

Jaana Leiviskä

Laboratory Diagnostics of Dyslipidemia

From Cholesterol to Apolipoproteins



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Jaana Leiviskä

**Laboratory Diagnostics of
Dyslipidemia
From Cholesterol to Apolipoproteins**

ACADEMIC DISSERTATION

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To my family

Abstract

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For decades cholesterol and low-density lipoprotein cholesterol (LDL-C) have been the cornerstones of cardiovascular risk assessment. The increasing prevalences in obesity, metabolic syndrome, and type 2 diabetes have challenged the diagnostics of dyslipidemia due to changed serum lipoprotein composition. The atherogenic dyslipidemia consists of decreased high-density lipoprotein cholesterol (HDL-C), increased triglycerides and small, dense low-density lipoprotein (sd-LDL) with increased number of apolipoprotein B (apoB) particles in serum. Apolipoprotein A-I (apoA-I) is the main apolipoprotein of HDL particles and apoB is the main apolipoprotein in LDL, intermediate-density lipoproteins (IDL), very-low density lipoproteins (VLDL), lipoprotein(a) [Lp(a)], and chylomicrons, respectively. The systematic errors in lipid and lipoprotein measurements may influence the accuracy of cardiovascular risk assessment and interpretations of population trends.

The first aim of this study was to assess the systematic errors in cholesterol, triglycerides, and HDL-C measurements. Secondly, the effect of fasting and non-fasting triglyceride values to the prevalences of high LDL-C and metabolic syndrome was estimated. The reference intervals for apoA-I, apoB, and apoB/apoA-I ratio were calculated and they were compared with the traditional lipid and lipoprotein concentrations in different pathophysiological conditions. Finally, two direct HDL-C methods and two turbidimetric apoA-I methods in two independent laboratories were also compared to discover the concordance between these methods.

The data for estimating accuracy of cholesterol, triglycerides, and HDL-C measurements was obtained from five different external quality assessment (EQA) programs, in which the Laboratory of Analytical Biochemistry at the National Institute for Health and Welfare has participated since 1978. The laboratory data for lipid and lipoprotein population trends was obtained from the FINRISK population-based health surveys during 1982 – 2012. The fresh serum samples for HDL-C and apoA-I method comparisons (n=413) were collected in the occupational health care laboratory.

The mean annual bias according to the EQAs for the cholesterol during 1978 – 2004 was -0.7 % (95% CI -0.9 to -0.6). For triglycerides the mean annual bias during 1978 – 2007 was -1.5 % (95% CI -2.3 to -0.8) and for HDL-C during 1982 – 2012 it was -2.3 % (95% CI -3.3 to -1.4).

The method comparison of two direct HDL-C methods showed a concentration-dependent difference between these methods. With low HDL-C concentrations (<1.0 mmol/l) difference between methods was negative -12.0 % (95% CI -13.5 to -10.0),

but with higher concentrations (>1.5 mmol/l) turned to positive: 9.0 % (95% CI 7.0 – 10.5).

We calculated conversion factors for transforming non-fasting triglyceride concentrations to true-fasting values according to the data from FINRISK studies in 1992, 1997, and 2002. The mean triglyceride concentration decreased 3.7 % for every fasting hour. Transforming non-fasting triglyceride values to true-fasting values increased the prevalence of high LDL-C (>3.0 mmol/l) from 51.3 % to 54.8 % and the prevalence of metabolic syndrome decreased from 39.7 % to 37.6 % in FINRISK 2007 study.

The reference intervals for apoA-I, apoB, and apoB/apoA-I ratio were calculated from FINRISK 2007 study. The therapeutic goal for apoB in both sexes was 0.9 g/l, which coincided with LDL-C concentration of 3.0 mmol/l. For apoA-I target values were >1.2 g/l in men and >1.4 g/l in women, which corresponded to HDL-C concentrations 1.0 mmol/l in men and 1.2 mmol/l in women. Obese men and women had the highest apoB concentrations and apoB/apoA-I ratios compared to the healthy reference group. Men with self-reported cardiovascular disease (CVD) or diabetes had lower apoB concentrations and apoB/apoA-I ratio than the averages in the healthy reference group, but on the contrary, in women apoB concentrations and apoB/apoA-I ratios were higher in groups with obesity, CVD, hypertension, or diabetes.

Participating in the external quality assessment programs with target values measured by the reference methods was essential, when interpreting the effects of the systematic errors on the population trends. The concentration-dependent differences in homogeneous HDL-C methods may cause misclassification in the risk assessment of cardiovascular disease. However, apoA-I methods showed better agreement than HDL-C in the method comparisons. ApoB measurements may produce more specific information in the risk assessment for CVD, especially in obesity, metabolic syndrome, and type 2 diabetes than total cholesterol and LDL-C measurements. Finally, the presented data supports the idea to incorporate apoA-I and apoB measurements to the national guidelines for assessment and treatment of dyslipidemia.

Keywords: dyslipidemia, cholesterol, triglycerides, HDL-C, LDL-C, apoA-I, apoB, systematic error, external quality assessment, homogeneous methods

Tiivistelmä

Jaana Leiviskä. Dyslipidemioiden laboratoriodiagnostiikka – kolesterolista apolipoproteiineihin. Terveyden ja hyvinvoinnin laitos (THL). Tutkimus 101. 120 sivua. Helsinki, Finland 2013.

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Vuosikymmenten ajan seerumin kokonaiskolesterolin ja low-density lipoproteiini (LDL)-kolesterolin määritykset ovat olleet sydän- ja verisuonitautien riskinarvioinnin kulmakiviä. Ylipainon, metabolisen oireyhtymän ja tyyppin 2 diabeteksen lisääntyminen suomalaisessa väestössä on muuttanut elimistön rasvaliukoisia yhdisteitä kuljettavien lipoproteiinien koostumusta, mikä on tuonut uusia haasteita dyslipidemioiden laboratoriodiagnostiikkaan. Aterogeenisessä dyslipidemiassa seerumin high-density lipoproteiini (HDL)-kolesterolin pitoisuus on alentunut, ja samanaikaisesti seerumin triglyseridipitoisuus sekä pienten, tiheiden LDL-partikkeleiden ja apolipoproteiini B:n määrä (apoB) on koholla. Apolipoproteiini A-I (apoA-I) on HDL-partikkeleiden tärkein apolipoproteiini ja apoB vastaavasti LDL-, IDL- (intermediate-density lipoproteiini), VLDL- (very low-density lipoproteiini) ja lipoproteiini(a)- [Lp(a)] partikkeleiden sekä kylomikronien tärkein apolipoproteiini. Systemaattiset virheet lipidi- ja lipoproteiiniäärityksissä voivat vaikuttaa sydän- ja verisuonitautien riskinarvioinnin tarkkuuteen ja väestötason muutosten tulkintaan.

Tutkimuksen tavoitteina oli arvioida systemaattisen virheen vaikutuksia kolesterolin-, triglyseridin- ja HDL-kolesterolimäärityksissä sekä paaston pituuden vaikutuksia triglyseridipitoisuuksiin ja korkean LDL-kolesterolin (> 3.0 mmol/l) ja metabolisen oireyhtymän esiintymiseen. Tavoitteena oli myös määrittää apoA-I:n, apoB:n ja apoB/apoA-I-suhteen viitearvot suomalaisessa väestössä ja verrata niitä kolesterolin-, LDL-kolesterolin-, HDL-kolesterolin- ja triglyseridipitoisuuksiin eri tautiryhmissä. Lopuksi verrattiin kahta suoraa HDL-kolesterolimenetelmää ja kahta immunoturbidimetristä apoA-I-menetelmää kahdessa eri laboratoriossa.

Aineistona kolesterolin-, triglyseridin- ja HDL-kolesterolimenetelmien systemaattisen virheen arviointiin käytettiin Terveyden ja hyvinvoinnin laitoksen (THL) analyttisen biokemian laboratorion (TLAB) viiden ulkoisen laadunarviointiohjelman tuloksia vuosilta 1978 – 2012. FINRISKI-tutkimusten lipidi- ja lipoproteiinituloksia vuosilta 1982 – 2012 käytettiin laboratoriotulosten systemaattisen virheen ja väestötasolla tapahtuneiden muutosten yhteisvaikutusten tulkinnassa. Kahden suoran HDL-kolesterolimenetelmän ja apoA-I-menetelmän vertailussa käytettiin yksityisen työterveyshuollon laboratorion kautta saatuja tuoreita seeruminäytteitä (n=413).

Keskimääräinen systemaattinen virhe kolesterolimäärityksissä ulkoisten laadunarviointikierron perusteella vuosina 1978 – 2004 oli -0.7 % (95 %:n luottamusväli -0.9, -0.6). Triglyseridimäärityksissä keskimääräinen systemaattinen

virhe vuosina 1978 – 2007 oli -1.5 % (-2.3, -0.8) ja HDL-kolesterolimäärityksissä vuosina 1982 – 2012 systemaattinen virhe oli -2.3 % (-3.3, -1.4).

Kahden suoran HDL-kolesterolimenetelmän vertailussa havaittiin konsentraatio-riippuvainen ero: matalilla HDL-kolesterolipitoisuuksilla (<1.0 mmol/l) menetelmien välinen ero oli negatiivinen -12.0 % (-13.5, -10.0), mutta korkeammilla (>1.5 mmol/l) HDL-kolesterolipitoisuuksilla ero oli keskimäärin +9.0 % (7.0, 10.5).

Jotta FINRISKI-tutkimuksissa käytettävän neljän tunnin paaston vaikutukset triglyseridipitoisuuksiin voitaisiin arvioida, kehitettiin muunnoskertoimet täydellisen paaston jälkeisen triglyseridipitoisuuden laskemiseksi vuosien 1992, 1997 ja 2002 FINRISKI-tutkimusten triglyseriditulosten perusteella. Seerumin triglyseridipitoisuus laski keskimäärin 3.7 prosentilla jokaisen paastotunnin aikana. Alkuperäisten triglyseridikonsentraatioiden muuntaminen paastoarvoiksi FINRISKI 2007 -aineistossa nosti korkean LDL-kolesterolin (>3.0 mmol/l) esiintymisosuuden 51.3 prosentista 54.8 prosenttiin. Metabolisen oireyhtymän esiintyminen FINRISKI 2007 -aineistossa puolestaan väheni 39.7 prosentista 37.6 prosenttiin.

ApoA-I:n, apoB:n ja apoB/apoA-I suhteen viitearvot laskettiin FINRISKI 2007 -aineistosta. ApoB:n tavoitearvoksi määritettiin sekä miehille että naisille 0.9 g/l, joka vastasi LDL-kolesterolipitoisuutta 3.0 mmol/l. ApoA-I:n tavoitearvo miehille oli 1.2 g/l ja naisille 1.4 g/l. Lihavilla miehillä ja naisilla oli korkeimmat apoB-pitoisuudet ja korkeimmat apoB/apoA-I suhteet verrattuna terveeseen viitearvoaineistoon. Niillä miehillä, joilla oli todettu sydän- ja verisuonitauti tai diabetes, oli matalammat apoB-pitoisuudet ja apoB/apoA-I suhde kuin terveillä, mutta naisilla tilanne oli päinvastainen.

Osallistuminen ulkoisen laadunarvioinnin kierroksiin, joissa käytetään referenssimenetelmiin jäljitettäviä tavoitearvoja, on välttämätöntä, jotta väestötason muutoksia pystytään tulkitsemaan oikein. Suorissa HDL-kolesterolimenetelmissä havaitut konsentraatiosta riippuvaiset tulostasoerot voivat aiheuttaa virheellisiä tulkintoja sydän- ja verisuonitautien riskinarvioinnissa. ApoA-I-menetelmissä vastaavaa eroa ei havaittu. ApoB:n määrittelyllä voidaan saada tarkempaa tietoa sydän- ja verisuonitautiriskistä kuin kolesteroli- tai LDL-kolesterolimäärityksillä erityisesti ylipainoisilla, metabolista oireyhtymää sairastavilla tai tyypin 2 diabeetikoilla. ApoA-I- ja apoB-määritykset olisivatkin syytä lisätä Dyslipidemioiden Käypä hoito –suositukseen.

Avainsanat: Dyslipidemia, kolesteroli, triglyseridit, HDL-kolesteroli, LDL-kolesteroli, apoA-I, apoB, systemaattinen virhe, ulkoinen laadunarviointi, kolesterolimääritykset

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List of original publications

This thesis is based on the following original articles referred to in the text by their Roman numerals I-V.

- I Sundvall J, Leiviskä J, Alfthan G, Vartiainen E. Serum cholesterol during 27 years: Assessment of systematic error and affecting factors and their role in interpreting population trends. *Clin Chim Acta* 2007;378:93-8.
- II Sundvall J, Laatikainen T, Hakala S, Leiviskä J, Alfthan G. Systematic error of serum triglyceride measurements during three decades and the effect of fasting on serum triglycerides in population studies. *Clin Chim Acta* 2008;397:55-9.
- III Sundvall J, Leiviskä J, Laatikainen T, Peltonen M, Salomaa V, Vanhala M, Korpi-Hyövälti E, Lauronen J, Alfthan G. The use of fasting vs. non-fasting triglyceride concentration for estimating the prevalence of high LDL-cholesterol and metabolic syndrome in population surveys. *BMC Med Res Methodol* 2011;11:63.
- IV Leiviskä J, Sundvall J, Alfthan G, Jauhiainen M, Salomaa V. Apolipoprotein A-I, apolipoprotein B, and apolipoprotein B/apolipoprotein A-I ratio: Reference intervals compared with values in different pathophysiological conditions from the FINRISK 2007 study. *Clin Chim Acta* 2011;412:1146-50.
- V Leiviskä J, Sundvall J, Alfthan G, Tähtelä R, Salomaa V, Jauhiainen M, Vartiainen E. What have we learnt about high-density lipoprotein cholesterol measurements during 32 years? Experiences in Finland 1980 – 2012. *Clin Chim Acta* 2013;415:118-123.

