLDLlipids are associated with both good cardiorespiratory and muscular fitness (Kosola et al. 2012). Levels of oxLDLlipids have shown to be increased by hypertension (Toikka et al. 2000) and age (Ahotupa, Ruutu & Mäntylä 1996). Finally, oxLDLlipids were recently suggested to act as a risk factor of total mortality in the elderly (Linna et al. 2013). Oxidative stress may therefore be associated with increased CVD risk caused by advanced age.

2.4.2 Fatty acids and risk for atherosclerosis

High dietary intake of polyunsaturated fatty acids (PUFAs) is associated with a lower risk for CVDs (Mozaffarian, Micha & Wallace 2010, Astrup et al. 2011). The most common PUFA in Western diets is linoleic acid and it is present in vegetable oils such as sunflower, corn, safflower, and soybean oils, and in margarines made from these oils (Calder 2013). High consumption of saturated fatty acids (SFAs) is suggested to be related to an increased CVD risk and thus dietary recommendations give instructions to reduce SFA intake (Vartiainen et al. 2010). There is, moreover, evidence suggesting that consumption of PUFAs in place of SFAs reduces the risk for CVDs (Mozaffarian, Micha & Wallace 2010). In line with those findings, a diet with high PUFA and low SFA proportions are associated with reduced levels of LDL and HDL cholesterol and the changes in cholesterol levels were reversed when subjects returned to the original diets rich in animal fats (Ehnholm et al. 1982). However, there is evidence suggesting that an elevated omega-6 PUFA (n6PUFA) intake in place of SFAs without a simultaneous omega-3 PUFA (n3PUFA) supply may elevate the risk for CVDs (Calder 2013, Ramsden et al. 2010). Rapeseed oil and fish products contain relevant amounts of n3PUFAs (Stimming et al. 2015). It is, moreover, suggested that n3PUFA consumption may reduce the risk of death from heart disease (Anonymous 1999, Chaddha, Eagle 2015) and it is speculated that replacement of n6PUFA with n3PUFA could be beneficial via the anti-inflammatory properties of n3PUFAs (Wall et al. 2010). Because of its beneficial effects on hemostasis, rapeseed oil is suggested to be the first n3PUFA containing oil to be used when correcting n6/n3 PUFA imbalances in the body (Seppänen-Laakso et al. 2010).

PUFAs are susceptible to oxidative modifications by free radicals or nonradical oxygen species due unstable hydrogen-carbon bonds (Bochkov et al. 2010). SFAs do not become oxidized, but their main source is red meat, which is rich in heme iron that is able to catalyze oxidative reactions (White, Collinson 2013). SFAs increase LDL cholesterol levels (Ehnholm et al. 1982, Dias, Wood & Garg 2016) and as discussed earlier, LDL oxidation is associated with elevated levels of LDL cholesterol (Toikka et al. 1999) and subclinical atherosclerosis measured with arterial IMT in a rather small population (N=38) (Toikka et al. 2000). One report has suggested that oxidized LDL may be an independent predictor of atherosclerosis (Salonen et al. 1992). However, in that study LDL oxidation was estimated by using the determination of autoantibodies to oxidized lipoproteins with known caveats in interpretation. The associations between serum fatty acid (FA) quality and lipoprotein lipid oxidation at the population level may therefore offer valuable information concerning the complex pathogenesis of atherosclerosis.

2.4.3 PON1

PON1 is an HDL –associated hydrolytic enzyme (Draganov et al. 2005) and it is known that PON1 rs662 polymorphism has a significant influence on PON1 activity levels (Wheeler et al. 2004, Charles-Schoeman et al. 2013). PON1 rs662 (A) encodes a glutamine (Q) and PON1 rs662 (G) encodes an arginine (R) (Adkins et al. 1993). In a group with PON1 rs662 A/A genotype (QQ genotype) PON1 activity is lower and in a group with PON1 rs662 G/G genotype (RR genotype) PON1 activity is higher (Charles-Schoeman et al. 2013). PON1 is suggested to be a key factor in the antioxidant capacity of HDL (Mackness, Arrol & Durrington 1991) and PON1 has been shown to regulate HDL-mediated cholesterol efflux from macrophages (Berrougui, Loued & Khalil 2012). The main atheroprotective mechanism of PON1 is, however, suggested to be its capability to be an antioxidant for LDL (Mackness, Mackness 2013). It is therefore proposed to be an anti-atherogenic enzyme (Reddy et al. 2008), but the ability of PON1 to inhibit oxidation of LDL is only shown *in vitro* (Mackness, Arrol & Durrington 1991).

The results of *in vivo* studies on the relationship between PON1 activity and cardiovascular risk are presently controversial. High activity of PON1 has been related to an increased risk for myocardial infarction (van Himbergen et al. 2008). Furthermore, genetically high PON1 activity has been associated with an increased risk for coronary heart disease (Wheeler et al. 2004). On the other hand, there is evidence that low PON1 activity is a risk factor for coronary events (Bhattacharyya et al. 2008). *In vivo*, it has been observed that increased PON1 activity is associated with decreased antioxidant capacity of HDL (Breton et al. 2014). The ability of PON1 to prevent HDL oxidation has not been confirmed *in vivo* and the importance of PON1 in the atherogenesis is therefore presently unclear. There is thus a need to clarify the role of PON1 in lipoprotein oxidation at the population level.

2.4.4 Fatty liver

Fatty liver is a risk factor for CVDs (Sattar, Forrest & Preiss 2014, Lu et al. 2013, Targher, Day & Bonora 2010). It is also a risk factor for type 2 diabetes (Sattar, Forrest & Preiss 2014, Targher, Day & Bonora 2010) as it is strongly associated with visceral obesity (Marchesini et al. 2008) and a common cause of abnormal liver function tests (Sattar, Forrest & Preiss 2014). Due to the high prevalence of obesity, non-alcoholic fatty liver disease is more common than alcoholic fatty liver disease (Marchesini et al. 2008) and it is estimated that approximately 20-30 % of the population in Western countries has nonalcoholic fatty liver disease (Marchesini et al. 2008). In subjects with type 2 diabetes and obesity its prevalence is from 70 % to 90 % (Marchesini et al.

2008). Its prevalence is shown to be 29 % in overweight/obese and 5 % in normal-weight adults in our study population (Suomela et al. 2015).

Fatty liver is a disease including conditions from steatosis to steatohepatitis and on to severe liver diseases such as cirrhosis (Sattar, Forrest & Preiss 2014). Steatosis is considered to be a benign condition, but due to its high prevalence it remains a cause of severe liver diseases (Vernon, Baranova & Younossi 2011). Steatohepatitis is a more severe steatosis coupled with inflammation and fibrosis (Powell et al. 1990). It is a significant risk factor of cirrhosis and hepatocellular carcinoma (Powell et al. 1990). Subjects with more severe forms of nonalcoholic fatty liver disease have increased risk of cardiovascular events before advanced liver disease is developed (Targher, Day & Bonora 2010). It is therefore essential to focus on prevention of CVDs, if nonalcoholic fatty liver disease has been diagnosed. It is notable, that all forms of fatty liver increase the risk of steatosis, steatohepatitis and hepatocellular carcinoma (Yki-Jarvinen 2014).

Fatty liver is a multi-factorial disease with regard to pathogenesis. The accumulation of excess triglycerides in the liver is the cause of this disease (Rolo, Teodoro & Palmeira 2012), which is dependent on recirculating free fatty acids and de novo lipogenesis (Rolo, Teodoro & Palmeira 2012). This accumulation of lipids may lead to mitochondrial dysfunction, inflammation and subsequently to oxidative stress and these events may result in the development of steatohepatitis (Rolo, Teodoro & Palmeira 2012). Steatohepatitis is associated with hepatic and circulating markers of oxidative stress (Sumida et al. 2013, Chalasani, Deeg & Crabb 2004). It is possible that these findings are only a consequence of the fatty liver disease itself or a finding indicating the causative role of lipid oxidation in the etiology of fatty liver. The association between oxidized lipoprotein lipids and fatty liver is therefore presently unclear at the population level.

3. AIMS

The Cardiovascular Risk in Young Finns Study is an ongoing epidemiological study to evaluate risk factors and precursors of CVD from childhood into adulthood. The present thesis is based on the findings from the 21-year follow-up study performed in 2001 and from the 31-year follow-up study performed in 2011. The major aims of the present thesis were as follows:

1. To study the associations of oxHDLlipids with risk factors for atherosclerosis at the population level (I).
2. To examine the associations of serum fatty acid quality with oxHDLlipids at the population level (II).
3. To determine the role of PON1 in HDL lipid oxidation at the population level (III).
4. To study the relationship of oxHDLlipids with future fatty liver (IV).

4. MATERIALS AND METHODS

4.1 DESCRIPTION OF THE CARDIOVASCULAR RISK IN YOUNG FINNS STUDY

The Cardiovascular Risk in Young Finns Study is an ongoing multicenter follow-up study in five cities and their rural surroundings with the university hospitals in Finland (Turku, Tampere, Helsinki, Kuopio and Oulu) to evaluate atherosclerotic risk factors from childhood into adulthood (Åkerblom et al. 1985, Raitakari et al. 2008). Participants were randomly chosen from the national register from the study areas. Two pilot studies were conducted in 1978 and 1979. The main study began in 1980 including 3,596 children and adolescents aged 3, 6, 9, 12, 15 and 18 years. These participants were invited to take part in the study in order to produce a representative sample of Finnish children and adolescents. The follow-up studies have been performed at 3-year intervals. The 21-year follow-up was conducted in 2001 with a total of 2,284 subjects (63.5 %) from the original cohort. In the follow-up in 2007, a total of 2,204 subjects were examined and 1,828 subjects participated both in 2001 and 2007. The latest follow-up was performed in 2010-2012 with a total of 2,063 participants. The design and participation rates in the follow-up studies in the Cardiovascular Risk in Young Finns Study are shown in Table 1. The analyses of oxHDLlipids were performed in 2001 to study the determinants of HDL oxidation at the population level.

Table 1. Design and cohorts of the Cardiovascular Risk in Young Finns Study

Year	N	Age															
1980	3596	3	6	9	12	15	18										
1986	2799			9	12	15	18	21	24								
2001	2284								24	27	30	33	36	39			
2007	2204										30	33	36	39	42	45	
2011	2063											33	36	39	42	45	48

4.2 STUDY DESIGN

Study I examined the associations of oxHDLlipids with risk factors for atherosclerosis. The cross-sectional study included subjects who had data on oxHDLlipids measured in 2001. A total of 1,395 participants aged 24 to 39 years were included in this analysis.

Cross-sectional data was used in study II to evaluate the associations of serum FA with oxHDLlipids. This study included 2,196 subjects with serum FA analyses available.

Study III focused on examining the associations of PON1 activity with oxHDLlipids at the population level. This cross-sectional setting included 1,895 subjects who had data on serum PON1 activity measured in 2001.

In study IV, the associations of oxHDLlipids with fatty liver measurements taken ten years later using ultrasound were investigated. This study included a total of 1,574 participants who had data on fatty liver status measured in 2011 and oxidation markers measured in 2001.

4.3 BIOCHEMICAL ANALYSES

4.3.1 Analysis of oxidation markers

All venous blood samples were collected after overnight fasting and all of the laboratory analyses were carried out on these samples.

The analysis of oxidized HDL and LDL lipids was based on the determination of the baseline level of conjugated dienes in lipoprotein lipids (Ahotupa et al. 1998). The serum HDL fraction (oxHDLlipids) was isolated with phosphotungstic acid precipitation (Väisänen et al. 1992). The precipitation reagent contained 0.55 M phosphowolframate and 25 M MgCI2. The serum LDL fraction (oxLDLlipids) was precipitated with buffered heparin (Ahotupa et al. 1998). Serum triglyceride-rich lipoproteins were isolated from the serum samples by spinning at 15,500 g for 20 min at 12°C. The precipitation buffer consisted of 0.064 M trisodium citrate adjusted to pH 5.05 with 5 N HCl, and contained 50,000 IU/L heparin. The lipids were extracted from the isolated lipoproteins by chloroform-methanol (2:1), dried under nitrogen and redissolved in cyclohexane. The amount of peroxidized lipids was assessed spectrophotometrically at 234 nm (Ahotupa et al. 2010). The isolation procedures were validated for this purpose and did not affect the level of oxidized lipids (Ahotupa et al. 1998). Validation studies for the assay have excluded interference by non-specific substances and shown that diene conjugation is able to detect oxidative HDL and LDL lipid modification found in all HDL and LDL lipid classes. Samples were stored at -80 °C until analyzed. The levels of oxidized lipoprotein lipids were not changed during the prolonged follow-up. The coefficient of variation for within-assay precision for determination of oxidized lipoprotein lipids was 4.4 and 5.2 %, and the coefficient of variation for the between-assay precision was 4.5 and 5.6 %, for LDL and HDL, respectively.

We additionally measured LDL oxidation with a method based on directing the mouse monoclonal antibody 4E6 against a conformational epitope in oxidized Apo-B-100 in serum (Holvoet et al. 2006). LDL particles containing oxidized Apo-B (oxLDLprot)

were evaluated with monoclonal antibody based enzyme-linked immunosorbent assay (Oxidized LDL ELISA kit, Mercodia, Sweden).

4.3.2 Other biochemical measurements

Serum cholesterol and triglyceride concentrations were analyzed by standard enzymatic methods (Olympus System Reagent; Germany) in a clinical chemistry analyzer (AU400, Olympus). HDL cholesterol was measured by the dextran sulphate 500,000 (Kostner 1976). LDL cholesterol was calculated with the Friedewald formula (Friedewald, Levy & Fredrickson 1972) for participants with triglycerides <4.0 mmol/L. Serum apolipoproteins A1 (Apo-A1) and B (Apo-B) were analyzed immunoturbidometrically (Orion Diagnostica, Espoo, Finland). All lipid measurements were performed in duplicate with similar results in the same laboratory and standard enzymatic methods were used for all lipid determinations. Fluorescence polarization immunoassays (Abbott Laboratories, Abbott Park, IL, USA) were used for measuring the serum insulin concentration. Serum glucose was measured enzymatically (Olympus Diagnostica GmbH, Hamburg, Germany). A highly sensitive turbidimetric immunoassay (Wako Chemicals, Neuss, Germany) was used to determine serum C-reactive protein (CRP) concentration. Serum testosterone was measured with Spectria testosterone RIA-kit (Orion Diagnostica) and sex hormone-binding globulin (SHBG) levels were determined with a Spectria SHBG IRMA-kit (Orion Diagnostica). Gamma-glutamyl transferase (GT) and alanine aminotransferase (ALAT) were measured with standard clinical laboratory methods (ALT and GGT System Reagent, Olympus, Ireland).

PON1 activity was determined with paraoxon (O,O-diethyl-O-p-nitrophenylphosphate) as the substrate and the increase in the absorbance at 412 nm, due to formation of 4-nitrophenol, was measured spectrophotometrically (Harangi et al. 2004). Genotyping was performed with the Illumina Bead Chip (Human 670K).

Serum proportions of total FAs were analyzed in a gas chromatography and flame ionization detector (Jula et al. 2002). Individual FAs were subdivided into SFAs (myristic acid, 14:0; pentadecanoic acid, 15:0; palmitic acid, 16:0 and stearic acid, 18:0), monounsaturated fatty acids (MUFAs) (palmitoleic acid, 16:1n7; octadecenoic acid, 18:1n7; oleic acid, 18:1n9; eicosenoic acid, 20:1n9 and docosenoic acid, 22:1n9) and PUFAs (linoleic acid, 18:2n6; gamma-linolenic acid, 18:3n6; eicosadienoic acid, 20:2n6; dihomo-gammalinolenic acid, 20:3n6; arachidonic acid, 20:4n6; docosatetraenoic acid, 22:4n6; alpha-linolenic acid, 18:3n3; eicosatetraenoic acid, 20:4n3; eicosapentaenoic acid, 20:5n3; docosapentaenoic acid, 22:5n3 and docosahexaenoic acid. 22:6n3 and 20:2n9). A high-throughput nuclear magnetic resonance platform was used to quantify serum LDL and HDL particle concentrations

(Soininen et al. 2009, Inouye et al. 2010, Kettunen et al. 2012). The total HDL particle concentration was the sum of small, medium, large and very large HDL particles. The total LDL particle concentration was sum of the variable of the small, medium and large LDL particles.

4.4 PHYSICAL EXAMINATION AND QUESTIONNAIRES

During the physical examination, height and weight were measured. Body mass index (BMI) was calculated with the formula weight (kg)/(height)(m))^2. Waist circumference was measured at the level of the umbilicus to an accuracy of 0.1 cm. Blood pressure was measured with a random zero sphygmomanometer (Hawksley & Sons Ltd) with an average of three measurements used in the analyses.

The information gathered was based on questionnaires concerning alcohol use, smoking habits (smoking/nonsmoking), physical activity, use of contraceptives (estrogen and/or progesterone, no vs. yes), energy and FA intake (Kaikkonen et al. 2013), energy-adjusted vitamin-E intake, and red meat (pork, beef, lamb and game) intake (Kaikkonen et al. 2013). Alcohol use was determined using a standard drink containing 12 g of pure alcohol per day. Subjects smoking daily were classified as smokers. Smoking habits were additionally calculated in pack years. One pack per year indicated smoking 20 cigarettes per day during one year. The physical activity index (PAI) was constructed by combining the information from questions concerning frequency and intensity of physical activity, frequency and hours spent on vigorous physical activity, the average duration of a physical activity session and participation in organized physical activity (Telama et al. 1985). The index score varied between 5 and 15.

4.5 ULTRASOUND STUDIES

Ultrasound studies were performed by trained sonographers following a standardized protocol. Measurements were made off-line from stored digital images.

The liver fat status was imaged with Acuson Sequoia 512 ultrasound mainframes (Acuson, Mountain View, CA, USA) using 4.0 MHz adult abdominal transducers in 2011. All the participants with acceptable image quality were included in the study. Fatty liver status was graded using the liver-to-kidney contrast (no or clear contrast), parenchymal brightness (normal, mild, intermediate or severe brightness), deep beam attenuation (clear, attenuated or no visible line of diaphragm), bright vessel walls (normal, partly visible or no visible vessel walls) and visibility of the neck of the gallbladder (normal=0, partly visible=1, no visible=2). A summarized fatty liver score with values from 0 to 9 was formed.

Carotid IMT was measured using ultrasound mainframes (Sequoia 512, Acuson, Mountain View, Calif) with 13.0-MHz linear array transducers (Raitakari et al. 2008). The mean was derived by using a minimum of four IMT measurements from the posterior wall of the left common carotid artery approximately 10 mm proximal to the carotid bifurcation.

4.6 STATISTICAL ANALYSES

The normality assumptions were calculated by examining histograms and normal probability plots. A T-test was used to examine differences in the characteristics between sexes. Statistical analyses were performed using a Statistical Analysis System (SAS, versions 9.2 and 9.3). Statistical significance was inferred at a 2-tailed P-value <0.05.

Study I

Values for CRP and insulin were log-transformed and values for glucose were square-root transformed before analyses because of skewed distributions. The effect of sex was analyzed separately, because the P-value for interaction was significant between oxHDLlipids and several risk factors, including oxLDLlipids, oxLDLprot, total cholesterol, HDL cholesterol, triglycerides, Apo-B, Apo-A1, CRP and alcohol use. To study the multivariate determinants of oxHDLlipids, HDL cholesterol, oxLDLlipids, LDL cholesterol, oxLDLprot and Apo-B, we analyzed multivariable models for men and for women. A set of priori selected explanatory variables were included in the multivariable regression analyses and the models were manually constructed by removing the least significant intercorrelating variables with the aid of multicollinearity diagnostics.

Study II

Values for vitamin E intake, BMI, and insulin were log-transformed and values for glucose were square root transformed. The effect of sex was analyzed separately, because P-value for interaction was significant between oxHDLlipids and PUFA, n6, MUFA and SFA. Pearson´s correlation coefficients were calculated to test the associations between oxidized lipoprotein lipids and FA determinants. Two-step linear regression models between FA variables and oxidized lipoprotein lipids were studied. Firstly, the FA variable and age were modeled as explanatory variables. In the second step, step 1 variables + physical activity, vitamin E intake, smoking, body mass index, alcohol intake, glucose, insulin, and systolic blood pressure were included in the model. HDL cholesterol was included in the models for oxHDLlipids and LDL cholesterol was included in the models for oxLDLlipids.

Study III

A normalized rank transformation was made for PON1 due to skewed distribution. Values for triglycerides, CRP and insulin were log-transformed and the value for glucose was square root transformed. Men and women were studied in combination, because there were no significant sex interactions between PON1 and other study variables. ANOVA was used to examine differences in characteristics between paraoxonase groups stratified by rs662 genotypes. P for trend was adjusted with age. In the multivariable models for oxHDLlipids, oxLDLlipids and oxLDLprot a set of priori selected explanatory variables were forced into the models simultaneously with the aid of multicollinearity diagnostics. In the model for oxHDLlipids, the explanatory variables were PON1, sex, Apo-A1, oxLDLlipids, age, and BMI. The explanatory variables in a model for oxLDLlipids were PON1, sex, Apo-B, oxHDLlipids, oxLDLprot, age and BMI, and in a model for oxLDLprot the explanatory variables were PON1, sex, Apo-B, oxHDLlipids, oxLDLlipids, age, and BMI. In addition, the multivariable model was constructed in order to study the multivariate determinants of PON1. A set of priori selected explanatory variables were included in the models and the construction was performed manually by removing the least significant intercorrelating variables with the aid of multicollinearity diagnostics.

Study IV

Variables with a skewed distribution were log-transformed prior to their statistical use. Associations between oxidation markers and fatty liver did not show any sex or alcohol consumption-related interactions. For this reason, all of the individuals were pooled together in the statistical models. A multivariable model was studied of oxHDLlipids with priori selected variables including fatty liver as an explanatory variable. Explanatory variables were forced into the model simultaneously with the aid of multicollinearity diagnostics. In this model, the fatty liver was modeled as an independent predictor. In addition, logistic regression models were constructed to study the multivariate determinants of a fatty liver. In these models, the fatty liver was modeled as an outcome variable. As explanatory variables, the fully adjusted logistic regression models consisted of a lipoprotein oxidation marker, age, sex, alcohol intake, leisure-time physical activity, smoking, and BMI. To achieve reasonable group sizes in Figure 7, the 10-level fatty liver score was stratified into 3 groups: Group 1: original score 0; Group 2: original scores 1-2; Group 3: original scores ≥3.