The original publications have been reproduced with the kind permission of their copyright holders. In addition, some unpublished data is presented.

Abbreviations

ABCA-I	ATP binding cassette transporter A-I
ApoA-I	Apolipoprotein A-I
ApoB	Apolipoprotein B
ATP	Adult treatment panel
CDC	Centers for Disease Control and Prevention
CETP	Cholesterol ester transfer protein
CHD	Coronary heart disease
CVD	Cardiovascular disease
DCM	Designated comparison method
DS	Dextran sulphate
EAS	European Atherosclerosis Society
EQA	External quality assessment
ESC	European Society of Cardiology
GC-IDMS	Gas chromatography-isotope dilution mass spectrometry
GPO	Glycerol phosphate oxidase
HDL	High-density lipoprotein
HDL-C	High-density lipoprotein cholesterol
HL	Hepatic lipase
IDL	Intermediate-density lipoprotein
LCAT	Lecithin-cholesterol acyltransferase
LDL	Low-density lipoprotein
LDL-C	Low-density lipoprotein cholesterol
Lp(a)	Lipoprotein(a)
LRP	LDL receptor-related protein
LPL	Lipoprotein lipase
MetS	Metabolic syndrome
MI	Myocardial infarction
NCEP	National cholesterol education program
Non-HDL-C	Non-high-density lipoprotein cholesterol
NMR	Nuclear magnetic resonance
PEG	Polyethylene glycol
PLTP	Phospholipid transfer protein
PTA	Phosphotungstic acid
RCT	Reverse cholesterol transport
Sd-LDL	Small, dense low-density lipoprotein
Sd-LDL-C	Small, dense low-density lipoprotein cholesterol
TG	Triglycerides
VLDL	Very low-density lipoprotein

1 Introduction

Cardiovascular disease (CVD) is still the leading cause for death in the developed countries and with globalized Western lifestyle these diseases are becoming more common also in the developing countries (Catapano et al. 2011). The basic risk factors for CVD are hypercholesterolemia, smoking, male gender, and hypertension, but increasing prevalences of obesity, metabolic syndrome (MetS), and type 2 diabetes can multiply the risk. The National Cholesterol Education Program (NCEP) Adult Treatment Panel (ATP) III in the US and the European Society of Cardiology (ESC) together with the European Atherosclerosis Society (EAS) have developed guidelines for the risk assessment and treatment of cardiovascular disease (Grundyl et al. 2004, Perk et al. 2012). Laboratory measurements to diagnose dyslipidemia – increased cholesterol, low-density lipoprotein cholesterol (LDL-C), or triglycerides concentrations, or decreased high-density lipoprotein cholesterol (HDL-C) concentration – have been a part of these guidelines since the beginning. The standardization and accuracy of analytical methods and the appropriate screening tools have been vital for the clinical laboratories to identify the relevant people in the risk.

The latest guidelines are based on the use of LDL-C as a primary target of prevention and treatment for CVD. The secondary target is non-HDL-C especially for hypertriglyceridemic subjects. Hypertriglyceridemia is a common feature in obesity, MetS, and type 2 diabetes and it belongs also to the atherogenic dyslipidemia. The other components of atherogenic dyslipidemia are low HDL-C and an increased number of small, dense low-density lipoprotein (LDL) particles (sd-LDL) (Austin et al. 1990). The common nutritional background in this condition has revealed to be a low-fat but high-carbohydrate diet (Volek et al. 2008). The liver produces apolipoprotein B (apoB)-containing very low-density lipoprotein particles from the extra free fatty acids and carbohydrates after their change to triglycerides to deliver cholesterol and triglycerides to the extrahepatic tissues.

Atherogenic lipoprotein particles consist of chylomicron remnants, very low-density lipoproteins (VLDL), VLDL remnants, intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), and lipoprotein(a), Lp(a). Chylomicrons deliver lipids from the diet, but the other atherogenic lipoproteins are derived from the liver and metabolized from the VLDL particles. All these particles contain one nonexchangeable apoB molecule and different composition of free cholesterol, cholesterol esters, phospholipids, triglycerides and other apolipoproteins. The measurement of apoB is a better estimate of the atherogenic lipoprotein particles than LDL-C concentration, which varies according to the size of LDL (Contois et al. 2009). The LDL-C concentration can be normal or even low in obesity, MetS, and

type 2 diabetes, resulting in the underestimation of CVD risk in these conditions (Sniderman et al. 2006).

Recently, the specificity of direct HDL-C and LDL-C methods has been challenged (Miller et al. 2010). HDL-C measurements are used to calculate LDL-C by the Friedewald formula and for non-HDL-C determination, which are the primary and the secondary targets for the prevention and treatment of CVD, respectively. The method-dependent differences in direct HDL-C and LDC-C measurements may impact on the accuracy of the risk assessment in the clinical practice.

The first aim of this study was to assess the systematic errors in the serum cholesterol, triglyceride, and HDL-C measurements during four decades in the Laboratory of Analytical Biochemistry (TLAB) at the National Institute for Health and Welfare; and the second aim was to assess the effect of systematic error to the interpretations of population trends in the FINRISK health examination studies. The third aim was to compare the use of fasting and non-fasting triglyceride values in population studies and when calculating the prevalences of high LDL-C or MetS. We also compared two direct HDL-C methods and two turbidimetric apolipoprotein A-I (apoA-I) methods in two separate laboratories to discover the concordance between these methods. Furthermore, the reference intervals for apoA-I, apoB, and apoB/apoA-I ratio were calculated and these reference intervals were compared with values in different pathophysiological conditions in the FINRISK 2007 study.

2 Review of the literature

2.1 Risk assessment of cardiovascular disease

For decades total cholesterol and LDL-C have been the primary targets for prevention and treatment of cardiovascular disease, but in 1993 NCEP launched new guidelines in ATP II, which recommended the addition of HDL-C measurements with total cholesterol to screen coronary heart disease (CHD) (Summary of the second report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel II 1993)). ATP III increased emphasis on the risk of high triglycerides by decreasing the cut-off value for normal triglycerides to 1.7 mmol/l and introduced non-HDL-C as a new secondary marker for hypertriglyceridemic patients (Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults 2001). In addition, a lower cut-off value for LDL-C (2.58 mmol/l) was recommended as a treatment goal for people already having CHD. ATP III recommended also screening with the fasting lipid profile: total cholesterol, LDL-C, HDL-C, and triglycerides. NCEP ATP is working on the next report (ATP IV) with the aim to simplify the recommendations for clinical practice. According to the latest evidence, the ideal LDL-C target for the high-risk patients should be as low as 1.8 mmol/l and the use of non-HDL-C and apoB are recommended for the follow-up of drug treatment (Martin et al. 2012).

The Finnish treatment goals shown in Table 1 are based on the European guidelines on cardiovascular disease prevention by the European Society of Cardiology (Graham et al. 2007). The Fifth Joint Task Force (JTF) of the European Societies on Cardiovascular Disease Prevention in Clinical Practice has updated the recommendations based on the latest scientific knowledge about cardiovascular disease risk assessment (Perk et al. 2012). Specific treatment goals for HDL-C have not been defined, but values under 1.0 mmol/l in men and 1.2 mmol/l in women increase risk for CVD. Treatment goal for triglycerides is the same <1.7 mmol/l as in the ATP III guidelines. According to these guidelines, apoB and apoB/apoA-I ratio do not add any further information to the CVD risk assessment, but they can be measured when available.

Table 1. Treatment goals for cholesterol, LDL-C, triglycerides, and HDL-C in Finland (Graham et al. 2007).

Target	Goal in normal population (mmol/l)	Goal for high risk subjects (mmol/l)
Cholesterol	< 5.0	<4.0 – 4.5
LDL-C	<3.0	<2.0 ^a – 2.5
Triglycerides	<2.0	<2.0
HDL-C	>1.0	>1.0

^a For the highest risk subjects LDL-C<1.8 mmol/l is recommended

2.2 Lipids

Lipids consist of the diverse groups of molecules, which are nearly insoluble in water, but soluble in organic solvents. The most important lipids in the human body are cholesterol and cholesterol esters, fatty acids, triglycerides, glycerophospholipids, sphingolipids, bile acids, steroid hormones, and fat-soluble vitamins. Lipids are structural components in biomembranes (glycerophospholipids, sphingolipids, and cholesterol); they function as hormones, precursors of hormones, signaling molecules, metabolic fuel (fatty acids), or energy storage (triglycerides), and aid in digestion of fat (bile acids) (Vance and Vance 2002).

Cholesterol is an essential molecule for maintenance of membrane fluidity and permeability, and it serves as a precursor of bile acids, steroid hormones and vitamin D. Cholesterol homeostasis in the cells is maintained by the dietary absorption, de novo synthesis, metabolism to bile acids and sterol hormones, and excretion as bile acids (Hu et al. 2010). All human cells are capable of synthesizing cholesterol from acetate, which can be originated from the metabolism of carbohydrates or fats, but the liver and intestine are the main organs for de novo synthesis of cholesterol (Vance and Vance 2002, Blom et al. 2011). Dietary lipids and endogenous lipids synthesized by the liver and extrahepatic tissues are transported in the circulation with apolipoproteins as lipoprotein complexes (Grundy 2011).

2.3 Lipoproteins

The classification of lipoproteins has traditionally been based on the different density of lipoprotein particles separated by ultracentrifugation. The main classes of lipoproteins are chylomicrons, very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), lipoprotein(a) [Lp(a)], and high-density lipoproteins (HDL)(Mahley et al. 1984). In spite of possible contaminations with adjacent lipoprotein fractions, ultracentrifugation has remained

as a golden standard for lipoprotein research. According to their electrophoretic mobility, lipoproteins are classified to α , pre β , and β lipoproteins that are HDL, VLDL, and LDL classes, respectively. Also chromatographic methods based on the size of lipoprotein particles or their immunoaffinity have been used for separation of different lipoprotein subclasses. High-resolution nuclear magnetic resonance (NMR) spectroscopy is a new technique to separate lipoprotein subclasses according to their different size (Langlois and Blaton 2006). Some of the small-size HDL and LDL particles are particularly atherogenic, therefore the possibility to separate these subclasses and estimate their proportion in the whole particle population could improve the risk assessment of CVD (Rizzo and Berneis 2006a, Movva and Rader 2008).

Because lipoproteins are a heterogeneous group of particle populations differing in density, size, and lipid compositions, the other alternative to classify them is according to their major apolipoproteins (Mahley et al. 1984). All other lipoproteins except HDL contain apoB-48 (chylomicrons) or apoB-100 (VLDL, IDL, LDL, Lp[a]), which makes it possible to assess the amount of atherogenic lipoprotein particles in the circulation by the apoB measurement. The major apolipoproteins of HDL particles are apoA-I and apoA-II. Apolipoproteins A-I, A-II, C-I, C-II, C-III, and E can exchange between lipoproteins during the metabolism whereas apoB-48 and apoB-100 are non-exchangeable. More details of lipoproteins' characteristics are shown in Table 2.

All lipoproteins share a similar particle structure: phospholipids, unesterified cholesterol, and apolipoproteins are on the surface and triacylglycerols, cholesterol esters and other neutral lipids are situated in the core of the particle. Apolipoproteins determine the metabolism of lipoproteins: they direct lipoprotein transport and redistribution between tissues, function as cofactors for a variety of enzymes, and maintain the structure of lipoproteins (Mahley et al. 1984).

2.4 Metabolism of lipoproteins

Lipoproteins transport lipids from the intestine and liver to the peripheral tissues. During their metabolism lipoproteins exchange apolipoproteins with each other and modify their lipid composition with the exception of apoB-48 and apoB-100 (Vance and Vance 2002). The role of VLDL and LDL particles is to deliver cholesterol, triglycerides, and other lipids to peripheral tissues in a process called forward cholesterol transport. Instead, HDLs transport excess cholesterol from the peripheral cells back to the liver in a process called reverse cholesterol transport (RCT) (Lewis and Rader 2005). Finally, all lipoproteins are catabolised in the liver, kidney or peripheral tissues via apolipoprotein-receptor-interactions or other mechanisms (Bamba and Rader 2007).

Table 2. The density, diameter, and apolipoprotein composition of lipoproteins.

Lipoprotein class	Density (kg/l)	Diameter (nm)	Apolipoproteins
Chylomicrons	<0.94 ^a	80 – 1200 ^d	B-48 , C-I, C-II, C-III, E
Very low-density lipoprotein	0.94 – 1.006 ^a	30 – 80 ^d	B-100 , C-I, C-II, C-III, E
Intermediate-density lipoprotein	1.006 – 1.019 ^c	23 – 35 ^d	B-100 , C-II, C-III, E
Low-density lipoprotein	1.006 – 1.063 ^a	18 – 25 ^d	B-100
Small dense low-density lipoprotein	1.050 – 1.063 ^e	22 – 25.5 ^f	B-100
Lipoprotein(a)	1.040 – 1.130 ^h	25 – 30 ^h	Apo(a) , B-100
High-density lipoprotein, total	1.063 – 1.210 ^a	5 – 12 ^d	A-I , A-II, C-II, C-III, E
High-density lipoprotein HDL ₂	1.063 – 1.125 ^b	9 – 12 ^g	A-I , A-II, C-II, C-III, E
High-density lipoprotein HDL ₃	1.125 – 1.210 ^b	6 – 9 ^g	A-I , A-II, C-II, C-III, E

^a (Vance and Vance 2002), ^b (Langlois and Blaton 2006), ^c (Nauck 2002), ^d (Contois et al. 2011),

^e (Campos et al. 1992), ^f (Sacks and Campos 2003), ^g (Betteridge et al. 1999), ^h (Burtis et al. 2008)

2.4.1 Chylomicrons and very low-density lipoproteins

Chylomicrons are secreted from the intestine after a fat-containing meal with apolipoprotein B-48 as a major protein component. They deliver fat as an energy source for muscles or to adipose tissue for storage. Chylomicrons contain also apoC-I, apoC-II, apoC-III, and apoE. For lipoprotein lipase (LPL) to function properly, they receive more apoC-II from HDL. After lipolysis of triglycerides with LPL, chylomicron remnants containing most of their cholesterol are taken up by the liver via LDL receptor pathway or LDL receptor-related protein (LRP), which both use apoE as a ligand on chylomicron remnant particle (Fujioka and Ishikawa 2009).

The liver uses free fatty acids from the circulation and excess carbohydrates, fat, and proteins from the meal to synthesize triacylglycerides, which form very low-density lipoproteins (VLDL) together with apolipoprotein B-100, cholesterol and other lipids. ApoB synthesis is highly regulated by the availability of core lipids, the type of dietary fatty acids, and by the hormonal balance (Ginsberg and Fisher 2009).

2.4.2 Low-density lipoprotein

The end-point of VLDL-metabolism is LDL, which is the most cholesterol-enriched (majority cholesterol esters) lipoprotein particle in the blood circulation. LDL is responsible for constant supply of essential cholesterol for tissues and cells. The only protein in LDL particles is apoB-100, which is derived from the VLDL and does not exchange between other lipoproteins during metabolism (Prassl and Laggner 2008). The LDL population is subdivided into large, medium, small, and very small particles (Rizzo and Berneis 2007). The prevalence of small particles varies with age, sex, genetic factors, and diet being 30 – 35 % in adult men, 5- 10 % in men under 20 years and premenopausal women, and 15 – 25 % in postmenopausal women (Rizzo and Berneis 2006b). LDL is endocytosed into the cells via the LDL or B/E receptors, or by the scavenger receptor pathway. Triglyceride-rich VLDL remnants are transformed in some cases to small, dense LDL (sd-LDL) particles, which are more atherogenic than the buoyant, large ones. Small LDL particles have lower affinity to normal LDL receptors, but increased binding to proteoglycans in arterial intima. This receptor-independent retention of LDL in subendothelial space overrides the normal regulation of cholesterol metabolism leading to the accumulation of cholesterol in extrahepatic tissues (Galeano et al. 1998).

LDL can be separated into phenotype A with large, buoyant particles and to phenotype B with small, dense particles. The proportion of phenotype B increases with increasing triglyceride levels and decreased levels of HDL-C. This lipid combination has been defined as atherogenic lipoprotein phenotype and is a typical feature connected to MetS (Austin et al. 1990). These small LDL particles are more easily oxidized; one possible reason could be the increased content of polyunsaturated fatty acids (Rizzo and Berneis 2006b).

2.4.3 High-density lipoprotein

High-density lipoproteins (HDL) are secreted from the liver (70 %) or intestine (30 %) as lipid-poor nascent particles that contain only apolipoprotein A-I and phospholipids. The maturation of HDL from nascent discoidal particle to larger, spherical HDL demands the action of lecithin-cholesterol acyltransferase (LCAT), which converts free cholesterol to cholesterol esters (Scanu and Edelstein 2008). These cholesterol esters are partly exchanged with triglycerides from apoB-containing lipoproteins via cholesterol ester transfer protein (CETP) function before transport to the liver for catabolism as a part of reverse cholesterol transport (Lewis and Rader 2005). HDLs are further divided into larger, cholesterol ester-rich HDL₂ particles and smaller HDL₃ particles (Movva and Rader 2008). Other details on interconversion of HDL, LDL, and VLDL are shown in Figure 1. Excess carbohydrates and sugar in the diet may cause endogenous hypertriglyceridemia, which activates CETP and hepatic lipase (HL) leading to formation of small-size HDL particles (Gou et al. 2005).

In addition to apolipoproteins A-I and A-II, HDL contains several other lipoproteins, enzymes, and other components, which are essential for its antiatherogenic, anti-inflammatory and antiapoptotic properties (Movva and Rader 2008, Gordon et al. 2011). When fasting, apolipoproteins C-I, C-II, and C-III are mainly associated with HDL, but they are exchanged to the surface of chylomicrons or VLDL after fat-containing meal. After the lipolysis of triglycerides in chylomicrons and VLDL, these apolipoproteins are recycled back to HDL pool.

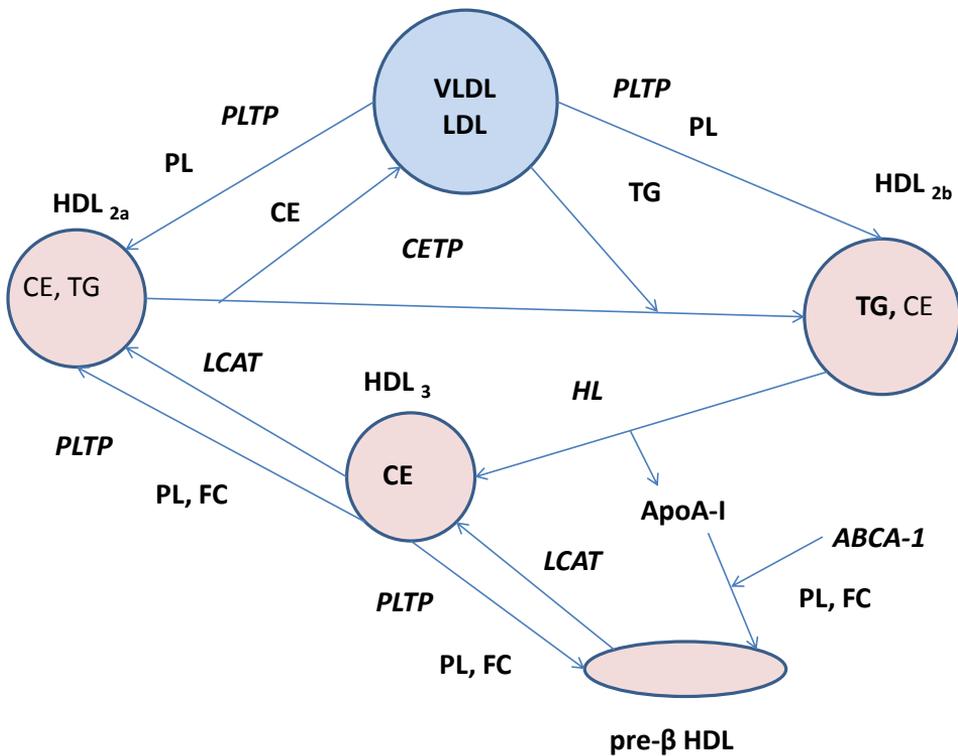


Figure 1. The metabolism of HDL particles in connection to LDL and VLDL (modified from Langlois and Blaton 2006). ABCA-1 = ATP binding cassette transporter A-1, CE = cholesterol esters, CETP = cholesterol ester transfer protein, FC = free cholesterol, HL = hepatic lipase, LCAT = lecithin-cholesterol acyltransferase, PLTP = phospholipid transfer protein, PL = phospholipids, TG = triglycerides

2.4.4 Atherosclerosis

Early signs of atherosclerosis are characterized by excessive apoB-containing lipoproteins – chylomicron remnants, VLDL remnants, LDL, or Lp(a) – accumulation in the subendothelial space of the arterial wall (Ginsberg and Fisher 2009). Under normal conditions, excess cholesterol is removed from subendothelial space via RCT. When the amount of atherogenic particles exceeds the RCT capacity of HDL particles, cholesterol starts to accumulate into monocyte-derived macrophages leading to the formation of foam cells (Stancu et al. 2012). The risk of CVD is directly associated to total cholesterol or LDL-C concentrations and inversely to the HDL-C concentration. However, high LDL-C is only one of the predicting factors for atherosclerosis; the increasing amount of sd-LDL is even more powerful risk factor (Superko 1996).

Raising the HDL-C by CETP-inhibition has clearly demonstrated that the mechanistic increase of total HDL-C does not necessarily lead to the risk reduction of CVD (Landmesser et al. 2012). The functionality of HDL in the reverse cholesterol transport and in the other atheroprotective mechanisms, including the stimulation of endothelial nitric oxide production and antioxidant activation, is more important than simply the HDL-C concentration in the circulation (Bandeali and Farmer 2012).

2.5 The role of obesity and metabolic syndrome

MetS is defined as a combination of obesity, hypertriglyceridemia, hyperglycemia, hypertension, and low HDL-C. Other features of MetS are endothelial dysfunction, insulin resistance, and proinflammatory and prothrombotic states. Various organizations have set different definitions for MetS with slightly different cutpoints (Alberti et al. 2009).

When energy supply exceeds the energy consumption, excess energy is stored into adipose tissue in a form of triglycerides. The first route to adipose tissue is directly from a fat-containing meal transported by chylomicrons. The second route is through the liver, where excess carbohydrates, proteins and free fatty acids are metabolized into triglycerides and packed to VLDL particles (Verges 2010). Triglyceride-rich VLDL particles are transformed into sd-LDL particles, which contain less cholesterol than normal, more buoyant LDL. A low-fat, high-carbohydrate diet has been revealed to be an important dietary factor behind atherogenic dyslipidemia triad: high triglycerides, low HDL-C, and increased small, dense LDL particles (Krauss 2001). This atherogenic triad is common for patients having CAD, MetS, insulin resistance, type 2 diabetes, and non-coronary forms of atherosclerosis (Carmena et al. 2004, Avramoglu et al. 2006, Bamba and Rader 2007, Rizzo and Berneis 2007, Choi and Ginsberg 2011). Sd-LDL particles have been shown to predict cerebro- and cardiovascular events in the future especially in connection with MetS (Rizzo et al. 2008).

2.6 Laboratory diagnostics of dyslipidemia

For decades the risk assessment for cardiovascular diseases has been based on the total serum cholesterol and calculated LDL-C by the Friedewald formula. After introducing HDL-C as a secondary treatment target in NCEP's guidelines, the precipitation-based assays for HDL-C have been replaced by less tedious homogenous methods. For precise and accurate measurements of cholesterol, HDL-C, and triglycerides, NCEP has introduced performance goals for laboratories (Table 3). To meet these performance goals, clinical laboratories should use methods which are traceable to the CDC reference methods.

Table 3. Performance goals according to NCEP recommendations (Warnick et al. 2002).

Lipid	Bias ^a	CV ^b	Total error
Cholesterol	3 %	3 %	9 %
HDL-C	5 %	4 % ^c	13 %
LDL-C	4 %	4 %	12 %
Triglycerides	5 %	5 %	15 %

^a Bias from the reference value, ^b Correlation of variation., ^c HDL-C \geq 1.1 mmol/l; when HDL-C < 1.1 mmol/l, SD < 0.044 mmol/l

The Centers for Disease Control and Prevention (CDC) has organized the Lipid Standardization Program since 1950s for total cholesterol, triglycerides, HDL-C, LDL-C, and recently also for apoA-I and apoB measurements. CDC also maintains the Cholesterol Reference Method Laboratory Network (CRMLN), which assists diagnostics manufacturers in validating their calibrators (Warnick et al. 2002).

2.6.1 Measurement of cholesterol

For fifty years CDC's reference method for cholesterol measurements has been a modification of Abell-Kendall colorimetric method (Abell et al. 1952). First cholesterol esters are released with alcoholic potassium hydroxide by saponification from lipoproteins. After hexane extraction, Liebermann-Burchard reagent is used to form a chromophore for colorimetric measurement (Zak 1977). Liebermann and Burchard discovered in 1890s that a mixture of sulfuric acid and acetic acid with acetic anhydride produced a characteristic colour with cholesterol in gallstones. Unfortunately, colorimetric methods with strong acids are not easily standardized and they are not well-suited for automated clinical chemistry analyzers. However,

for ninety years cholesterol measurements were based on this hazardous technology in clinical laboratories (Rifai et al. 2004).

The next step in the evolution for analyzing cholesterol in serum was the discovery of specific enzymatic reagents in the early 1970s (Zak 1977, Rifai et al. 2004). Cholesterol is distributed in serum between different lipoprotein fractions as free cholesterol and in esterified form. Thus, first cholesterol esters must be hydrolyzed by cholesterol esterase enzymes to receive all cholesterol available for analysis. In the next phase, cholesterol is oxidized to cholestenone and hydrogen peroxide is liberated. This hydrogen peroxide is then quantified usually in a reaction producing a chromophore.

The concern about the comparability of cholesterol measurements emerged already in the 1950s and CDC established a cholesterol methodology development laboratory and standardization office in the end of 1950s (Rifai et al. 2004). Since then CDC has organized a Lipid Standardization Program for laboratories and reagent manufacturers. CRMLN has maintained the Abell-Kendall cholesterol reference method, which has been standardized by CDC. Recently, a new gas chromatography-isotope dilution mass spectrometry (GC-IDMS) procedure has been proposed to replace the former colorimetric reference method (Edwards et al. 2011).