4.7 ETHICS

The Cardiovascular Risk in Young Finns Study was approved by local ethics committees. Participants signed written informed consent forms during the follow-up studies and their parents gave their consent in 1980.

5. RESULTS

5.1 CLINICAL CHARACTERISTICS

Table 2. Oxidized lipoprotein lipids and serum lipids in 2001.

	Men (N=629)		Women (N=766)	
	Mean	SD	Mean	SD
oxHDLlipids (µmol/L)	27.9	7.4	29.8	7.9
oxLDLlipids (µmol/L)	29.0	11.3	24.6	8.4
oxLDLprot (U/L)	88.4	25.3	77.8	21.7
Total cholesterol (mmol/L)	5.3	1.0	5.0	0.9
HDL cholesterol (mmol/L)	1.2	0.3	1.4	0.3
LDL cholesterol (mmol/L)	3.4	0.9	3.1	0.8
Triglycerides (mmol/L)	1.6	1.0	1.1	0.6
Apo-B (g/L)	1.1	0.3	1.0	0.2
Apo-A1 (g/L)	1.4	0.2	1.6	0.3

All: $p<0.05$ between sexes, SD=standard deviation, oxLDLlipids= oxidized LDL lipids, oxHDLlipids=oxidized HDL lipids, oxLDLprot= oxidized LDL proteins, Apo-B= apolipoprotein-B, Apo-A1=apolipoprotein-A1. Values are presented for participants who had data on oxHDLlipids.

Mean levels of oxidized lipoprotein lipids and serum lipids are presented in Table 2. The mean level of oxHDLlipids was higher in women (29.8 µmol/L) than in men (27.9 µmol/L) and men had higher levels of oxLDLlipids (29.0 µmol/L) than women (24.6 µmol/L). There were significant differences in the levels of oxLDLprot and serum lipids between males and females.

Table 3 shows the characteristics of the study subjects in 2001. The mean age of the study participants was 31.6 in males and 31.5 in females. The values of BMI, waist circumference, blood pressure, glucose levels, alcohol use, and daily smoking were higher in men than in women.

Table 3. Characteristics of the study subjects in 2001.

	Men (N=629)		Women (N=766)	
	Mean	SD	Mean	SD
Age (yrs)	31.6	5.1	31.5	5.0
BMI (kg/m²)	25.7	4.2	24.3	4.5
Waist circumference (cm)	89.8	10.8	78.7	11.0
Systolic BP (mmHg)	124.0	12.4	114.2	12.5
Diastolic BP (mmHg)	75.7	10.8	70.7	9.6
Insulin (mU/L)	7.5	5.6	7.6	5.6
Glucose (mmol/L)	5.2	0.6	4.9	0.9
PAI (unitless, range 5-15)	10.0	2.5	10.1	2.1
CRP (mmol/L)	1.5	3.5	2.2	4.5
Alcohol use (drinks per day)	1.3	1.6	0.6	0.7
Pack years (years)	4.6	7.1	2.0	4.3
Daily smoking (%)	32.8		19.4	
SHBG (nmol/L)	30.6	11.9	-	-
Testosterone (nmol/L)	18.5	7.8	-	-
Use of contraceptives (%)	-	-	28.0	
Serum PUFA (%)	39.3	4.8	40.6	4.1
Serum n3 (%)	4.7	1.4	4.8	1.6
Serum n6 (%)	34.4	4.5	35.7	3.9
Serum MUFA (%)	28.3	3.3	27.1	2.8
Serum SFA (%)	32.4	2.4	32.2	2.3
Vitamin E (mg/1000 kJ/day)	1.23	0.4	1.26	0.4
ALAT (U/L)	23.9	20.5	13.2	9.9
GT (U/L)	48.3	54.8	23.9	26.7

$p<0.05$ between sexes, except for age, insulin, PAI, vitamin E, n3 and SFA (all $p>0.05$). SD=standard deviation, BMI= body mass index, PAI= physical activity index, CRP= C-reactive protein, SHBG= sex hormone-binding globulin, serum PUFA=polyunsaturated fatty acid, serum MUFA=monounsaturated fatty acid, serum n3=omega-3 PUFA; serum n6=omega-6 PUFA, serum SFA=saturated fatty acid, BP=blood pressure, ALAT= alanine aminotransferase, GT=gamma-glutamyl transferase. Values are presented for participants who had data on oxHDLlipids. Values for ALAT and GT were measured in 2011.

5.2 OXIDIZED HDL LIPIDS AND THE RISK FACTORS FOR ATHEROSCLEROSIS

To study the multivariate determinants of oxidized lipoprotein lipids, oxLDLprot, HDL cholesterol, LDL cholesterol, and Apo-B, multivariable models for men (Table 4) and for women (Table 5) were analyzed.

In men, oxHDLlipids were directly associated with oxLDLlipids, and inversely with insulin levels and age. The variables explained 17.0 % (model R^2) of the total variation in oxHDLlipids. In women, oxHDLlipids were directly associated with oxLDLlipids, CRP and alcohol use, and inversely with waist circumference, age and daily smoking. The variables explained 33.7 % (model R^2) of the total variation in oxHDLlipids. Both models for oxHDLlipids were adjusted with Apo-A1. IMT was not significantly associated with oxHDLlipids when added to the multivariable models.

Independent predictors of oxLDLlipids in men were oxHDLlipids and insulin (direct associations) as well as systolic blood pressure and SHBG (inverse associations). The variables explained 53.4 % (model R^2) of the total variation in oxLDLlipids. In women, independent predictors of oxLDLlipids were oxHDLlipids and insulin with a direct association, and systolic blood pressure with an inverse association. The variables explained 53.6 % (model R^2) of the total variation in oxLDLlipids. Both models for oxLDLlipids were adjusted with Apo-B.

In men, oxLDLprot were directly associated with oxLDLlipids, age, BMI, insulin and SHBG, and inversely with oxHDLlipids and PAI. In women, oxLDLprot were directly associated with oxLDLlipids, age, BMI and systolic blood pressure, and inversely with oxHDLlipids.

Table 4. Multivariable models of oxHDLlipids, HDL cholesterol, oxLDLlipids, LDL cholesterol, oxLDLprot and Apo-B with risk factors in men.

	oxHDLlipids (N=628), R^2=17.0 %		HDL cholesterol (N=618), R^2=30.3 %		oxLDLlipids (N=622), R^2=53.4 %		LDL cholesterol (N=605), R^2=22.6 %		oxLDLprot (N=581), R^2=26.2 %		Apo-B (N=626), R^2= 56.6 %	
Variables	ß (SE)	Partial R^2	ß (SE)	Partial R^2	ß (SE)	Partial R^2	ß (SE)	Partial R^2	ß (SE)	Partial R^2	ß (SE)	Partial R^2
Apo-A1	11.763 (1.368) *	9.6 %										
Apo-B					27.311 (1.242) *	43.4 %						
oxHDLlipids			0.012 (0.001) *	6.4 %	0.417 (0.042) *	7.4 %	-0.018 (0.005) *	2.6 %	- 0.561 (0.127) *	4.4 %	-0.008 (0.001) *	6.8 %
oxLDLlipids	0.158 (0.025) *	3.4 %	-0.007 (0.001) *	5.8 %			0.035 (0.004) *	11.4 %	0.794 (0.091) *	15.4 %	0.015 (0.001) *	43.6 %
Age	-0.262 (0.055) *	2.9 %	0.008 (0.002) *	1.7 %			0.312 (0.006) *	5.2 %	0.383 (0.193) §	0.3 %	0.008 (0.002) *	2.1 %
BMI							0.033 (0.009) †	1.7 %	0.982 (0.281) †	3.1 %	0.013 (0.002) *	
Insulin	-1.480 (0.521) ‡	1.1 %	-0.050 (0.019) §	9.4 %	1.622 (0.654) §	0.5%			4.153 (2.108) §	0.5 %		
Systolic blood pressure					-0.081 (0.026) ‡	0.5%						
SHBG			0.003 (0.001) *	2.4 %	-0.113 (0.028) *	1.6%	0.014 (0.003) *	1.6 %	0.171 (0.086) §	0.5 %		
CRP			-0.027 (0.009) §	1.0 %								4.0 %
Alcohol use			0.036 (0.006) *	3.5 %								
PAI									-1.116 (0.376) ‡	1.8 %		

ß, regression coefficient; SE, standard error; *P≤ 0.0001; †P≤ 0.0005; ‡P≤ 0.005; §P< 0.05; Insulin and CRP were log-transformed. oxLDLlipids= oxidized LDL lipids, oxHDLlipids=oxidized HDL lipids, oxLDLprot= oxidized LDL proteins, Apo-B= apolipoprotein-B, Apo-A1=apolipoprotein-A1, HDL=high-density lipoprotein, LDL=low-density lipoprotein, BMI= body mass index, SHBG= sex hormone-binding globulin, CRP= C-reactive protein, PAI= physical activity index.

Table 5. Multivariable models of oxHDLlipids, HDL cholesterol, oxLDLlipids, LDL cholesterol, oxLDLprot and Apo-B with risk factors in women.

	oxHDLlipids (N=742), R^2=33.7 %		HDL cholesterol (N=755), R^2=28.4 %		oxLDLlipids (N=760), R^2=53.6 %		LDL cholesterol (N=757), R^2=22.5 %		oxLDLprot (N=760), R^2=25.7 %		Apo-B (N=755), R^2= 53.1 %	
Variables	ß (SE)	Partial R^2	ß (SE)	Partial R^2	ß (SE)	Partial R^2	ß (SE)	Partial R^2	ß (SE)	Partial R^2	ß (SE)	Partial R^2
Apo-A1	11.867 (0.987) *	25.2 %										
Apo-B					24.015 (0.981) *	42.6 %						
oxHDLlipids			0.017 (0.001) *	19.2 %	0.343 (0.026) *	9.6 %	-0.014 (0.003) *	2.6 %	-0.319 (0.093) †	1.4 %	-0.005 (0.001) *	2.7 %
oxLDLlipids	0.217 (0.029) *	4.2 %	-0.007 (0.001) *	4.7 %			0.037 (0.003) *	13.5 %	1.182 (0.087) *	20.1 %	0.017 (0.001) *	42.7 %
Age	-0.131 (0.048) ‡	0.8 %	0.005 (0.002) ‡	0.6 %			0.021 (0.005) *	3.6 %	0.415 (0.139) ‡	0.9 %		
Waist circumference	-0.117 (0.025) *	1.8 %	-0.005 (0.001) *	1.1 %							0.004 (0.001) *	6.2 %
BMI							0.024 (0.006) *	0.8 %	0.579 (0.169) †	2.9 %		
Insulin					1.582 (0.409) *	0.7 %	-0.141 (0.053) ‡	0.7 %				
Systolic blood pressure			0.002 (0.001) §	0.5 %	-0.054 (0.017) ‡	0.6 %			0.118 (0.058) §	0.4 %	0.002 (0.001) *	0.9 %
CRP	0.531 (0.216) §	0.5 %	0.021 (0.008) §	0.6 %								
Alcohol use	0.740 (0.326) §	0.5 %	0.054 (0.012) *	1.6 %			-0.126 (0.033) *	1.3 %			-0.020 (0.007) ‡	0.4 %
Daily smoking	-1.743 (0.598) ‡	0.7 %										

ß, regression coefficient; SE, standard error; *P≤ 0.0001; †P= 0.0007; ‡P< 0.009; §P< 0.05; Insulin and CRP were log-transformed. oxLDLlipids= oxidized LDL lipids, oxHDLlipids=oxidized HDL lipids, oxLDLprot= oxidized LDL proteins, Apo-B= apolipoprotein-B, Apo-A1=apolipoprotein-A1, HDL=high-density lipoprotein, LDL=low-density lipoprotein, BMI= body mass index,, CRP= C-reactive protein.

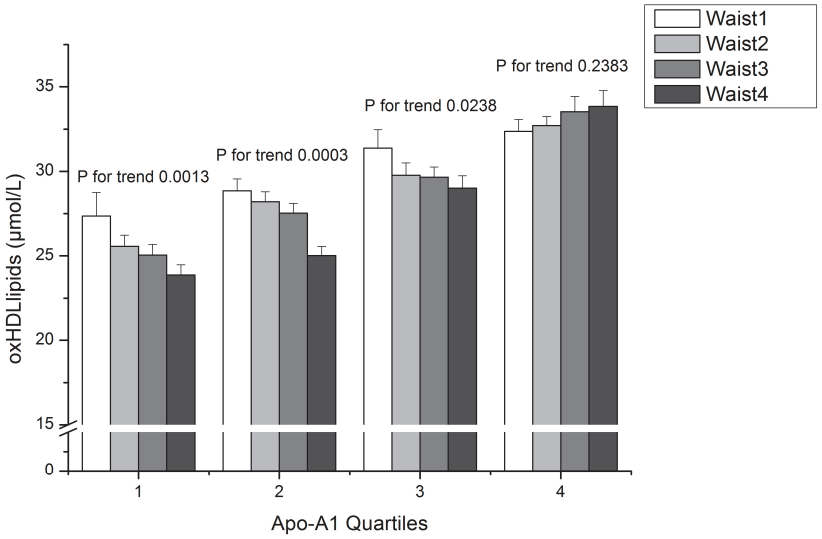

Figure 4. OxHDLlipids concentrations according to Apo-A1 quartiles stratified by waist circumference quartiles.

Fig. 4 and Fig. 5 are presented in order to demonstrate that waist circumference and age are significant independent predictors of the oxHDLlipids levels.

Figure 4 shows that waist circumference is a negative correlate of oxHDLlipids in the three lowest Apo-A1 quartiles. In the highest Apo-A1 quartile, the correlation between oxHDLlipids and waist circumference is not significant.

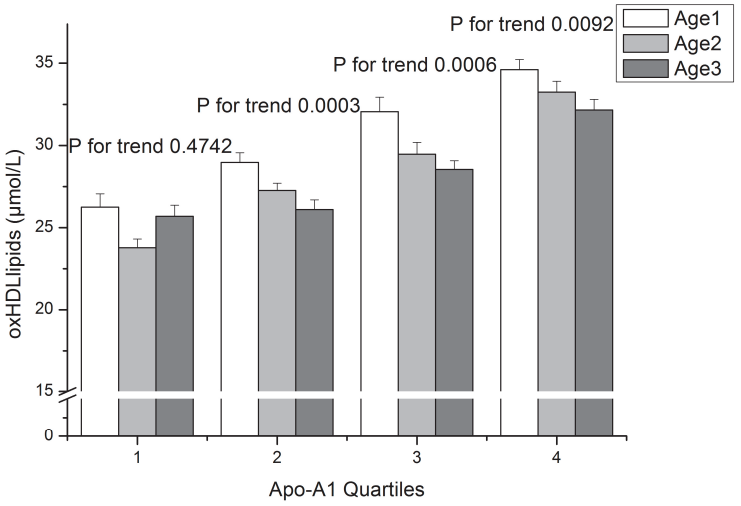

Figure 5. oxHDLlipids concentrations according to Apo-A1 quartiles stratified by age tertiles.

Figure 5 demonstrates that oxHDLlipids are inversely associated with age in the three highest Apo-A1 quartiles.

5.3 FATTY ACIDS AND OXIDIZED HDL LIPIDS

To study the bivariate correlations between oxidized lipoprotein lipids and serum FA proportions, we correlated oxHDLlipids and oxLDLlipids with PUFA, n3, n6, MUFA and SFA (Table 6).

In women, oxHDLlipids correlated inversely with PUFA and n6, and directly with MUFA and SFA. There was a borderline significant association between oxHDLlipids and n3. In men, there were no significant associations between oxHDLlipids and serum FAs.

In both sexes, oxLDLlipids correlated inversely with PUFA and n6, and directly with MUFA and SFA. There was additionally a borderline significant association between oxLDLlipids and n3 in men.

Table 6. Pearson´s correlations between oxidized lipoprotein lipids and serum fatty acid proportions.

	oxHDLlipids				oxLDLlipids			
	Men		Women		Men		Women	
Serum total FA %	r	P-value	r	P-value	r	P-value	r	P-value
PUFA	0.002	0.97	-0.17	<.0001	-0.63	<.0001	-0.53	<.0001
n3	-0.05	0.16	-0.06	0.08	-0.07	0.06	-0.05	0.21
n6	0.02	0.67	-0.16	<.0001	-0.65	<.0001	-0.53	<.0001
MUFA	-0.006	0.87	0.12	0.0007	0.60	<.0001	0.48	<.0001
SFA	0.005	0.89	0.16	<.0001	0.43	<.0001	0.34	<.0001

N is 628 in men and 763 in women. FA= fatty acid, PUFA=polyunsaturated fatty acid, MUFA=monounsaturated fatty acid, n3=omega-3 PUFA; n6=omega-6 PUFA, SFA=saturated fatty acid, oxLDLlipids= oxidized LDL lipids, oxHDLlipids=oxidized HDL lipids.

To study the multivariable determinants between serum FAs and oxidized HDL and LDL, we constructed linear regression models for men (Table 7) and for women (Table 8).

In both sexes, the results in the multivariable models were very similar to those in Pearson´s correlations between oxidized lipoprotein lipids and serum FA proportions. In men, oxHDLlipids were not associated serum FAs. In women, oxHDLlipids associated inversely with PUFA and n6, and directly with MUFA and SFA. Adjustment with additional CVD risk factors in step 2 diluted the association between oxHDLlipids and SFA into statistical nonsignificance.

In men, oxLDLlipids were inversely correlated with PUFA, n3 and n6, and directly with MUFA and SFA. The association between oxLDLlipids and n3 was diluted into statistical nonsignificance in step 2. In women, oxLDLlipids were inversely correlated with PUFA and n6, and directly with MUFA and SFA.

Table 7. Linear regression models between fatty acid variables and oxidized lipoprotein lipids in men.

		oxHDLlipids		oxLDLlipids	
Serum FA %	Step	*Beta*	*P*	*Beta*	*P*
PUFA	1	0.002	0.98	-1.49	<.00011
	2	-0.15	0.13	-1.29	<.000111
n3	1	-0.25	0.24	-0.74	0.03
	2	-0.27	0.32	-0.49	0.15
n6	1	0.02	0.73	-1.61	<.0001
	2	-0.13	0.22	-1.33	<.0001
MUFA	1	-0.02	0.83	2.05	<.0001
	2	0.21	0.13	1.72	<.0001
SFA	1	0.03	0.81	1.99	<.0001
	2	0.15	0.42	1.45	<.0001

Linear regression models contained 2 steps. Step 1: the FA variable and age were included in the model. Step 2: step 1+physical activity, vitamin E intake, smoking, body mass index, alcohol intake, glucose, insulin, and systolic blood pressure were included in the model. HDL cholesterol was included in the models for oxHDLlipids and LDL cholesterol was included in the models for oxLDLlipids. Values for vitamin E intake, body mass index and insulin were log-transformed and the value for glucose was square root transformed. FA=fatty acid, MUFA=monounsaturated fatty acid; n3=omega-3 PUFA; n6=omega-6 PUFA; PUFA=polyunsaturated fatty acid; SFA=saturated fatty acid.

Table 8. Linear regression models between fatty acid variables and oxidized lipoprotein lipids in women.

		oxHDLlipids		oxLDLlipids	
Serum FA %	Step	*Beta*	*P*	*Beta*	*P*
PUFA	1	-0.29	<.0001	-1.09	<.0001
	2	-0.42	0.0008	-1.14	<.0001
n3	1	-0.24	0.33	-0.27	0.31
	2	-0.78	0.06	-0.14	0.73
n6	1	-0.29	<.0001	-1.12	<.0001
	2	-0.34	0.006	-1.08	<.0001
MUFA	1	0.31	0.0017	1.43	<.0001
	2	0.62	0.0003	1.45	<.0001
SFA	1	0.47	0.0002	1.27	<.0001
	2	0.27	0.23	1.02	<.0001

Linear regression models contained 2 steps. Step 1: the FA variable and age were included in the model. Step 2: step 1+physical activity, use of contraceptives, vitamin E intake, smoking, body mass index, alcohol intake, glucose, insulin, and systolic blood pressure were included in the model. HDL cholesterol was included in the models for oxHDLlipids and LDL cholesterol was included in the models for oxLDLlipids. Values for vitamin E intake, body mass index, and insulin were log-transformed and value for glucose was square root transformed. FA=fatty acid, MUFA=monounsaturated fatty acid; n3=omega-3 PUFA; n6=omega-6 PUFA; PUFA=polyunsaturated fatty acid; SFA=saturated fatty acid.

5.4 PON1 AND OXIDIZED HDL LIPIDS

Table 9. Characteristics of the study subjects stratified by rs662 genotypes.