2.6.2 Measurement of triglycerides

Most of the serum triglycerides (TG) are in triglyceride-rich lipoproteins: chylomicrons and VLDL. Triglycerides are measured as the amount of glycerol in serum, so also mono- and diglycerides are included. The first in-house method for triglyceride measurements was introduced in 1966 at CDC (Stein and Myers 1995). First the serum lipids were extracted with chloroform and then phospholipids and free glycerol were removed by silicic acid. An aliquot of the sample was saponified and the released glycerol was oxidized to formaldehyde. Finally, formaldehyde was reacted with chromotropic acid to form a chromogen, whose absorbance can be measured.

Since 1970s enzymatic methods have replaced chemical methods in clinical laboratories. In the first step triglycerides are hydrolyzed to glycerol and fatty acids by lipase. Glycerol is then transformed to glycerol-3-phosphate with release of adenosine 5-diphosphate (ADP), which both can be used for measuring triglycerides. Most of the TG assays use glycerol phosphate oxidase (GPO) to generate hydrogen peroxide, which is reacted with peroxidase to give a red dye. Free glycerol in specimen may cause erroneous results in both chemical and enzymatic methods. To avoid the use of obsolete instrumentation and toxic chemicals CDC has developed a new reference method procedure by isotope dilution GC-MS (ID-GC-MS) (Edwards et al. 2012).

Blood samples for triglyceride measurements should be taken after at least a nine hours fasting in order to avoid chylomicrons in the sample, which may increase TG

concentration. In the Friedewald equation it is assumed that most of the triglycerides are in the VLDL fraction so when post-prandial chylomicrons are abundant, this equation should not be used (Friedewald et al. 1972).

2.6.3 Measurement of HDL-C

High-density lipoproteins (HDL) are a heterogeneous group of lipoproteins that are the densest and smallest of all lipoprotein particles. These physical and chemical characteristics have been used to separate HDL particles from the other apoB-containing lipoproteins (Langlois and Blaton 2006). The combination of apolipoproteins in HDL particles has an impact on the measurement of HDL-C: the HDL subclass enriched with apoE may or may not be included in the concentration of HDL-C in different methods (Warnick et al. 2001). ApoE-rich HDL being larger and less dense than other HDL particles precipitate together with apoB-containing particles, when dextran sulphate is used, but not with heparin/manganese (Gibson et al. 1984).

First generation HDL-C methods used polyanions with or without divalent cations to precipitate all other lipoproteins except HDL (Finley et al. 1978). After a centrifugation step, cholesterol was analyzed from the supernatant. The early applications used heparin with $MnCl_2$ for precipitation, but this combination emerged to be inadequate for proper measurements of HDL-C. The next methods used dextran sulphate or phosphotungstic acid (PTA) in combination with Mg^{2+} ions. A major interfering issue with precipitation techniques has been the increased amount of triglycerides in lipoproteins, which make them lighter and cause incomplete precipitation.

When HDL-C measurements became more common in clinical chemistry laboratories in 1990s, attempts to eliminate the tedious precipitation step led to second-generation methods. One of these methods used encapsulated iron beads linked to dextran sulphate to separate apo B-containing lipoproteins from HDL by a magnet. When compared to precipitation methods, a small negative difference to the dextran sulphate method and a positive difference to PTA method were observed (Warnick et al. 2001).

The third generation HDL-C assays are homogeneous, direct methods without a precipitation step. The first innovations used polyethylene glycol to separate apo B-containing chylomicrons, VLDL, and LDL particles from HDL particles. The next finding was to introduce sulphate α -cyclodextrin together with Mg^{2+} for blocking chylomicrons and VLDL particles (Sugiuchi et al. 1995). After this step, polyethylene glycol (PEG)-modified enzymes reacted with cholesterol in HDL particles leaving larger LDL particles intact. Other homogeneous assays have used antibodies against apoB and apoC, synthetic detergents, or selective enzymes which react only with cholesterol in other lipoproteins than HDL. One example of the latter modification of homogeneous methods utilizes an accelerator for the reaction of

cholesterol oxidase with non-HDL cholesterol. In the other reaction a specific detergent solubilizes specifically HDL particles for the cholesterol measurement. These commercial homogeneous assays have improved the precision via the better repeatability in pipetting and better control of the reaction temperature and timing, but specificity on specimens with unusual lipoproteins is still a problem (Miller et al. 2010).

The CDC's reference method for HDL-C combines ultracentrifugation of chylomicrons and VLDL particles with precipitation of apoB-containing lipoproteins by heparin and $MnCl_2$ (Warnick et al. 2001). Because few laboratories have possibilities for ultracentrifugation, CRMLN developed a designated comparison method (DCM) without the ultracentrifugation step (Kimberly et al. 1999). This method is based on dextran sulphate (50 000 kDa) and $MgCl_2$ as a precipitation reagent.

NCEP has set the analytical performance goal for measuring HDL-C with $\leq 5\%$ bias from the reference value (Warnick and Wood 1995). However, dyslipidemia and other conditions with atypical lipoprotein characteristics (size, density, and charge) may cause discrepant results with different HDL-C methods. High triglyceride, haemoglobin, bilirubin and monoclonal paraprotein concentrations may also interfere with some HDL-C methods (Langlois and Blaton 2006).

2.6.4 Measurement of LDL-C

The first methods for LDL separation have been the same – ultracentrifugation and electrophoresis – as for HDL (Nauck et al. 2002). ApoB is an arginine- and lysine-rich, positively charged protein, which has been the basis for most of the precipitation and homogeneous HDL-methods. Because the LDL particle population is diverse containing large size-variation, the reference method for LDL-C has been difficult to develop. The current basis for accuracy of LDL-C measurements is a combination of ultracentrifugation and polyanion precipitation, so called β -quantification (Bachorik and Ross 1995). First chylomicron and VLDL fractions are removed by ultracentrifugation at density 1.006 kg/l. LDL, IDL and Lp(a) are then precipitated by heparin- Mn^{2+} and the cholesterol concentration is measured by the Abell-Kendall reference method. LDL-C is calculated by the difference of these fractions.

The Friedewald formula has been the basic method for calculating LDL-C concentrations in many epidemiological studies and the cut-off values are based on these data (Friedewald et al. 1972). The Friedewald formula combines total cholesterol, HDL-C and triglycerides in the following equation: total cholesterol (mmol/l) – HDL-C (mmol/l) – triglycerides (mmol/l)/2.2. Because the presence of chylomicrons affects the estimation of the triglyceride fraction, fasting samples are needed for calculated LDL-C. The accuracy of Friedewald formula decreases

already when TGs are >2.6 mmol/l, but adequate estimates can still be obtained with TGs received up to 4.0 mmol/l (Marniemi et al. 1995).

Homogeneous assays for LDL-C have been developed and they should be used especially when the triglyceride concentration is higher than 4.5 mmol/l (Warnick et al. 2002). Currently, there are several different homogeneous LDL-C assays based on selective detergents or other elimination methods to separate chylomicrons, VLDL, and HDL from LDL (Contois et al. 2011). A precipitation assay for sd-LDL cholesterol (sd-LDL-C) has also been developed (Hirano et al. 2009). According to the latest evidence, homogeneous LDL-C assays have limited specificity against unusual specimens, which may cause misclassification of patients (Miller et al. 2010). Different LDL-C assay kits may react partially also to VLDL, IDL, Lp(a), or apoE-rich HDL particles producing variations in LDL-C concentrations (Nauck et al. 2002, Iizuka et al. 2012).

NCEP ATP III has accepted the sd-LDL as a novel cardiovascular risk factor, but the measurement of LDL particle size has not been transformed into clinical practice. However, the amount of all apoB-containing particles is possible to estimate with apoB measurements instead of LDL-C (Davidson 2008).

Lipoprotein(a) [Lp(a)] consists of a LDL-like particle with an additional apolipoprotein(a) attached via a disulfide bond to apoB-100. Apo(a) is synthesized in the liver with structural homology with plasminogen. Lp(a) increases the CVD risk via macrophage foam cell formation and prothrombotic effects (Dube et al. 2012). The origin of Lp(a) synthesis is still obscure, but apo(a) is apparently attached to apolipoprotein B-containing lipoproteins extracellularly (Nordestgaard et al. 2010).

2.6.5 Measurement of non-HDL-C

ATP III included non-high-density lipoprotein cholesterol (non-HDL-C) as a new secondary target for therapy in the NCEP ATP III guidelines (Warnick et al. 2002). The aim was to increase the impact of triglyceride-rich lipoproteins to the screening and treatment guidelines. Non-HDL-C combines all apoB-containing atherogenic particles: VLDL, IDL, LDL, and Lp(a). Non-HDL-C is calculated by subtracting the concentration of HDL-C from the total cholesterol. The non-HDL-C should be measured if the LDL-C treatment goal has been achieved but the triglyceride concentration is still high. However, according to a meta-analysis of LDL-C, non-HDL-C, and apoB as markers for cardiovascular risk, non-HDL-C was reported to be better than LDL-C, but apoB was superior to both LDL-C and HDL-C (Sniderman et al. 2011).

2.6.6 Measurement of apoA-I and apoB

The analytical difficulties with homogeneous HDL-C and LDL-C measurements have inspired the debate on apoA-I and apoB as alternatives for the risk assessment of cardiovascular disease. ApoB as a major protein of LDL and apoA-I of HDL

could be used to measure the amounts of these atherogenic and anti-atherogenic lipoprotein particles. Also measuring apoB concentration in serum is a better estimate of the number of atherogenic, sd-LDL particles than LDL-C (Davidson et al. 2011). The increasing number of these particles is prevalent in patients with abdominal obesity, MetS, diabetes, hypertriglyceridemia, and low HDL-C; LDL-C concentration, however, is generally normal (Brunzell et al. 2008).

Apolipoproteins have been measured by different methods: immunodiffusion, radioimmunoassays, enzyme-linked immunoassays, immunonephelometry, or immunoturbidimetric assay (Albers and Marcovina 1989). The lack of standardization for apoA-I and apoB measurements has prevented their use in cardiovascular risk assessment guidelines, but nowadays proper reference materials for apoA-I and apoB are available (Marcovina et al. 1993, Marcovina et al. 1994).

Because total cholesterol, LDL-C and apoB are highly correlated, the same value of the 75th percentile as for LDL-C has been suggested to be the target value also for apoB. Respectively, because HDL-C concentrations below the 10th percentile have been classified as risk for CVD, the same cut-off value has been used for apoA-I (Albers and Marcovina 1989). Since the reference material for apoA-I and apoB was introduced, some population-based reference values have been reported (Leino et al. 1995, Contois et al. 1996a, Contois et al. 1996b).

CDC adopted apoA-I and apoB measurements as a part of the Lipid Standardization Program in 2010 with designated comparison methods, because real reference methods have not been implemented at the present. The proper measurement of apolipoproteins demands co-operation of reference laboratories, reagent manufacturers, and national advisory boards responsible for guidelines for risk assessment of CVD (Albers and Marcovina 1989).

The national guidelines have emphasized the use of total cholesterol and LDL-C for CVD risk assessment. However, the latest evidence has revealed that after reaching the therapeutic target for LDL-C, a substantial risk for CVD still remains. Many epidemiological studies have shown that apoB or apoB/apoA-I ratio might be a better predictor for cardiovascular risk than traditional cholesterol measurements (Walldius et al. 2001, Yusuf et al. 2004, Sniderman et al. 2006, Barter et al. 2006, Contois et al. 2009).

2.6.7 Other techniques to analyse lipoprotein subpopulations

Nuclear magnetic resonance (NMR) spectroscopy or gradient gel electrophoresis has been used to separate lipoprotein particles according to their size (Rizzo and Berneis 2006a, Rizzo and Berneis 2006b). Also the efficiency of reverse cholesterol transport as facilitated by HDL particles has been investigated. The total number of particular LDL or HDL subpopulations or assessment of their functionality may be an even better approach than the apoB or apoA-I measurements, but the use of NMR or electrophoresis demands special equipment and so far are not suitable for clinical

practise. However, precipitation methods for sd-LDL-C and HDL-C in HDL₂ and HDL₃ subpopulations have been developed (Hirano et al. 2008, Hirano et al. 2009).

3 Aims of the study

The first aim of this study was to assess the accuracy of the laboratory methods used in the Finnish population-based surveys for the cardiovascular risk factors during the last four decades. The second aim was to calculate reference intervals for apoA-I, apoB, and apoB/apoA-I ratio and compare them with traditional cholesterol-based risk markers in different subgroups. The third aim was to assess the possibility to add apoA-I and apoB measurements to the diagnostics arsenal of dyslipidemia.

Specific aims of the study were to answer to the following questions:

- Is it possible to measure accurately cardiovascular risk factors (cholesterol, HDL-C, and triglycerides) during several decades and what is the effect of systematic error to the interpretations of population trends (I, II, V)?
- What is the effect of fasting and non-fasting on the triglyceride concentration when estimating the prevalence of high LDL-C and metabolic syndrome in population-based surveys (II, III)?
- Do different homogeneous HDL-C methods classify people into different cardiovascular risk groups depending on the used methods (V)?
- What new information does apoA-I, apoB, and apoB/apoA-I ratio give to the risk assessment for cardiovascular disease and metabolic syndrome (IV, V)?