	A/A (N=1071)		G/A (N=707)		G/G (N=117)		P for
Variable	Mean	SD	Mean	SD	Mean	SD	trend
Women/men	576/495		387/320		60/57		0.93
Age (yrs)	31.49	4.94	31.92	5.03	32.36	5.12	0.02
BMI (kg/m²)	24.98	4.51	25.20	4.36	25.59	4.44	0.21
PON1 (U/L)	51.84	21.58	126.57	31.14	152.5	49.54	<.0001
oxHDLlipids (µmol/L)	28.94	7.43	28.68	6.55	29.31	8.13	0.94
oxLDLlipids (µmol/L)	26.75	9.76	27.01	9.56	26.96	8.96	0.74
oxLDLprot (U/L)	82.54	23.74	83.25	25.89	82.58	24.79	0.96
Apo-A1 (g/L)	1.49	0.25	1.47	0.24	1.45	0.25	0.02
Apo-B (g/L)	1.11	0.25	1.06	0.27	1.07	0.27	0.24
Total cholesterol (mmol/L)	5.10	0.95	5.13	0.95	5.12	0.95	0.92
HDL cholesterol (mmol/L)	1.29	0.31	1.26	0.30	1.24	0.33	0.005
LDL cholesterol (mmol/L)	3.22	0.81	3.28	0.85	3.27	0.86	0.32
Triglycerides (mmol/L)	1.31	0.83	1.33	0.82	1.30	0.71	0.65
IMT (mm)	0.578	0.089	0.588	0.095	0.588	0.094	0.10
Waist circumference (cm)	83.87	12.46	84.44	12.22	85.19	12.61	0.38
Systolic BP (mmHg)	116.8	12.82	116.5	12.86	117.8	15.12	0.95
Diastolic BP (mmHg)	71.07	10.39	70.81	10.60	72.35	12.29	0.97
Insulin (mU/L)	7.65	5.61	7.88	5.87	7.86	7.19	0.39
Glucose (mmol/L)	5.05	0.69	5.07	1.10	5.21	0.94	0.19
PAI (unitless, range 5-15)	10.02	2.36	9.98	2.31	9.72	2.38	0.43
CRP (mmol/L)	1.89	3.89	1.78	3.88	2.33	4.84	0.71
Alcohol use (drinks per day)	0.87	1.19	0.85	1.89	0.92	1.60	0.91
Daily smoking (%)	23.99		25.07		25.44		0.51
Testosterone (nmol/L)	9.67	10.5	9.01	9.02	9.48	9.05	0.37
SHBG (nmol/L)	59.55	55.2	57.94	52.3	57.4	55.4	0.77

P for trend was adjusted with age; SD=standard deviation, PON1= paraoxonase, oxHDLlipids= oxidized HDL lipids, oxLDLlipids= oxidized LDL lipids, oxLDLprot= oxidized LDL proteins, Apo-B= apolipoprotein-B, Apo-A1=apolipoprotein-A1, BMI= body mass index, IMT= intima-media thickness, BMI= body mass index, PAI= physical activity index, CRP= C-reactive protein, BP=blood pressure, SHBG= sex hormone-binding globulin.

The characteristics of the study subjects stratified by the rs662 genotype are shown in Table 9. There was a statistically significant difference across rs662 genotypes in PON1 levels: the mean activity of serum PON1 was 51.8 U/L in a group with A/A genotype (QQ genotype), 126.6 U/L in a group with G/A genotype (QR genotype) and 152.5 U/L in a group with G/G genotype (RR genotype) (P-value <.0001). There were also statistically significant differences in age, Apo-A1, and HDL cholesterol, but not in any other of the variables studied.

Multivariable models were constructed to study whether PON1 is a significant explanatory variable for oxHDLlipids, oxLDLlipids and oxLDLprot (Table 10). PON1 correlated in multivariable models inversely with oxLDLlipids (P<.0001), but not with oxHDLlipids. There was a borderline significant association between PON1 activity and oxLDLprot (p=0.08).

Table 10. Multivariable models of oxHDLlipids, oxLDLlipids and oxLDLprot with priori selected variables including PON1 as an explanatory variable.

	oxHDLlipids (N=1255), R^2= 28.1 %			oxLDLlipids (N=1254), R^2= 53.6 %			oxLDLprot (N=1254), R^2= 60.4 %		
Variables	ß (SE)	P-value	Semi-partial R^2	ß (SE)	P-value	Semi-partial R^2	ß (SE)	P-value	Semi-partial R^2
PON1	-0.01 (0.16)	0.93	0.07	-0.68 (0.17)	0.0001	0.09 %	-0.69 (0.39)	0.08	0.04 %
Sex	-0.20 (0.37)	0.59	1.6 %	0.54 (0.41)	0.18	4.8 %	-0.47 (0.90)	0.60	5.2 %
Apo-A1	13.65 (0.75)	<.0001	20.4 %						
Apo-B				32.08 (1.25)	<.0001	26.3 %	81.35 (2.55)	<.0001	34.6 %
oxHDLlipids				0.34 (0.03)	<.0001	4.4 %	0.09 (0.06)	0.15	0.2 %
oxLDLlipids	0.14 (0.02)	<.0001	4.5 %				-0.34 (0.06)	<.0001	18.0 %
oxLDLprot				-0.07 (0.01)	<.0001	17.6 %			
Age	-0.17 (0.04)	<.0001	1.2 %	-0.06 (0.04)	0.12	0.3 %	0.10 (0.08)	0.23	2.3 %
BMI	-0.11 (0.04)	0.01	0.4 %	0.02 (0.05)	0.60	0.01 %	0.01 (0.11)	0.91	0.0004 %

ß, regression coefficient; SE, standard error, PON1= paraoxonase, oxHDLlipids= oxidized HDL lipids, oxLDLlipids= oxidized LDL lipids, oxLDLprot= oxidized LDL proteins, Apo-B= apolipoprotein-B, Apo-A1=apolipoprotein-A1, BMI= body mass index. Normalized rank transformation was made for PON1.

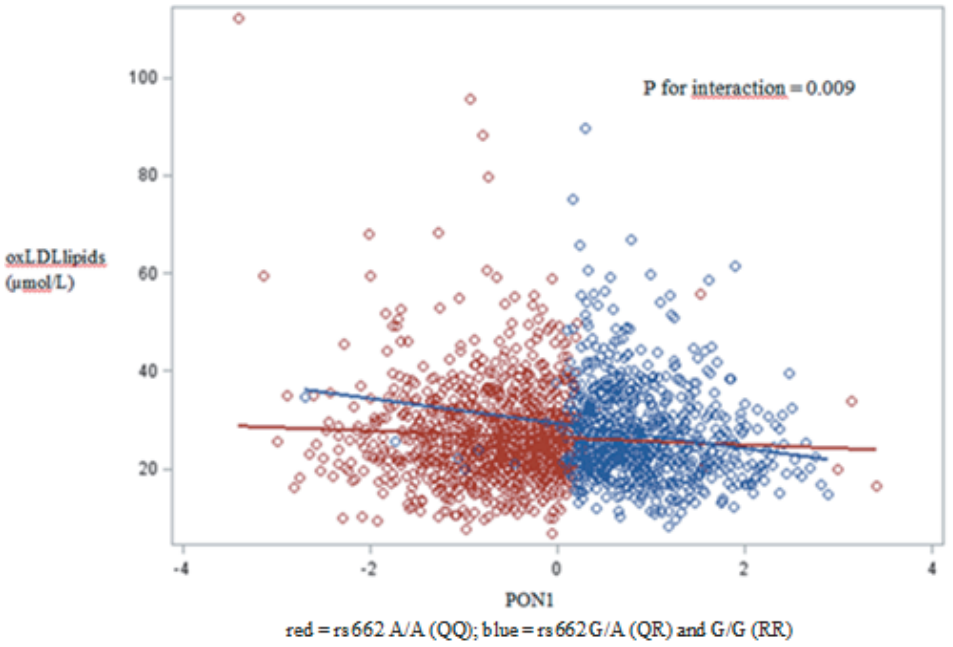

Figure 6. Scatterplot of oxLDLlipids and PON1 stratified by rs662 genotypes.

Figure 6 presents a scatterplot of oxLDLlipids and PON1 stratified by the rs662 genotypes. The inverse association between PON1 and oxLDLlipids was stronger in the subgroup with the G/A (QR) or G/G (RR) genotype. There was no significant association between PON1 and oxLDLlipids within individuals with the A/A genotype (QQ genotype).

Table 11. Multivariable model of PON1 with other study variables.

Variable	ß	SE	P-value	Semi-partial R^2
Systolic BP	0.01	0.002	0.0006	0.9 %
LDL cholesterol	0.10	0.03	0.0009	0.7 %
Insulin	0.14	0.04	0.0015	0.5 %
HDL cholesterol	0.27	0.07	0.0005	0.4 %
oxLDLlipids	-0.008	0.003	0.0061	0.08 %

R^2= 2.6 %, N=1895; ß, regression coefficient; SE, standard error; Values for triglycerides, CRP and insulin were log-transformed and the value for glucose was square root transformed. A normalized rank transformation was made for PON1. PON1=paraoxonase-1, BP=blood pressure, LDL=low-density lipoprotein, HDL=high-density lipoprotein, oxLDLlipids= oxidized LDL lipids.

To study the multivariate determinants of serum PON1, we analyzed the multivariable model shown in Table 11. PON1 associated directly with systolic blood pressure (P=0.0006, semi-partial R^2=0.9 %), LDL cholesterol (P-value=0.0009, semi-partial R^2=0.7 %), insulin (P-value=0.0015, semi-partial R^2=0.5 %), HDL cholesterol (P-value=0.0005, semi-partial R^2=0.4 %), and inversely with oxLDLlipids (P-value=0.0061, semi-partial R^2=0.08 %).

5.5 FATTY LIVER AND OXIDIZED HDL LIPIDS

Table 12. Multivariable model of oxHDLlipids with priori selected variables including fatty liver as an explanatory variable.

	oxHDLlipids (N=1029), R^2= 25.3 %		
Variables	ß (SE)	P-value	Semi-partial R^2
Fatty liver	-1.08 (0.44)	0.013	1.0 %
Sex	0.03 (0.45)	0.95	0.7 %
Apo-A1	12.95 (0.9)	<.0001	17.4 %
oxLDLlipids	0.17 (0.02)	<.0001	4.2 %
Age	-0.21 (0.04)	<.0001	1.9 %
BMI	-0.05 (0.05)	0.38	0.05 %

ß, regression coefficient; SE, standard error, oxHDLlipids= oxidized HDL lipids, oxLDLlipids= oxidized LDL lipids, Apo-A1=apolipoprotein-A1, BMI= body mass index.

To study whether a fatty liver is a significant explanatory variable for oxHDLlipids, we analyzed the multivariable model shown in Table 12. OxHDLlipids correlated inversely with a fatty liver (P=0.013, semi-partial R^2=1.0 %).

Table 13. Logistic regression analysis of LDL lipid and protein oxidation and HDL lipid oxidation markers (2001) associated with the odds of a fatty liver (2011).

	n					
	Normal liver	Fatty liver	OR	95%CI Lo	Up	*P*
A: age and sex adjustment						
Model 1: oxLDLprot (U/L)	1286	288	1.37	1.18	1.58	<0.001
Model 2: oxLDLlipids (µmol/L)			1.53	1.32	1.76	<0.001
Model 3: Oxidation score (sum of oxLDLprot+oxLDLlipids)			1.54	1.33	1.79	<0.001
Model 4: oxHDLlipids (µmol/L)	843	193	0.79	0.67	0.93	0.004
Model 5: oxLDLlipids/oxHDLlipids			1.67	1.40	1.99	<0.001
*B: A+adjustment for the risk factors of fatty liver**						
Model 1: oxLDLprot (U/L)	1286	288	1.07	0.91	1.25	0.40
Model 2: oxLDLlipids (µmol/L)			1.22	1.05	1.43	0.011
Model 3: Oxidation score (sum of oxLDLprot+oxLDLlipids)			1.18	1.00	1.38	0.047
Model 4: oxHDLlipids (µmol/L)	843	193	0.83	0.70	0.99	0.038
Model 5: oxLDLlipids/oxHDLlipids			1.34	1.11	1.63	0.002

Data are odd ratios (OR) and their confidence intervals (CIs) for a one SD change in the explanatory variables indicating LDL or HDL oxidative status. *The risk factors included were alcohol consumption (categorized 1 to 4), leisure-time physical activity (1 to 15), smoking (no vs. yes) and body mass index. Further adjustment of the models for serum CRP did not dilute any of the statistically significant associations into statistical non-significance. oxHDLlipids= oxidized HDL lipids, oxLDLlipids= oxidized LDL lipids, oxLDLprot= oxidized LDL proteins.