4 Materials and methods

4.1 The systematic error in measuring cholesterol, triglycerides, and HDL-C in population studies (I, II, V)

4.1.1 External quality assessment programs

The Laboratory of Analytical Biochemistry (TLAB) at the National Institute for Health and Welfare has participated in several external quality assessment programs since 1978. The Lipid Reference Program of World Health Organization (WHO) in 1978 – 1997 and the national Labquality since 1978 have offered regulary external quality assessment (EQA) samples to the laboratory. TLAB has also attended to the EQA of the Canadian External Quality Assessment Laboratory (CEQAL) during the ten-year period 1993 – 2004 and CDC’s Lipid Standardization Program since 2001. The reference materials and source of target values in different EQA programs are shown in Table 4. We used data from these programs to assess precision and accuracy for serum cholesterol, triglycerides, and HDL-C measurements during four decades.

Table 4. Reference materials and source of target values in external quality assessment programs (EQA).

EQA	Reference material	Target value
WHO	Lyophilized human sera	CDC’s reference methods
CEQAL	Frozen human sera	CDC’s reference methods
CDC	Frozen human sera	CDC’s reference methods
Labquality	Lyophilized or fresh human sera or lyophilized animal sera	Average of participants’ results

WHO: World Health Organisation, CEQAL: Canadian External Quality Assessment Laboratory, CDC: Centers for Disease Control and Prevention

4.1.2 Population surveys

The FINRISK population health surveys for cardiovascular risk factors have been carried out in five-year intervals since 1972 in Finland. Independent random samples stratified for age (25 – 64) and sex have been drawn from the population register.

The sample size has varied from 8000 to 13 500 subjects from 2 - 6 different regions. The protocol for sample collection has been the same in all surveys: venous blood samples have been drawn in a sitting position after at least a four-hour fasting between 11 am and 6 pm. After 30 min clotting time, samples have been centrifuged at 2200 g within one hour and the sera mailed daily to the laboratory until 2002. Since 2007, serum samples have been stored frozen at -20°C in the field centers for a few weeks and thereafter at -70°C before analyses.

4.1.3 Laboratory analyses

The methods, instruments, calibrators, and reagents have varied during four decades, but total cholesterol, triglycerides, and HDL-C measurements have been performed in the same laboratory of Analytical Biochemistry at the National Institute for Health and Welfare since 1970s. The reagent suppliers, instruments, and methods used in the laboratory are shown in Table 5 and Table 6.

Table 5. Reagent suppliers and instruments for cholesterol, triglycerides, and HDL-C measurements from 1972 at the National Institute for Health and Welfare.

Time period	Reagent supplier	Instrument
1972 - 1979	Self-made	Auto Analyzer II, Technicon, USA
1979 – 1998	Boehringer-Mannheim, Germany Nyegaard and Co AS Nyco-test, Norway ^a	Olli-C, Thermo Fisher Scientific, Finland
1998 - 2006	Thermo Fisher Scientific, Finland	Optima 909, Thermo Fisher Scientific, Finland
2006 ->	Abbott Laboratories, USA	Architect c8000, Abbott Laboratories, Abbott Park, IL, USA

^a 1980 – 1981

4.1.4 Bias corrections for cholesterol and HDL-C

The interpretation of changes in mean serum cholesterol and HDL-C concentrations in the Finnish population demands accurate information of real biases during the population surveys. Because EQA programs of WHO and CDC did not overlap in our laboratory, we had to estimate bias for cholesterol according to the bias between WHO/CDC versus Labquality. HDL-C results have been corrected according to the

biases at the time of FINRISK analyses. Details of bias corrections are reported in publications I and V.

Table 6. Methods for cholesterol, triglycerides, and high-density lipoprotein cholesterol (HDL-C) measurements from 1972 at the National Institute for Health and Welfare.

Cholesterol	Triglycerides	HDL-C
Direct Liebermann-Burchard (1972 – 1979)	Kessler and Lederer extraction/saponification/ fluorometry (1972 – 1977)	Dextran-MgCl ₂ precipitation, enzymatic CHOD-PAP (1979 – 1998)
Enzymatic CHOD-PAP (1979 ->)	Fully enzymatic, UV-test (1977 – 1982)	Homogeneous, polyethylene glycol-modified enzyme (PEG) (1998 – 2006)
	Enzymatic GPO-PAP, colorimetric test (1982 – 2006)	Homogeneous, accelerator selective detergent (2006 ->)
	Enzymatic GPO (2006 ->)	

4.1.5 Statistical analyses

Continuous variables have been presented as means with 95% confidence intervals (CIs) or standard deviation (SD). For method comparisons we used Bland-Altman difference plots, linear regression analyses, and paired t-tests. P-values <0.05 were considered statistically significant. All statistical analyses have been performed using Excel Analyse It –software or SPSS.

4.2 Effects of fasting and non-fasting on serum triglyceride concentration (II, III)

The effects of fasting on serum triglycerides were investigated from FINRISK population surveys in 1992, 1997, and 2002 from which triglyceride concentrations were available. The final data included subjects whose triglyceride concentration was <10 mmol/l and fasting time 2 – 15 hours. We compared non-fasting (2 – 8 hours for men, 2 – 7 hours for women) to overnight fasting with samples from FINRISK 2007 study followed by a substudy of the same participants later during the same year. Subjects were categorized as healthy when their BMI was <35 kg/m², alcohol consumption was <90th percentile, they reported no CVD, cancer, or

medication for hypercholesterolemia, and they had normal blood pressure. The population health surveys were approved by the Ethical Committee of the Hospital District of Helsinki and Uusimaa and all participants gave a written informed consent.

4.2.1 The kinetics of fasting

We hypothesized that serum triglyceride concentration decreases after the last meal until it reaches a constant plateau. The first linear regression model was used to calculate the rate of decreasing triglyceride concentration and the second model represented the time after sufficient fasting time. The linear regression model $\log(\text{tg}) = a + b * \text{fasting hours} + c * \text{sex} + e$ was simplified to the relative change $(\exp(b) - 1) * 100$. Estimates of relative change were calculated for all subjects and different subgroups: healthy, serum triglyceride concentration above 90th percentile or below 25th percentile, and BMI > 35 kg/m².

4.2.2 Correction of non-fasting triglycerides to fasting values

Non-fasting triglyceride concentrations were transformed to fasting values using the correction factors for all subjects and different subgroups. Those subjects which had >7 hours fast for women and >8 hours fast for men in the first visit were chosen as the reference group. They also had to participate in the second study visit with a true fasting time. The correction factors for per hour of fasting were: 3.7 % for all, 4.3 % for healthy and 6.5 % for BMI > 35 kg/m². The correction was calculated to 7 hours fasting for women and 8 hours fasting for men.

4.2.3 Statistical analyses

The differences between corrected non-fasting and true-fasting triglyceride values were calculated with the Altman-Bland method showing the mean and 95% confidence intervals. Triglyceride concentrations were expressed as mean, median, and 25th and 75th percentiles. The Wilcoxon matched-pairs signed-rank test was used to calculate differences between groups and chi-square test for differences between prevalences. The total intraindividual biological variation for triglycerides was calculated in the reference group and for the whole group from both the non-fasting and true-fasting visits. P-values < 0.05 were considered statistically significant. The statistical analyses were performed using Microsoft Excel Analyse-It software, Stata statistical package 10.1 (Stata Corporation 2007, Stata Statistical Software: Release 10.1 College Station, TX; StataCorp LP.), and IBM SPSS Statistics 19.

4.3 Reference intervals for apoA-I, apoB, and apo B/apoA-I ratio (IV)

4.3.1 Reference population

The FINRISK 2007 population-based health survey was used to calculate reference intervals for apoA-I, apoB, and apoB/apoA-I ratio. The reference population was selected from the total sample (n=6733) by excluding subjects without laboratory data (n=497) and subjects with self-reported CVD or cholesterol-lowering medication (n=1036), self-reported diabetes (n=198), obesity with BMI >30 kg/m² (n=608), or self-reported hypertension or antihypertensive medication (n=1566). The final reference population sample was 1528 women and 1300 men stratified by age with ten-year intervals from 25 to 74.

4.3.2 Laboratory analyses

The blood samples were taken according to the FINRISK protocol after at least a four-hour fasting period. ApoA-I and apoB were measured with Abbott Architect reagents by immunoturbidimetric methods (Abbott Laboratories, Abbott Park, IL, USA). The methods were standardized with WHO/IFCC reference materials SP1-01 for apoA-I and SP3-07 for apoB. Cholesterol, triglycerides, and HDL-C were measured by Abbott reagents. LDL-C was calculated by the Friedewald formula, when the triglyceride concentration was <4.0 mmol/l. The inter-assay coefficients of variations (CV) and biases for cholesterol, triglycerides, HDL-C, apoA-I, and apoB are represented in Table 7.

The final reference intervals, cut-off value, and therapeutic goals for apoB were bias-corrected with 8 %. We analyzed also Certified Reference Material BCR-393 (European Commission, Joint Research Centre Institute for Reference Materials and Measurements, Geel, Belgium) for apoA-I resulting in a bias of 2.6 % (\pm 0.17). Certified reference material for apoB was not available. Because of the opposite results between CDC and reference material, ApoA-I results were not bias-corrected.

Table 7. Interassay coefficients of variation (CV) and biases for cholesterol, triglycerides, HDL-C, apoA-I, and apoB.

Analyte	CV	Bias
Cholesterol	0.9 % ^a	1.0 % ^a
HDL-C	2.2 % ^a	-0.8 % ^a
Triglycerides	1.1 % ^a	-0.9 % ^a
ApoA-I	1.4 – 2.0 % ^b	-5 % ^c
ApoB	2.1 – 3.8 % ^b	-8 % ^c

^a According to the reference samples from CDC, ^b Depending on the concentrations of daily quality control samples, ^c According to the reference samples from CDC and in-house long-term quality control sample

4.3.3 Statistical analyses

Continuous variables were expressed as mean (\pm SD) and differences between the mean values were assessed with t-test. The reference intervals for apoA-I, apoB, and apoB/apoA-I were calculated using 2.5th and 97.5th percentiles from the reference population using a nonparametric method. Outlier testing was done according to IFCC recommendations (Solberg 2004) with and without Horn algorithm (Solberg and Lahti 2005). Differences between the age groups were determined by analyses of variance (ANOVA) with Tukey's post-hoc analysis. The healthy reference group and groups with selected diseases were compared with one-way ANOVA. For apoA-I, apoB and apoB/apoA-I cutoff values we used 10th, 25th, 50th, 75th, and 90th percentiles in the reference group. The linear regression analysis was used to calculate correlation between LDL-C and apoB. Statistical analyses were performed by PASW Statistics for Windows version 18.0 (SPSS Inc. Chicago, IL, USA) and Excel Analyse-It software version 2.07 (Analyse-It software Ltd., City West Business Park, Leeds, United Kingdom).

4.4 Comparisons of two HDL-C and apoA-I assays (V)

Two homogeneous HDL-C and two immunoturbidimetric apoA-I methods were compared using freshly collected serum samples between the Laboratory of Analytical Biochemistry at the National Institute for Health and Welfare (THL) and the laboratory of Mehiläinen Laboratory Services (Mehiläinen) in Helsinki, Finland. Anonymous samples were received from the occupational health care of Mehiläinen. The protocol of method comparison was approved by the local Ethics Committee.

4.4.1 Laboratory analyses

Cholesterol, HDL-C, and apoA-I were measured by Abbott reagents (Abbott Laboratories, Abbott Park, IL, USA) at THL and by Thermo reagents (Thermo Fisher Scientific, Vantaa, Finland) at Mehiläinen. For HDL-C measurements, THL used a homogeneous, accelerator selective detergent (ASD) method and Mehiläinen used a PEG-modified enzyme method. ApoA-I concentrations were measured by immunoturbidimetric assays in both laboratories. The interassay coefficients of variation (CVs) are shown in Table 8. During the time of method comparison THL attended in CDC's Lipid Standardization Program and biases compared to CDC's reference methods were -0.6 % (\pm 0.4) for cholesterol and -0.3 % (\pm 2.5) for HDL-C.

Table 8. Interassay coefficients of variation (CV) for cholesterol, HDL-C, and apoA-I at THL and Mehiläinen.

Analyte	CV (THL)	CV (Mehiläinen)
Cholesterol	0.6 %	1.5 %
HDL-C	1.5 %	2.9 %
ApoA-I	1.1 – 1.3 % ^a	2.4 – 2.7 % ^a

^a Depending on the concentrations of daily quality control samples

4.4.2 Statistical analyses

The differences between methods were estimated with the Bland-Altman difference plot, Wilcoxon's test for paired samples, and with Kappa statistic. For Kappa calculation we used the cut-off values of 1.0 mmol/l and 1.2 g/l for men and 1.2 mmol/l and 1.4 g/l for women in HDL-C and apoA-I assays, respectively. Statistical analyses were done with Excel Analyse-It software version 2.07 (Analyse-It software Ltd., City West Business Park, Leeds, United Kingdom).

5 Results

5.1 The systematic error of cholesterol, triglycerides, and HDL-C measurements (I, II, V)

5.1.1 Changes in cholesterol methods

The different methods and instruments used during four decades at TLAB are introduced in Tables 5 and 6 in the Material and methods section. Auto Analyzer II (Technicon, USA) demonstrated no difference between the Liebermann-Burchard method and enzymatic CHOD-PAP-method, but applying the enzymatic method to an Olli-C analyzer (Thermo Fisher Scientific, Vantaa, Finland) decreased cholesterol concentrations by 2.3 %. The next change to an Optima analyzer (Thermo Fisher Scientific, Vantaa, Finland) did not result in significant differences during the method comparison.

5.1.2 Changes in triglyceride methods

Triglycerides have been measured with similar enzymatic methods since 1977, so only the instrument changes have caused possible systematic errors to the results (Tables 5 and 6). The change from Olli-C to Optima showed an average difference of 1.1 % (95% CI -8.2 to 10.3) in triglyceride values. The method for Optima analyzer had a secondary calibration instead of the previously used glycerol standards in methanol. The latest instrument change to an Architect analyzer demonstrated a difference of -3.1 % (95% CI -8.7 to 2.5) against the Optima analyzer.

5.1.3 Changes in HDL-C methods

The assays for measuring HDL-C have developed from the precipitation methods to direct, homogeneous ones during the last two decades (Tables 5 and 6). A new dextran sulphate (DS) lot purchased in 1987 decreased the HDL-C concentrations with 5.2 % and the difference was dependent on the HDL-C concentration. When HDL-C concentration was below 1.0 mmol/l, difference was -2.5 % (95% CI -3.8 to -1.4) compared to -12.8 % (95% CI -15.4 to -10.9) at HDL-C levels >1.5 mmol/l. The homogeneous PEG method yielded 4.0 % higher HDL-C concentrations than the DS precipitation method, and the difference was only slightly dependent on the HDL-C concentration. The final change to the ASD method had only a minor effect on the systematic error.

5.1.4 External quality assessment and population surveys

TLAB has participated in both national and international external quality assessment programs since 1978. Details on the annual cholesterol, triglycerides, and HDL-C biases are presented in the publications I, II, and V, but the mean annual biases and biases during the FINRISK years are shown in Table 9 and Figure 2.

Table 9. Mean annual biases of cholesterol, triglycerides, and HDL-C measurements.

Lipid	Years	Bias	95% CI
Cholesterol	1978 - 2004	-0.7 %	-0.9 – -0.6
Triglycerides	1978 - 2007	-1.5 %	-2.3 – -0.8
HDL-C	1980 - 2012	-2.3 %	-3.3 – -1.4

The cholesterol bias was quite stable until the end of 1990s, when the mean bias changed from negative to positive according to the CDC's reference samples. A new calibrator lot introduced in 2000 increased cholesterol values, but since that EQA results have reached an almost zero bias during the FINRISK 2012 survey. The annual triglyceride bias has remained rather stable between the acceptance limit for different EQA programs: 8 – 18 % for WHO's Lipid Reference Program and 5 – 15 % for CDC's Lipid Standardization Program. The precipitation methods for HDL-C showed an average bias of -4.7 % (95% CI -5.9 to -3.6), which changed to a slightly positive bias of +0.1 % (95% CI -0.7 to 1.0) after the homogeneous methods were adapted in the laboratory since 1998.

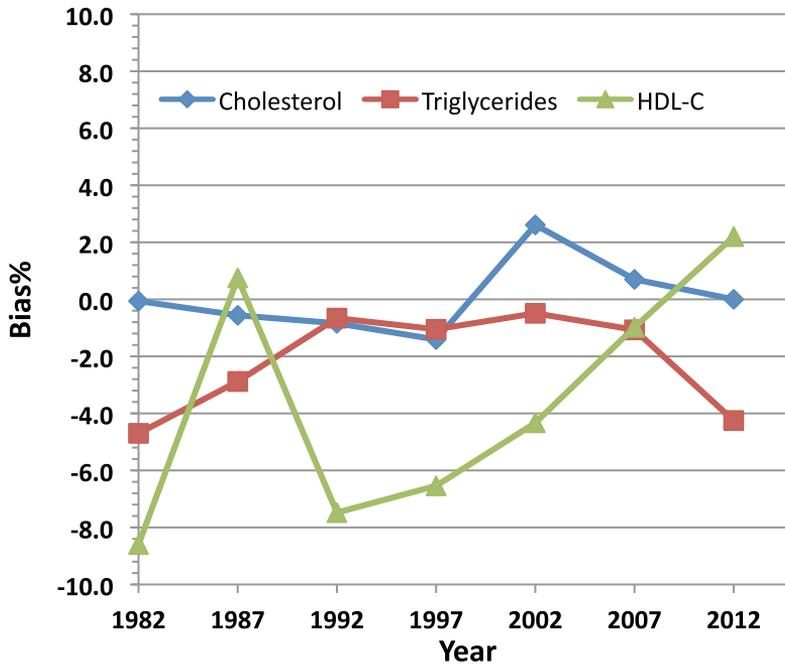


Figure 2. Mean annual biases for cholesterol, triglycerides, and HDL-C during the FINRISK years 1982 – 2012. Results are from EQAs organized by WHO (1982 – 1997) and by CDC (2002 – 2012).

The decreasing trend for mean serum cholesterol concentration continued from 1982 until 1997 in men and until 2007 in women (Tables 10 and 11). After bias-correction in 2002, the same trend continued also in men until 2007. However, the mean cholesterol concentrations increased in the latest FINRISK 2012 survey, 1.7 % in men and 3.1 % in women compared to the previous survey 2007 (Vartiainen et al. 2012). The mean HDL-C concentrations during the same FINRISK surveys have demonstrated positive trends with original data ($P=0.053$ for men and $P=0.069$ for women), but no trend was observed after bias-correction ($P=0.816$ for men and $P=0.625$ for women). The difference in HDL-C concentrations between men and women was on average 0.28 mmol/l (± 0.02) during the entire observed period.

Table 10. Mean cholesterol and HDL-C concentrations and cholesterol/HDL-C ratio without and with bias-correction for men in FINRISK surveys 1982 -2012.

Year	Chol (mmol/l)	Bias-corrected Chol	HDL-C (mmol/l)	Bias-corrected HDL-C	Chol/HDL-C	Chol/HDL-C Bias-corrected
1982	6.14	6.07	1.22	1.33	5.0	4.6
1987	6.12	6.07	1.30	1.29	4.7	4.7
1992	5.79	5.77	1.26	1.35	4.6	4.3
1997	5.52	5.52	1.26	1.34	4.4	4.1
2002	5.62	5.47	1.35	1.41	4.2	3.9
2007	5.29	5.25	1.30	1.31	4.1	4.0
2012	5.34 ^a	5.34	1.33	1.31	4.0	4.1

^a Vartiainen et al 2012

Table 11. Mean cholesterol and HDL-C concentrations and cholesterol/HDL-C ratio without and with bias-for women in FINRISK surveys 1982 -2012.

Year	Chol (mmol/l)	Bias-corrected Chol	HDL-C (mmol/l)	Bias-corrected HDL-C	Chol/HDL-C	Chol/HDL-C Bias-corrected
1982	6.06	5.98	1.46	1.58	4.2	3.8
1987	5.95	5.91	1.58	1.57	3.8	3.8
1992	5.58	5.55	1.52	1.63	3.7	3.4
1997	5.47	5.47	1.54	1.64	3.6	3.3
2002	5.44	5.29	1.64	1.71	3.3	3.1
2007	5.19	5.15	1.56	1.58	3.3	3.3
2012	5.31 ^a	5.31	1.62	1.59	3.3	3.3

^a Vartiainen et al 2012

We compared also the mean HDL-C concentrations from the precipitation method (1982 – 1997) and from the direct methods (2002 – 2012) using the results from the FINRISK surveys. Before bias-correction HDL-C mean concentrations

were 5 % lower by the precipitation method compared to direct methods for both sexes, but after bias-correction no difference was observed (Table 12).

Table 12. Mean HDL-C concentrations in men and women during 1982 -1997 (precipitation method) and 2002 – 2012 (direct methods) without and with bias-correction.

HDL-C (precipitation), mmol/l	HDL-C (precipitation, bias- corrected), mmol/l	HDL-C (direct), mmol/l	HDL-C (direct, bias- corrected), mmol/l
Men			
1.26 ^a	1.33	1.33	1.34
Women			
1.52 ^a	1.61	1.61	1.63

^a Differed significantly ($p < 0.05$) from the bias-corrected by one-way Anova.

5.2 Correction of non-fasting triglycerides to fasting triglycerides (II, III)

5.2.1 Calculations of factors for transforming non-fasting to fasting triglycerides

The fasting time before blood sampling in FINRISK surveys has been defined as a minimum of 4 hours, so most of the participants have fasted for 4 – 6 hours. The mean serum triglyceride concentrations for a fasting time of 2 – 8 hours from FINRISK surveys in 1992, 1997, and 2002 for all and healthy men and women are shown in Table 13. Triglyceride concentrations decreased during the first 8 fasting hours in men and the first 7 hours in women. We used this data to calculate the factors for relative changes per hour as shown in Table 14.

Table 13. Mean serum triglyceride concentrations for all and healthy men and women in FINRISK surveys 1992, 1997 and 2002 per fasting time.

Fasting time	All men (n=10 460), mmol/l	Healthy men (n=4246), mmol/l	All women (n=11 624), mmol/l	Healthy women (n=5017), mmol/l
2	1.96	1.95	1.53	1.38
3	1.93	1.67	1.42	1.22
4	1.77	1.71	1.31	1.22
5	1.71	1.59	1.24	1.14
6	1.68	1.55	1.21	1.10
7	1.56	1.44	1.14	1.02
8	1.53	1.42	1.16	1.04

Table 14. Relative change (95% CI) of serum triglyceride concentration per hour in different groups in FINRISK surveys in 1992, 1997, and 2002.

All	25th percentile	90th percentile	Healthy	BMI >35
-3.7 [-4.2,-3.1]	-2.3 [-2.7,-1.8]	-5.3 [-6.2,-4.3]	-4.3 [-5.2,-3.5]	-6.5 [-8.7,-4.3]

5.2.2 Converting non-fasting triglycerides to fasting values with factors

We transformed non-fasting triglyceride concentrations to fasting values using the correction factors for all and different subgroups (Table 15 and Figure 3) and compared these results with true fasting values in FINRISK 1992, 1997, and 2002 studies.

We also compared statistically corrected non-fasting triglyceride values with true fasting values in the FINRISK 2007 survey. Using this data from the same subjects, we estimated the misclassification of subjects into the categories of high LDL-C or MetS with and without triglyceride correction (Table 16). The definition for MetS according to the NCEP (ATP III) uses triglyceride concentration > 1.7 mmol/l as one criterion for MetS (Grundy et al. 2005).

Table 15. Comparison of original and calculated 8 hour fasting triglyceride concentrations for different subgroups in FINRISK 1992, 1997, and 2002 studies.

Subgroup	Original 8 h fasting TG, mmol/l	Calculated 8 h fasting TG, mmol/l	Original – calculated TG, mmol/l	Difference, %
25th percentile	0.68	0.68	0.00	0.0
Healthy	1.29	1.30	-0.01	-0.6
All	1.39	1.42	-0.03	-2.1
BMI >35	1.78	1.66	0.12	6.9
90th percentile	3.34	3.16	0.18	5.4

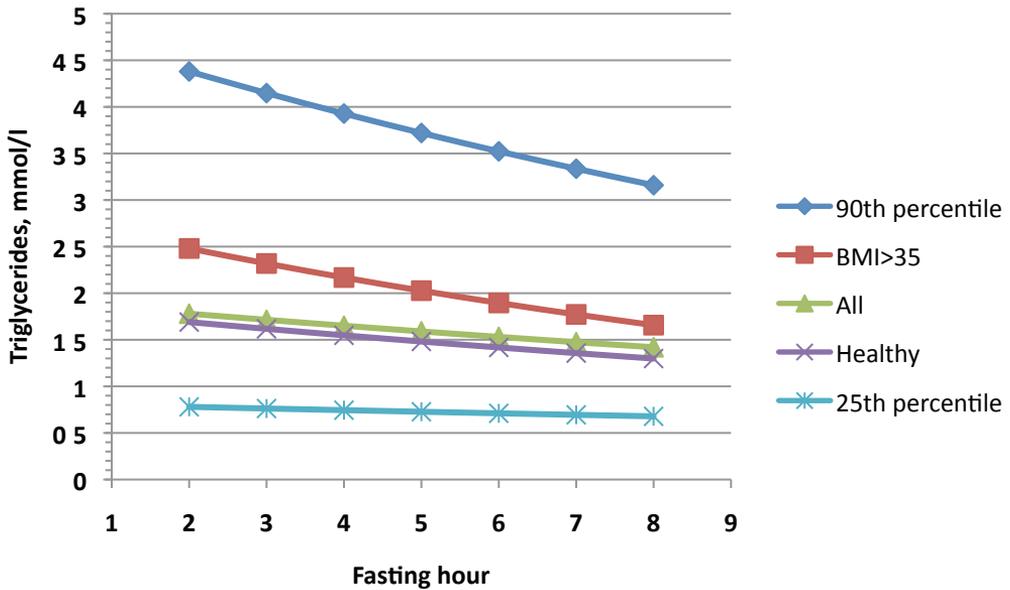


Figure 3. The mean serum triglyceride concentration per hour using factors (Table 14) for different subgroups.

Table 16. Comparison of non-fasting, corrected non-fasting and true-fasting triglyceride median concentrations [25th, 75th], and the prevalences of LDL-C>3.00 mmol /l and metabolic syndrome in the FINRISK 2007 survey.