To gain insights into the associations between fatty liver disease and lipoprotein oxidation, we constructed the logistic regression analyses shown in Table 13. Oxidized HDL and LDL lipids associated significantly with the prevalence of a fatty liver in

2011. These associations remained significant after adjustment for the risk factors of a fatty liver including alcohol consumption, leisure-time physical activity, smoking, and BMI. The OR for oxHDLlipids were 0.83 after these adjustments (P-value=0.038). The association between oxLDLprot, and future fatty liver disease was not significant after adjustment for the risk factors of fatty liver disease.

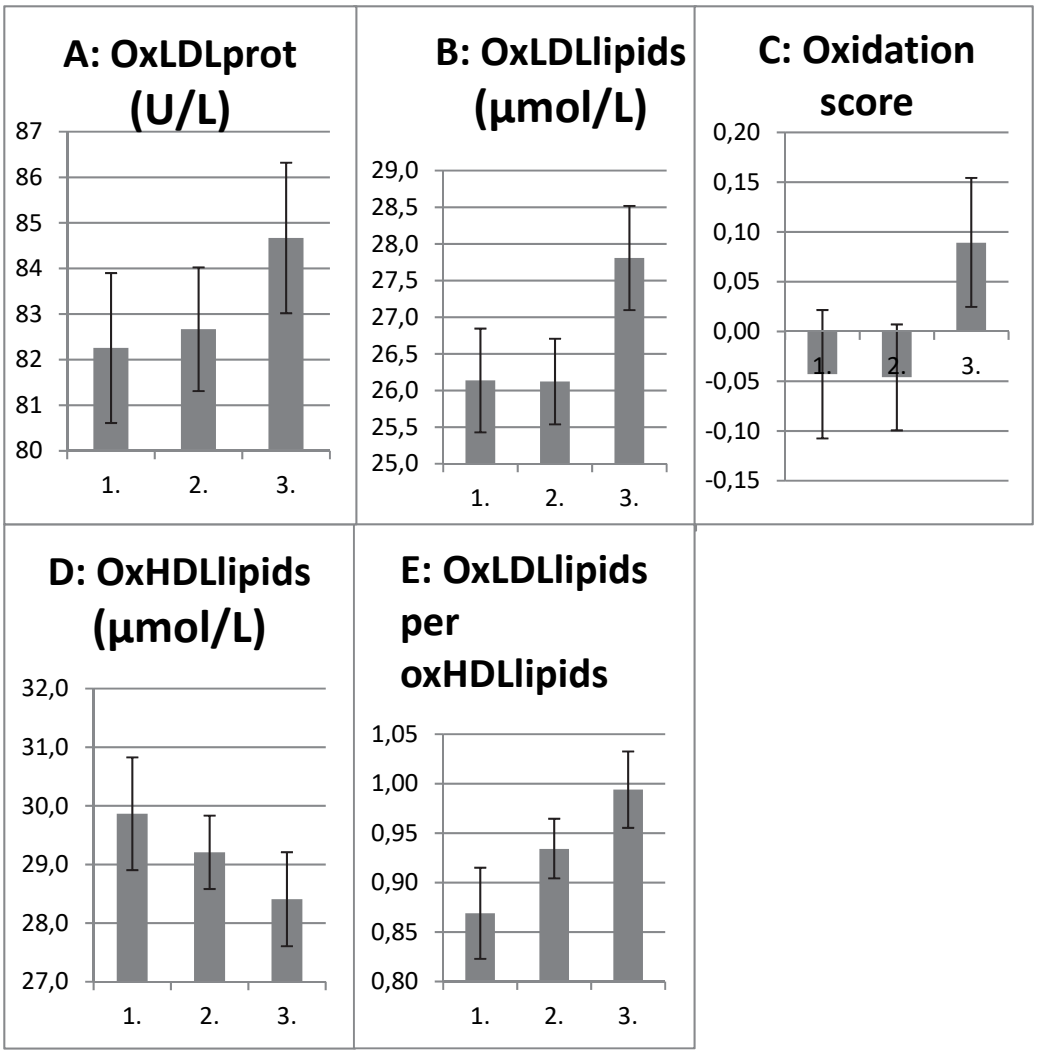

Figure 7. Mean levels of lipoprotein oxidation markers according to the severity of fatty liver disease. Error bars denote 95%CIs. The markers of LDL oxidation were adjusted for serum LDL cholesterol and serum LDL particle concentrations, and correspondingly, the marker of HDL oxidation for HDL cholesterol and HDL particle concentrations. The oxLDLlipids/oxHDLlipids ratio was adjusted for the ratios of serum LDL cholesterol/HDL cholesterol and serum LDL particle/HDL particle concentration. To achieve reasonable group sizes, the 10-level fatty liver score was stratified into 3 groups: Group 1: original score 0; Group 2: original scores 1-2; Group 3: original scores ≥3. Number of subjects per group is given next to the error bars.

Figure 7 presents the fact that there is a significant inverse association between oxHDLlipids and fatty liver status, and a direct association between fatty liver disease and the markers of LDL oxidation.

6. DISCUSSION

Information concerning the associations of oxidized HDL lipids with risk factors for atherosclerosis is limited. The present study has shown that an elevated cardiovascular risk profile characterized primarily by advanced age is associated with lower oxHDLlipid levels. It was found that high oxHDLlipid levels were associated with high oxLDLlipid levels, but by contrast with low oxLDLprot levels. When studying the associations between oxidized lipoprotein lipids and serum FAs, it was shown that in women oxHDLlipids were negatively associated with PUFA and positively with MUFA. Serum PUFA and particularly n6PUFA was inversely associated with oxLDLlipids. MUFA and SFA were directly associated with levels of oxLDLlipids. PON1 activity was inversely associated with oxLDLlipid levels, but not with oxHDLlipid levels. It may, therefore, be suggested that PON1 could have a role in the oxidation of LDL lipids. It was additionally shown that oxidized HDL and LDL lipids are associated with a future fatty liver. However, oxHDLlipids were not associated with IMT. Therefore, the role of oxHDLlipids as a possible cardiovascular risk factor needs further studies. The main findings of the study are presented in the Figure 8.

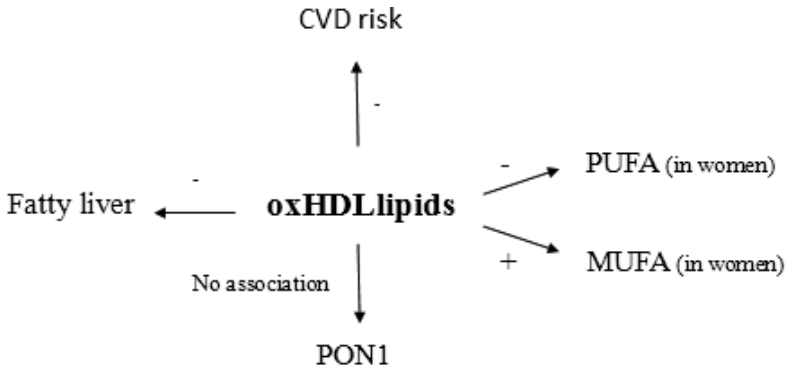

Figure 8. Main findings of the study.

6.1 PARTICIPANTS

The participants in this thesis are from the Cardiovascular Risk in Young Finns Study, which is on-going epidemiological study of CVD risk factors in children and young adults launched in 1980. The participants were randomly selected from different parts of the country, equally selected from both sexes, and from both urban and rural areas in order to represent Finnish children and adolescents as closely as possible. A total of 3,596 subjects (83.2 % of those invited) aged 3 to 18 years participated in the study in

1980 and were considered to be representative of the total random sample (Åkerblom et al. 1985). A total of 2,284 subjects in 2001 and 2,204 subjects in 2007 participated from the original study cohort.

Lost-to follow-up is inevitable in longitudinal studies. In the follow-up studies the participation rates have, however, been satisfactory and childhood risk factors in 1980 were similar between participants and non-participants. The study cohort in this study seems therefore to be representative of the original study cohort. The sample size of this study is sufficient for statistical analyses and the follow-up bias does not seem to influence the representativeness of the original study population. The results of the present study may therefore be generalized in white populations.

6.2 METHODS

6.2.1 Lipoprotein lipid oxidation

The analysis of oxidized HDL and LDL lipids in this thesis was based on the determination of the baseline level of conjugated dienes in lipoprotein lipids (Ahotupa et al. 1998). The analysis measures mildly oxidized lipoproteins in circulation (Ahotupa, Vasankari 1999). The isolation procedures were validated for the purpose and did not affect the level of oxidized lipids (Ahotupa et al. 1998). Diene conjugation is able to detect oxidative HDL and LDL lipid modification found in all HDL and LDL lipid classes. Despite there being relatively little knowledge concerning the associations of oxHDLlipids with risk factors for atherosclerosis, it is known that oxLDLlipids are associated with several risk factors for atherosclerosis such as HDL and LDL cholesterol (Vasankari et al. 2000, Toikka et al. 1999), BMI (Vasankari et al. 2000) and hypertension (Toikka et al. 2000). The comparison between oxidized HDL and LDL in our study is reliable due the fact that we have measured oxidative modification of HDL and LDL using the same method.

The baseline diene conjugation method enables to measure oxidative modifications of lipids in lipoprotein particles *in vivo* (Ahotupa et al. 1998), and the measurement with this method is fast and simple to perform (Ahotupa, Vasankari 1999). The advantage of measuring oxidized lipoproteins is that it directly enables the investigation of the role of lipid oxidation in atherosclerosis in large epidemiologic studies. A baseline diene conjugate assay is therefore well suited to clinical purposes such as our present study.

We additionally measured heavily oxidized LDL using a method that was determined with an enzyme-linked immunosorbent assay with antibodies directed against the oxidized Apo-B molecule (Oxidized LDL ELISA kit, Mercodia, Sweden) (Holvoet et al. 2006). This measurement for oxLDLprot is suggested to be an independent predictor of atherosclerosis (Salonen et al. 1992). A limitation of this method is that the

preparation of similar antigens between laboratories is not possible, which may result in unreliable outcomes. It is, however, valuable that we were able to compare the associations between two methods measuring oxidized LDL and CVD risk factors in our present study; one being oxLDLlipids, which is a measure for mildly oxidized lipids in LDL, and the other oxLDLprot, a measure for heavily oxidized LDL.

6.2.2 Cardiovascular risk factors

The methods for measuring the risk factors for atherosclerosis such as HDL and LDL cholesterol levels, blood pressure, BMI and glucose levels are well-standardized and therefore reliable assessments (Raitakari et al. 2008). Self-reported questionnaires for alcohol use, smoking, and physical activity are valid measurements for studying these health factors although these questionnaires may also involve limitations (Raitakari et al. 2008).

Serum total FA proportions were used instead of dietary FA intake, because the intake data is based on a short-term 48-h recall and is available for only half of the subjects (Kaikkonen et al. 2013). With this method of measuring serum FA proportions it is possible to consider factors influencing fatty acid metabolism (Murakami et al. 2008).