Group	Tg, mmol/l	[25th, 75th]	LDL>3.0 mmol/l	Prevalence of metabolic syndrome ^b
Non-fasting	1.18 ^a	[0.87, 1.72]	51.3 % ^a	39.7 % ^a
Non-fasting, corrected	1.06 ^a	[0.78, 1.52]	54.8 % ^a	37.6 % ^a
True-fasting	1.00 ^a	[0.75, 1.38]	56.4 % ^a	35.5 % ^a

^a $p < 0.001$ compared to the other groups; ^b According to the National Cholesterol Education Program ATP III (Grundy et al. 2005)

The prevalences of MetS according to the criteria of International Diabetes Federation (IDF) and International Diabetes Federation Task Force (IDFTF) (Alberti et al. 2009, Alberti et al. 2005) were higher: 47.1 % and 49.8 % for non-fasting, 45.6 % and 47.9 % after statistical correction compared to 44.8 % and 46.3 % in true-fasting subjects in the same FINRISK 2007 study ($P < 0.0001$ for all comparisons). The difference between the prevalences of MetS decreased from 4.2 % to 2.1 % with ATP III criteria and from 2.3 % and 3.5 % to 0.8 % and 1.6 % with IDF and IDFTF, respectively ($P < 0.0001$ for all comparisons).

5.3 Reference intervals for apoA-I, apoB and apoB/apoA-I ratio (IV)

Reference intervals for apoA-I, apoB, and apoB/apoA-I ratio were calculated according to the 2.5th percentile and 97.5th percentile from the healthy reference group in the FINRISK 2007 study. The mean apoA-I and apoB concentrations, and apoB/apoA-I ratio in different age groups are shown in Figures 4, 5, and 6. The mean apoA-I concentrations were higher in women than in men in all age groups, but women reached the male apoB concentration by the age of 65. The apoB/apoA-I ratio increased in women during the whole observed time; whereas in men the ratio was quite stable after 35 years.

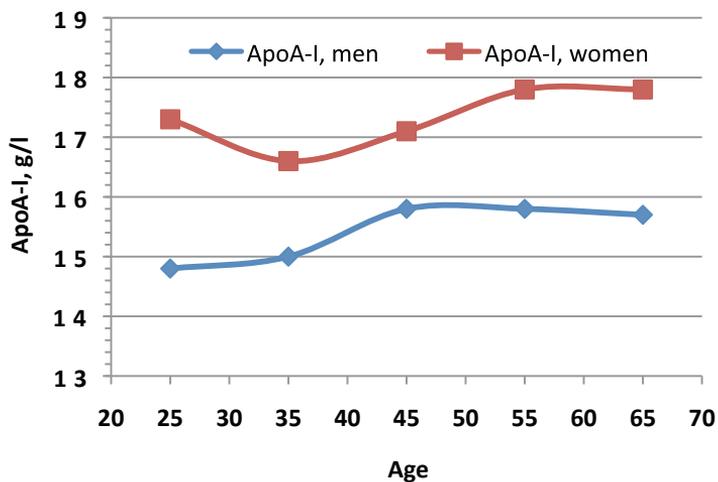


Figure 4. The mean serum apoA-I concentration for men and women in different age groups in the healthy reference group from FINRISK 2007 study.

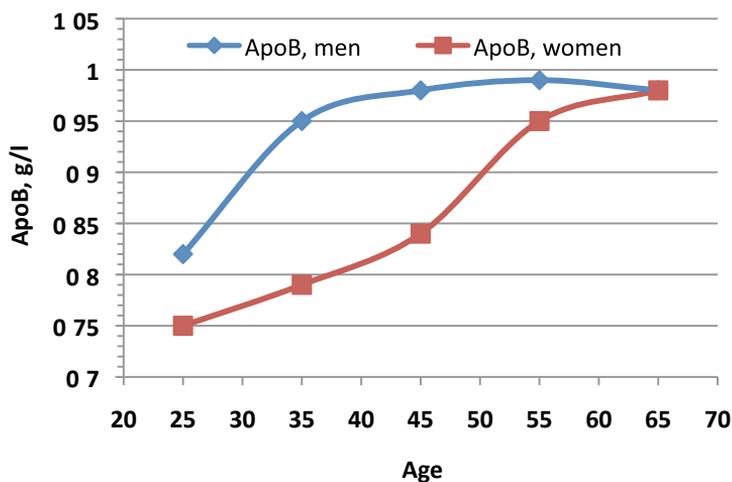


Figure 5. The mean serum apoB concentration for men and women in different age groups in the healthy reference group from FINRISK 2007 study.

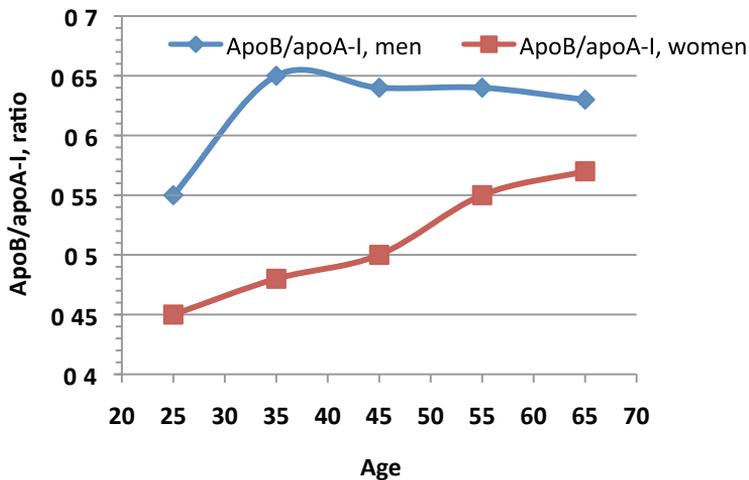


Figure 6. The mean serum apoB/apoA-I ratio for men and women in different age groups in the healthy reference group from FINRISK 2007 study.

The final reference intervals for apoA-I, apoB, and apoB/apoA-I are shown in Table 17. ApoB values were bias-corrected (see Material and methods 4.3.2).

Table 17. Reference intervals for apoA-I, apoB, and apoB/apoA-I ratio from the healthy reference group from FINRISK 2007 study.

Sex	ApoA-I, g/l	ApoB, g/l ^a	ApoB/apoA-I
Men	1.1 – 2.0	0.6 – 1.5	0.3 – 1.0
Women	1.2 – 2.3	0.6 – 1.3	0.3 – 0.8

^a Bias-corrected

5.4 Comparison of lipids, lipoproteins, and apoA-I and apoB in different pathophysiological conditions (IV)

The same groups, which were excluded from the healthy reference group in the FINRISK 2007 study, were used to assess the differences in mean concentrations of lipids, lipoproteins, and apoA-I and apoB between the healthy and non-healthy groups.

The mean cholesterol concentrations were 11 % and 12 % lower in men when having CVD or diabetes, but in women mean cholesterol concentrations had only a minor 1 % difference compared to the healthy reference group (Figure 7). The lowest HDL-C concentrations were observed in subjects with diabetes and obesity in both sexes: 14 % lower in men and 15 % lower in women in contrast to the reference group (Figure 8). LDL-C concentration was higher in men than in women in the healthy reference group, but in all disease groups women had higher LDL-C concentrations than men (Figure 9). The mean LDL-C concentrations were on average 20 % lower in men with self-reported CVD or diabetes, but in women LDL-C was only 4 % lower in these disease groups compared to the healthy reference group. Triglyceride concentrations were 27 – 67 % higher in all disease groups compared to the healthy reference group in both sexes (Figure 10). The triglyceride concentrations were on average 55 % higher in men and 60 % higher in women with diabetes and BMI>30 than in the reference group.

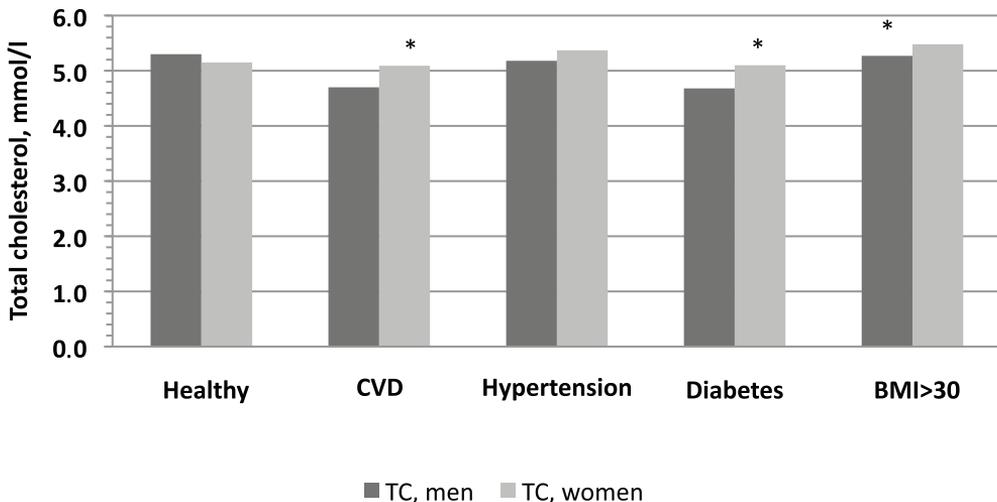


Figure 7. The comparison of mean total cholesterol (TC) for men and women in the healthy reference group and different disease groups. * Did not differ significantly from the healthy group ($p < 0.05$; one-way ANOVA). Others differed significantly from the healthy reference group.

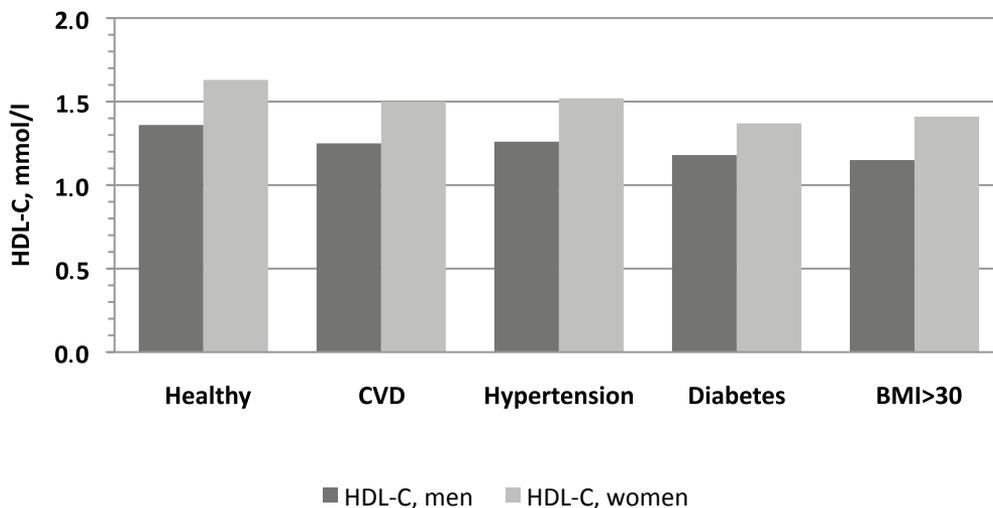


Figure 8. The comparison of mean HDL-C for men and women in the healthy reference group and different disease groups. All groups differed significantly from the healthy reference group ($p < 0.05$; one-way ANOVA).

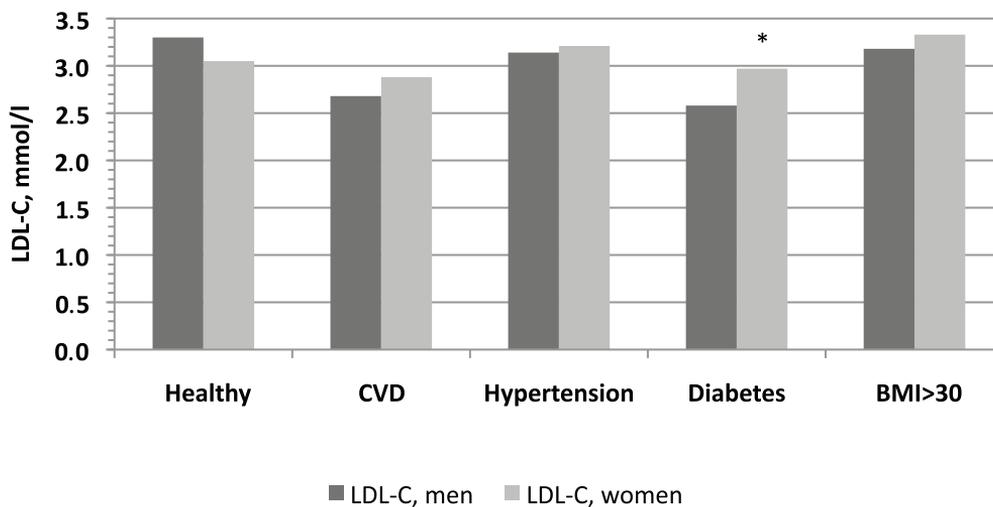


Figure 9. The comparison of mean LDL-C for men and women in the healthy reference group and different disease groups. * Did not differ significantly from the healthy group ($p < 0.05$; one-way ANOVA). Others differed significantly from the healthy reference group.