Paraoxon was used as the substrate when measuring PON1 activity. It is not an endogenous substrate, but it is assumed to reflect the catalytic activity of PON1 (Tang et al. 2012).

Fatty liver status was determined with ultrasonography. It is a common cause of abnormal liver function tests and is a risk factor for CVDs (Sattar, Forrest & Preiss 2014). It is known that ultrasound has only a low sensitivity to steatohepatitis (Saadeh et al. 2002), but it is, however, an appropriate method for population studies such as the Young Finns Study (Suomela et al. 2015). It is estimated that imaging with ultrasound may detect steatosis when more than 30 % of the liver is affected (Saadeh et al. 2002). It is notable that magnetic resonance imaging techniques are more accurate to detect liver fibrosis than ultrasound methods (Tan, Venkatesh 2016).

6.3 RESULTS

6.3.1 Oxidized HDL lipids and the risk factors for atherosclerosis

It was shown that oxHDLlipids were inversely associated with age and directly associated with oxLDLlipids. These results remained significant after adjustment for Apo-A1. In women, oxHDLlipids levels were additionally associated with several other known risk factors for atherosclerosis as there were inverse associations with waist circumference and daily smoking and direct associations with CRP levels and

alcohol use. In men, oxHDLlipids were also inversely associated with insulin levels. The associations of oxHDLlipids with risk factors for atherosclerosis are presented in Figure 9.

The results in this thesis suggest that an elevated cardiovascular risk profile is associated with lower oxHDLlipids levels. OxHDLlipids were not, however, associated with IMT, which is a marker of subclinical atherosclerosis. OxHDLlipids were additionally directly associated with oxLDLlipids. There is only a limited amount of previous information concerning the associations of oxidized HDL and risk factors for atherosclerosis at the population level. However, it may be possible to hypothesize why low oxHDLlipid levels seems to be a risk factor for CVDs. The main previous finding is that HDL is shown to be active in the reverse transport of lipid peroxides (Ahotupa et al. 2010). It has been demonstrated that oxidized cholesterol esters are removed to the liver from HDL but not from LDL (Christison et al. 1996) and that products of lipid oxidation are transferred from LDL to HDL (Sattler, Stocker 1993) with the aid of CETP (Christison, Rye & Stocker 1995). It is notable that transfer of oxidized and unoxidized lipids between LDL and HDL, facilitated by CETP, proceeds in opposite directions (Christison, Rye & Stocker 1995). This is in line with the finding in this thesis that oxHDLlipid levels were directly associated with oxLDLlipid levels. When considered together, the available data may suggest that the lipid peroxide transport function of HDL may be one mechanism explaining the atheroprotective effects of HDL. This study, however, failed to provide direct evidence that oxHDLlipids could be a possible marker of early stages of atherosclerosis, as there was no association between oxHDLlipids and IMT. Therefore, the role of oxHDLlipids as a possible biomarker of various stages of atherosclerosis as well as cardiovascular disease outcomes needs further studies.

In this study, oxLDLlipids and oxLDLprot had similar trends as regards associations with CVD risk factors, which is in line with an earlier finding that there is an association between oxLDLlipids and autoantibodies against oxidized LDL (Ahotupa et al. 1998). When comparing these two methods used to measure oxidized LDL, the main difference was that high oxHDLlipids levels were associated with high oxLDLlipids, but, in contrast, with low oxLDLprot. There are a few possible explanations for this finding. OxLDLlipids measures mildly oxidized lipids in LDL and therefore may represent acute lipid oxidation, and oxLDLprot is a measure for heavily oxidized LDL and may thus indicate chronic lipid oxidation (Ahotupa et al. 1998). Another explanation may be that oxHDLlipids may reflect the capacity of HDL to remove oxidized lipids from LDL particles. This hypothetical capacity may increase in an acute phase as the concentration of oxidized lipids is increased in LDL particles: thus, the positive correlation between oxHDLlipids and oxLDLlipids may be explained with this hypothesis. However, if this capacity of HDL is dysfunctional, it may lead to

accumulation of oxidized lipids in LDL and to oxidation of Apo-B in the long term, which may explain the inverse association between oxHDLlipids and oxLDLprot in this thesis. Both these explanations are in agreement with the reverse transport of lipid peroxides theory (Ahotupa et al. 2010) and the antioxidant function of HDL theory (Navab et al. 2011). It may be hypothesized that the ratio of oxHDLlipids to oxLDLlipids may be a potential marker to detect an impaired HDL oxidized lipid transport function and therefore an elevated risk for CVDs.

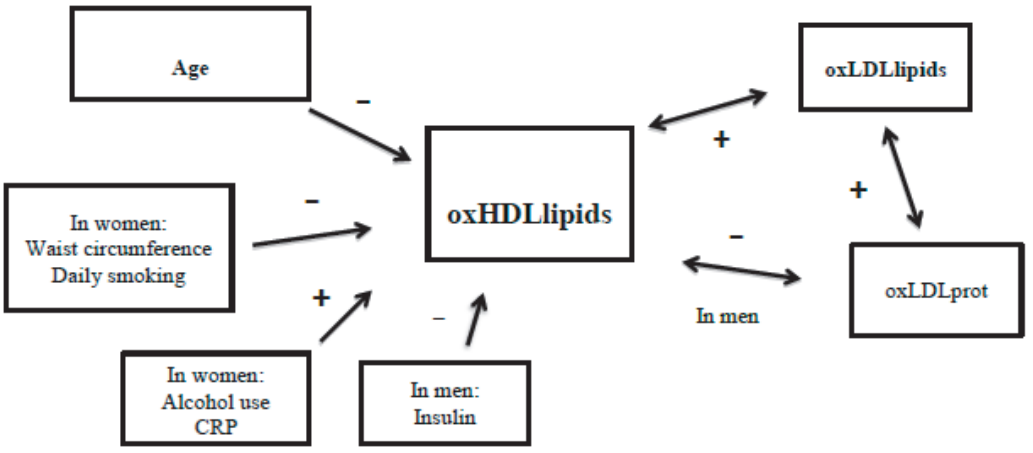

Figure 9. The associations of oxidized HDL lipids (oxHDLlipids) with known risk factors for atherosclerosis.

6.3.2 Fatty acids and oxidized HDL lipids

In this study, oxHDLlipids were negatively associated with PUFA and positively with MUFA in women. There was no significant association between serum FA and oxHDLlipids in men. Serum PUFA and particularly n6PUFA was inversely associated with oxLDLlipids. MUFA and SFA were directly associated with levels of oxLDLlipids. These results remained significant after adjustment with CVD risk factors.

PUFAs are known to be susceptible to oxidative modifications due to unstable hydrogen-carbon bonds (Bochkov et al. 2010) and high intake of PUFA is traditionally associated with lower risk for CVDs (Mozaffarian, Micha & Wallace 2010). It is, however, suggested that an elevated PUFA intake without simultaneous n3PUFA intake could increase the risk for CVD (Ramsden et al. 2010). Moreover, n3PUFA consumption is suggested to reduce the risk of death from CVDs (Anonymous 1999, Chaddha, Eagle 2015). It has also been presented that replacement of n6PUFA with n3PUFA could be beneficial via the anti-inflammatory properties of n3PUFAs (Wall et al. 2010). Childhood n3PUFA has controversially shown to be directly related to

adulthood blood pressure and subclinical atherosclerosis (Kaikkonen et al. 2013, Kaikkonen et al. 2012). There is also evidence that high PUFA/SFA and n6/n3 PUFA concentration ratios associate negatively with F2-isoprostanes (Kaikkonen et al. 2013), which is in line with our present findings regarding the relationship between oxLDLlipids and FA, suggesting that PUFAs are associated with a lowered LDL oxidation and therefore with lowered risk for CVD. It is notable that the PUFAs were measured in this study from serum reflecting the situation *in vivo* and not from supplementation as a part of clinical trial.

In this study, oxLDLlipids were directly associated with SFA. In line with this, SFAs are shown to be directly associated with lipid peroxidation (Kaikkonen et al. 2013). A mechanism explaining these findings may be that a major dietary source for SFAs is red meat, which is capable of catalyzing oxidative reactions as it is rich in heme iron (White, Collinson 2013). Increased LDL oxidation is additionally linked with red meat consumption (Cocate et al. 2015). It may, therefore, be suggested that SFAs are related to a higher CVD risk. MUFAs were similarly directly associated with oxidized lipoprotein lipids. A major source of MUFAs in the Western diet is red meat (Alonso, Ruiz-Gutierrez & Martinez-Gonzalez 2006) as in the case of SFAs. Our present findings are in agreement with a previous study where childhood MUFAs were associated with adulthood subclinical atherosclerosis (Kaikkonen et al. 2013).

Interestingly, we found in this study that oxHDLlipids associated inversely with PUFA and directly with MUFA. There was no significant association between n3PUFA and oxHDLlipids. These findings could be explained by the oxidized-lipid transporting capacity of HDL (Ahotupa et al. 2010). When the amount of oxidized lipids is elevated i.e. with higher proportions of SFA and MUFA, the HDL may be able to increase its capacity to transport oxidized lipids and this may be seen as a direct correlation between oxHDLlipids and MUFA. Direct correlation between PUFA and oxHDLlipids may reflect the reduced need of HDLs to transport oxidized lipids in circulation. These results concerning the associations between oxidized lipoprotein lipids and serum FAs are supportive of the oxidized-lipid transporting theory of lipoproteins (Ahotupa et al. 2010).

It is difficult to explain why there were no significant associations between oxHDLlipids and serum FAs in men. One possible explanation may be that the associations between oxHDLlipids and cardiovascular risk factors seem to be stronger in women than in men in the present study as the model R^2 for oxHDLlipids was higher in women (33.7%) than in men (17.0%). These findings may be related to the suggestion that the inverse association between HDL cholesterol and cardiovascular risk is stronger in women than in men (Gordon et al. 1977, Cooney et al. 2009). The

HDL-raising effect of estrogen may also partly explain these results (Cifkova, Krajcoviechova 2015).

6.3.3 PON1 and oxidized HDL lipids

Serum PON1 activity was negatively associated with oxLDLlipids suggesting that PON1 may have a role in the oxidation of LDL lipids. PON1 activity was, however, not associated with oxHDLlipids. It was also found that PON1 rs662 polymorphism is strongly associated with PON1 activity, but not with oxidized lipoprotein lipids.

PON1 has been presented as having a role in HDL antioxidant activity *in* vitro (Mackness, Arrol & Durrington 1991). In contrast, it has been shown *in vivo* that PON1 activity is associated with decreased HDL antioxidant capacity (Breton et al. 2014). We observed in the present study that PON1 activity and genotype were not associated with oxHDLlipids. There is thus no evidence that PON1 would have the ability to retard HDL oxidation *in vivo.* These facts are in line with the hypothesis that the oxidized lipid sequestering and transporting capacity of HDL may be an independent mechanism that explains the atheroprotective effects of HDL (Ahotupa et al. 2010).

The suggestion that PON1 is anti-atherogenic (Reddy et al. 2008) is mostly based on *in vitro* studies. The results from *in vivo* studies are, however, inconsistent. Some studies suggest that PON1 is atheroprotective (Bhattacharyya et al. 2008, Zhou et al. 2013) and others that higher PON1 activity is associated with an increased risk for atherosclerosis (van Himbergen et al. 2008, Birjmohun et al. 2009). The main atheroprotective mechanism of PON1 is suggested to be the capability to inhibit LDL oxidation (Mackness, Mackness 2013). These results are partly in line with our present study as we observed that higher PON1 activity is associated with lower levels of oxLDLlipids. A borderline significant association between PON1 activity and oxLDLprot was also found. A significant genotype interaction in the association between PON1 activity and oxLDLlipids is shown in this thesis, as there was a stronger inverse correlation in the G/G genotype (RR genotype) than in the A/A genotype (QQ genotype). This is in agreement with the findings that the effect of rs662 polymorphism on PON1 activity is substrate dependent (Adkins et al. 1993), and that oxidized lipids are hydrolyzed faster by the G/G genotype (RR genotype) than by the A/A genotype (QQ genotype) (Mackness et al. 1997).

We show in this study that higher PON1 activity is associated with an elevated cardiovascular risk profile characterized by higher LDL cholesterol levels, systolic blood pressure, and insulin levels; this finding in line with previous results (van Himbergen et al. 2008). Contradictory results were also observed as lower PON1 activity is described as being associated with diabetes and hypertension (Zhou et al.

2013). The results in our study may, in part, explain why the association between PON1 activity and cardiovascular risk seems to be currently controversial: consequently, it may support the results presenting that PON1 is not atheroprotective (van Himbergen et al. 2008) as PON1 seems to be associated with an elevated cardiovascular risk. This available evidence thus supports the hypothesis that measuring PON1 activity may not be useful in identifying patients with CVD risk (Macharia et al. 2014), and the fact that this subject, at least, needs further studies.

PON1 rs662 polymorphism has a strong effect on PON1 activity levels (Wheeler et al. 2004), which is in line with the results in this thesis. We found that subjects with A/A genotype (QQ genotype) had lower serum PON1 levels than those with G/A (QR) or G/G (RR) genotypes as presented earlier (Charles-Schoeman et al. 2013). There were no significant differences in CVD risk factors between PON1 groups stratified by rs662 genotypes except for age, which seems to support the hypothesis that PON1 rs662 is not associated with a risk for CVDs. In line with this, it has been shown that PON1 rs662 polymorphism is not associated with the risk for future coronary artery disease (Birjmohun et al. 2009) or with major adverse cardiovascular events (Tang et al. 2012).

6.3.4 Fatty liver disease and oxidized HDL lipids

In this study, we found that oxidized lipoprotein lipids associated significantly with the risk of future fatty liver disease, independently from any common risk factors for fatty liver. Higher levels of oxHDLlipids were associated with a lower risk for future fatty liver disease, and elevated oxLDLlipid levels were associated with a higher risk for fatty liver. The association between oxLDLprot and fatty liver was not significant after adjustment for the risk factors of fatty liver.

Oxidative stress may be involved in the pathogenesis of fatty liver (Chalasani, Deeg & Crabb 2004). In the present study, oxidized lipoprotein lipids were independently related to the risk of future fatty liver disease. However, it remains unclear whether the association between lipid oxidation markers and fatty liver is causative or a consequence of the fatty liver disease. Oxidatively modified HDL and LDL may thus precede the development of fatty liver or are present in the early phases of the pathogenesis based on our present findings.

Two different oxidation markers were used in this study i.e. oxLDLlipids and oxLDLprot. These two markers are closely linked to each other as 4-hydroxynonenal-mediated protein carbonylation is one of the important mechanisms in protein oxidation (Fritz, Petersen 2011). In this thesis, oxLDLlipids were directly associated with fatty liver, but there was no significant association between oxLDLprot and fatty liver. There was simultaneously a negative association between oxHDLlipids and fatty

liver. The different associations between fatty liver and oxLDLlipids and oxLDLprot may be explained by the impaired oxidized lipid transport function i.e. high levels of oxLDLlipids and low levels of oxHDLlipids. Such levels of oxidized lipoprotein lipids may indicate the reduced capacity of HDL to remove oxidized lipids from LDL, which may lead to accumulation of oxidized lipids in LDL and eventually to oxidation of Apo-B. It can, therefore, be hypothesized that early phases of lipid oxidation may play a role in the early-phases of fatty liver instead of the more chronic lipid oxidation, which may account for the differences found in these two methods measuring oxidized LDL. It can be hypothesized that the oxHDLlipids/oxLDLlipids ratio may be a potential marker to detect an impaired HDL oxidized lipid transport function and therefore elevated risk for fatty liver.

6.4 STRENGHTS AND LIMITATIONS

An important strength of this study is the population-based design in the Young Finns Study, which provided sufficient numbers of participants for statistical analyses. In addition, all the methods used including the laboratory and the physical and ultrasound examinations were properly established. The analytical method determining the baseline level of conjugated dienes in lipoprotein lipids was validated in detail and found suitable for its intended use (Ahotupa et al. 1998).

There are limitations in this thesis. It is a fact that the determination of lipoprotein oxidation is challenging. A baseline diene conjugation assay may recognize other lipoproteins than HDL and LDL with a similar composition. There are also problems concerning the oxLDLprot method: the preparation of identical antigens is, in practice, impossible and antibodies may recognize oxidized Apo-B in all Apo-B containing lipoproteins.

FAs were measured as proportions of the total amount and therefore the actual concentrations were not known: thus, it is a generally used approach to measure FAs. However, the FAs were measured from the serum total fraction, which accounts for both FA intake and metabolism and is more stable than the free fraction.

Paraoxon is not an endogenous substrate, but it is assumed to reflect the catalytic activity of PON1 (Tang et al. 2012).

There was, additionally, no information concerning the fatty liver status of the participants in 2001 and, as mentioned, ultrasonography is only a semi-quantitative method. It is noteworthy that studies I-III were cross-sectional and thus we may not have been able to include data on all confounding factors. However, a significant amount of variables were measured and the study population is relatively large.

6.5 CLINICAL IMPLICATIONS AND FUTURE RESEARCH PROSPECTIVES

The main atheroprotective functions of HDL are presently unclear. In this thesis we hypothesized that the ability of HDL to transport oxidized lipids could be an important risk-related mechanism in the atherogenesis, as it logically combines both the oxidation and reverse cholesterol transport theories. Therapies focusing on these HDL functional properties may provide, in the future, a strategy for addressing the residual cardiovascular risk in high-risk CVD patients.

Reliable determination of oxidized lipoproteins is demanding due to the complexities concerning the nature of the chemistry of lipoprotein oxidation. The results concerning lipoprotein oxidation should therefore be considered with caution, and the necessity acknowledged of the need to conduct more studies in relation to this topic. An issue for future research is a study focusing on lipoprotein oxidation in the light of lipoprotein particle composition and size. Mendelian randomization studies or interventions by pharmacological compounds, if available, could additionally be potentially relevant topics to focus on in the future. Results from these studies could assist in the confirmation that the measurement for oxidized lipoprotein lipids could be a novel biomarker for clinical use in the future. Based on available information, there is presently not sufficient data to confirm that the baseline diene conjugation method could be clinically useful instead of the traditionally measured cholesterol levels in CVD patients.

7. CONCLUSIONS

1. An elevated cardiovascular risk profile characterized primarily by advanced age is associated with lower oxHDLlipid levels in a population of young Finnish men and women.

2. In women, higher levels of oxHDLlipids were associated with a lower PUFA proportion and a higher MUFA proportion. Serum n6PUFA was inversely associated with oxLDLlipids. MUFA and SFA were directly associated with oxLDLlipid levels.

3. PON1 activity was inversely associated with oxLDLlipids levels, but it was not demonstrated that PON1 was associated with oxHDLlipids levels. Therefore, it can be suggested that PON1 may have a role in the oxidation of LDL lipids.

4. Oxidized HDL and LDL lipids are associated with the risk of developing fatty liver disease.

ACKNOWLEDGEMENTS

This study was carried out at the Research Centre of Applied and Preventive Cardiovascular Medicine, University of Turku.

This study was financially supported by the Academy of Finland, the Social Insurance Institution of Finland, the Aarne and Aili Turunen Foundation, Paavo Ilmari Ahvenainen Foundation, The Finnish Medical Foundation, Emil Aaltonen Foundation, University of Turku, Turku University Foundation, Kuopio, Tampere and Turku University Hospital Medical Funds, the Juho Vainio Foundation, the Paavo Nurmi Foundation, the Finnish Foundation of Cardiovascular Research, the Finnish Cultural Foundation, the Sigrid Juselius Foundation, the Tampere Tuberculosis Foundation and the Yrjö Jahnson Foundation

I owe my deepest gratitude to my supervisor, Professor Olli Raitakari. His excellent knowledge in the fields of medicine and statistics has been invaluable for this study. His challenging scientific questions have been a great value for my study. Similarly, I am also deeply grateful to my other supervisor, Professor Tommi Vasankari. His enormous knowledge, dedication and support have been essential for my study. I appreciate his fast and detailed responses whenever I needed comments on a manuscript. I also express my sincere thanks to Docent Markku Ahotupa. His expertise in the field of lipoprotein oxidation is incomparable. I am grateful for Docent Jari Kaikkonen. His expertise and help in writing publications have been invaluable. It has been a priviledge to work with these gentlemen.

I am grateful to Professor Jorma Viikari for the constructive comments and scientific advice during preparation of this thesis. Professor Markus Juonala is acknowledged for providing support and valuable comments.

The official reviewers, Docent Hannu Vanhanen and Docent Mustafa Atalay are warmly acknowledged for their valuable comments and constructive criticism, which improved the quality of this study.

This thesis would not have been possible without the work of several scientists during the previous decades in the Cardiovascular Risk in Young Finns Study. Former coordinators of the study are appreciated for their extensive work. I am very thankful for co-authors of the original publications in this thesis: Mika Ala-Korpela, Nina Hutri-Kähönen, Antti Jula, Antti Kangas, Mika Kähönen, Terho Lehtimäki, Vera Mikkilä, and Pasi Soininen for their important collaboration.

I wish to thank late Mervi Oikonen for encouraging comments, motivation, and for always being helpful. I warmly thank Irina Lisinen and Ville Aalto for their statistical advice and teaching me in data handling. I warmly thank Nina Ruotsalainen for being helpful whenever I needed assistance. I am grateful to the staff of the Research Centre of Applied and Preventive Cardiovascular Medicine for providing great work atmosphere. I wish to thank to the volunteer study subjects who made this study possible. Elizabeth Nyman is acknowledged for reviewing the language of this thesis.

I warmly thank the investigators of the Young Finns Study: Ari Ahola-Olli, Jonna Juhola, Tomi Laitinen, Noora Mattsson, Juha Koskinen, Olli Hartiala, Costan Magnussen, Paula Jääskeläinen, Lauri Vähämurto, Atte Voipio, Sonja Firtser, Joel Nuotio, Jarkko Heiskanen, Miia Lehtovirta, Mari Nupponen, Emmi Suomela, Juha Mykkänen, Suvi Rovio, Niina Pitkänen, Harri Helajärvi, and Saku Ruohonen for the happy company and countless inspiring discussions during these years.

Thank you to all my family members and friends. I owe special gratitude for Kaija, Igor and Pia for always being there for me. Finally, Paula and Olive, you are the most important peoples in my life.

Turku, September 2016

Petri Kresanov

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