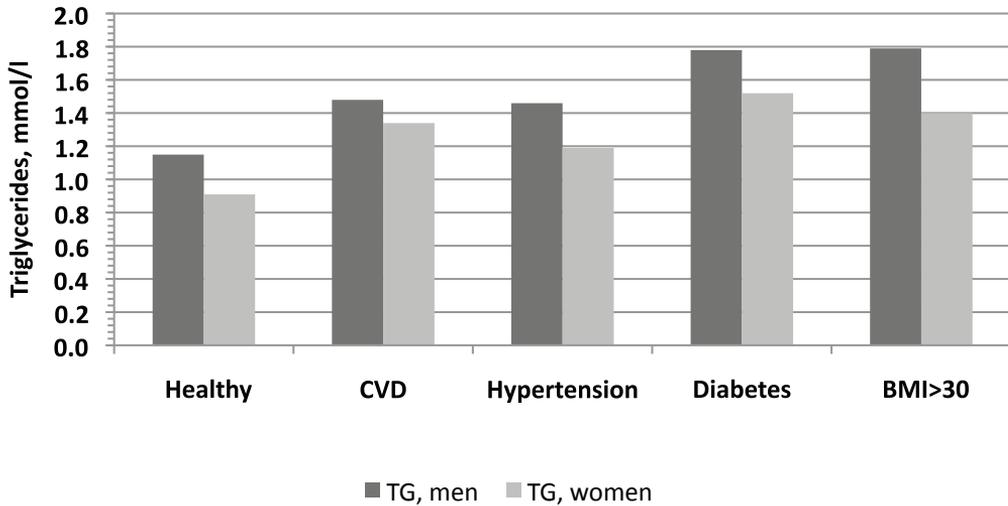


Figure 10. The comparison of median triglycerides (TG) for men and women in the healthy reference group and different disease groups. All groups differed significantly from the healthy reference group ($p < 0.05$; one-way ANOVA).

ApoA-I concentrations showed a similar pattern as the HDL-C concentrations for men and women: the lowest concentrations were observed in subjects with diabetes and obesity (Figures 8 and 11). Mean LDL-C concentrations were 6 % and 3 % lower in women having CVD or diabetes than in the healthy reference group, but both apoB and apoB/apoA-I ratios were on average 10 % higher in these disease groups (Figures 12 and 13). However, men had lower concentrations for both LDL-C and apoB when having CVD. Obese men had the lowest apoA-I concentrations, the highest apoB concentrations, and the highest apoB/apoA-I ratio compared to the reference group. Obese women had 16 % higher apoB concentration and 24 % higher apoB/apoA-I ratio than women in healthy reference group. In addition, women had higher apoB concentrations and apoB/apoA-I ratios in all disease groups compared to the reference group.

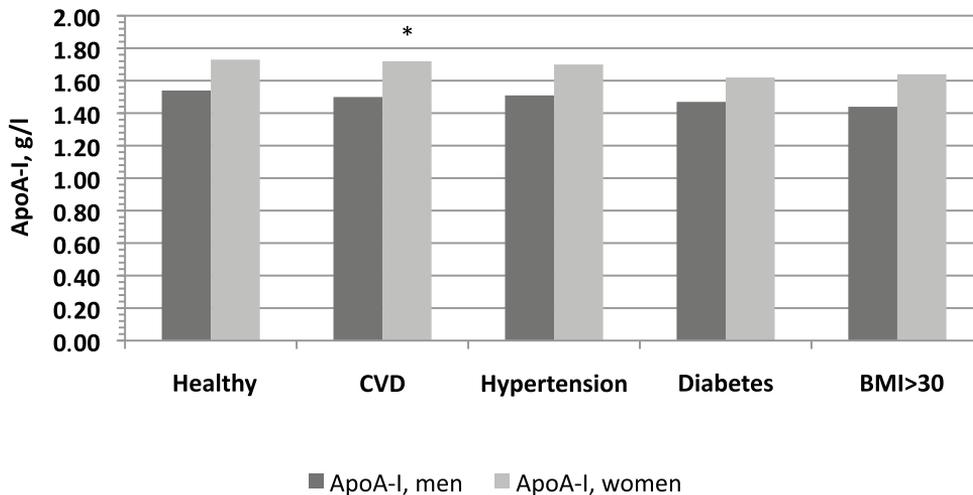


Figure 11. The comparison of mean apoA-I for men and women in the healthy reference group and different disease groups. * Did not differ significantly from the healthy group ($p < 0.05$; one-way ANOVA). Others differed significantly from the healthy reference group.

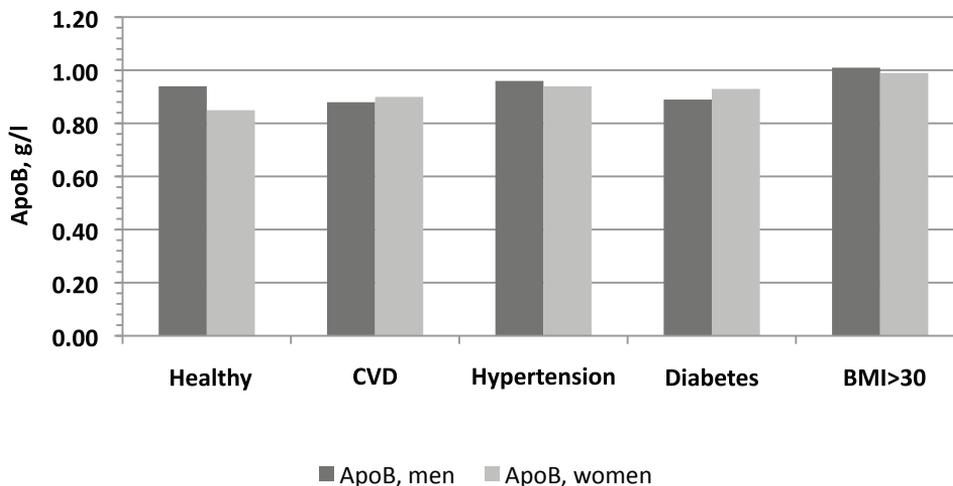


Figure 12. The comparison of mean apoB for men and women in the healthy reference group and different disease groups. All groups differed significantly from the healthy reference group ($p < 0.05$; one-way ANOVA).

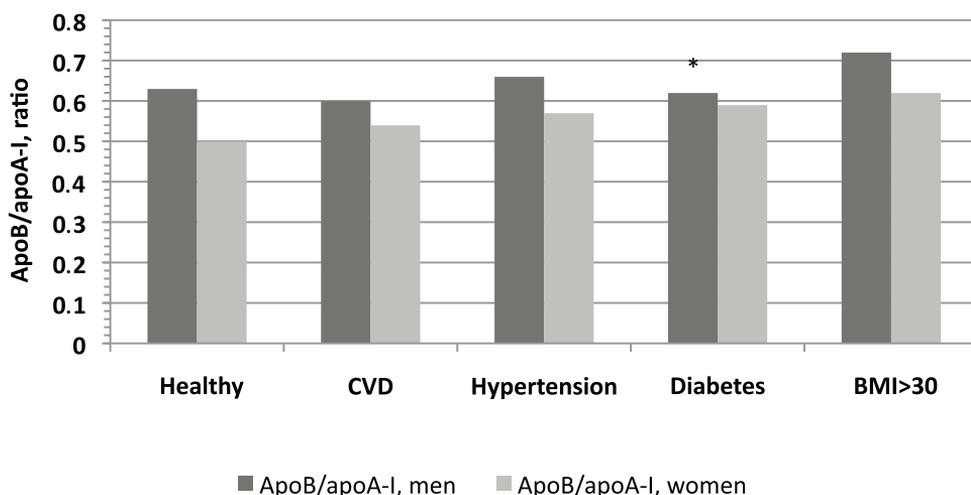


Figure 13. The comparison of apoB/apoA-I ratio for men and women in the healthy reference group and different disease groups. * Did not differ significantly from the healthy group ($p < 0.05$; one-way ANOVA). Others differed significantly from the healthy reference group.

5.5 Defining cut-off values for apoA-I, apoB, and apoB/apoA-I ratio (IV)

The comparison of 10th, 25th, 50th, 75th, and 90th percentiles for HDL-C, apoA-I, LDL-C, apoB, and apoB/apoA-I ratio are shown in Figures 14 and 15. The 10th percentile was used as a cut-off value for low HDL-C: 1.0 mmol/l for men and 1.2 mmol/l for women (Albers and Marcovina 1989). The same 10th percentile for apoA-I caused cut-off values 1.2 g/l for men and 1.4 g/l for women. The 50th percentile for LDL-C was almost equivalent to the concentration of 3.0 mmol/l, which is a target value in the risk assessment for CVD in Europe (Graham et al. 2007). At the same 50th percentile level apoB concentrations were the same 0.9 g/l for both sexes, and apoB/apoA-I ratios were 0.6 for men and 0.5 for women.

The linear regression equations for calculating apoB from known LDL-C concentration are as follows:

$$\text{Men: } \text{apoB} = 0.2393 * \text{LDL-C} + 0.1466 \quad (R^2 = 0.87)$$

$$\text{Women: } \text{apoB} = 0.2298 * \text{LDL-C} + 0.1460 \quad (R^2 = 0.87)$$

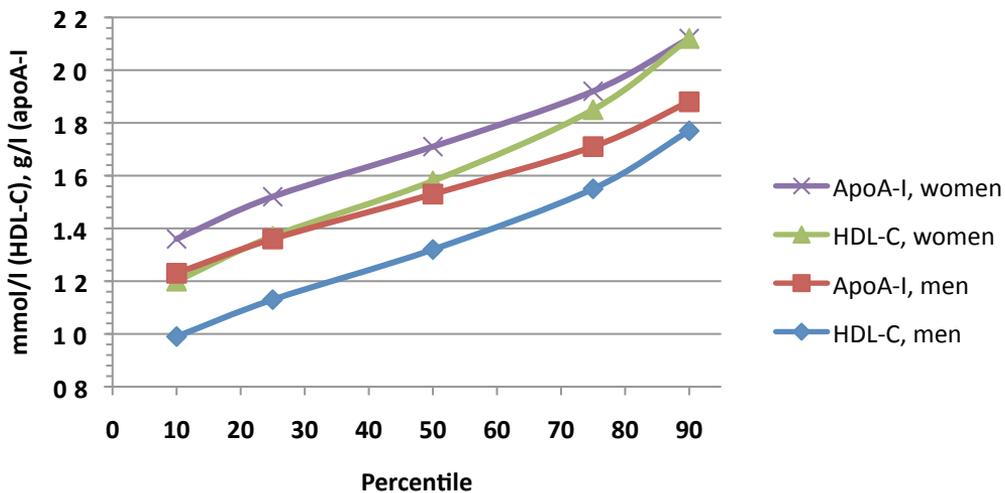


Figure 14. The comparison of HDL-C and apoA-I percentiles for women and men in the healthy reference group in FINRISK 2007 study.

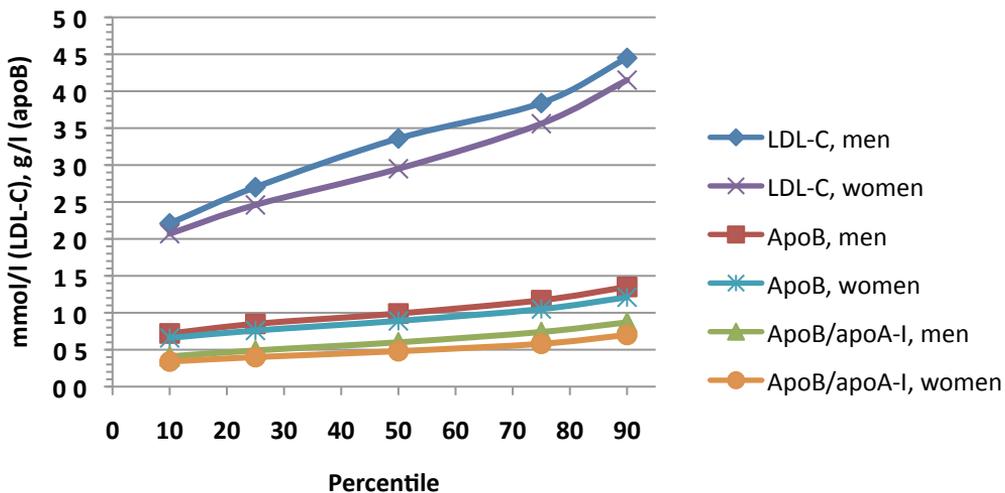


Figure 15. The comparison of LDL-C, apoB, and apoB/apoA-I ratio percentiles for women and men in the healthy reference group in FINRISK 2007 study.

The therapeutic goals for apoB (bias-corrected) are calculated according to the linear regression analyses corresponding to the serum LDL-C concentrations in different risk groups (Table 18).

Table 18. The therapeutic goals for LDL-C and apoB. Goals are the same for men and women (Graham et al. 2007).

Analyte	Low risk ^b	High risk ^c	Highest risk ^d
LDL-C, mmol/l	<3.0	<2.5	<2.0
ApoB, g/l ^a	<0.9	<0.8	<0.7

^a Bias-corrected, ^b 0 – 1 risk factors, ^c Individuals with more than 1 risk factor or with CVD or diabetes

^d Individuals with CDV and diabetes

5.6 Method comparisons of two direct HDL-C methods and immunoturbidimetric apoA-I methods (V)

Two direct HDL-C methods and two immunoturbidimetric apoA-I methods were compared between two independent laboratories. The other laboratory used a PEG-method (Thermo) and TLAB used an ASD-method (Abbott) for analysing HDL-C concentrations. The mean concentrations for cholesterol (5.06 mmol/l vs. 5.16 mmol/l) and apoA-I (1.54 g/l vs. 1.61 g/l) differed between these two laboratories ($P < 0.001$), but mean HDL-C concentrations (1.45 mmol/l vs 1.46 mmol/l) were almost the same ($P = 0.266$).

In spite of the same mean HDL-C concentrations, these two methods demonstrated concentration-dependent difference (Figure 16). The difference was negative with low HDL-C concentrations and turned to positive with higher concentrations (Table 19). The difference was significant at all concentration ranges ($P < 0.0001$, Wilcoxon's test).

The agreements between PEG- and ASD-method for HDL-C and two immunoturbidimetric methods for apoA-I were calculated with Kappa statistic using previously defined cut-off values (Results 5.5). The results are shown in Tables 20 - 23.

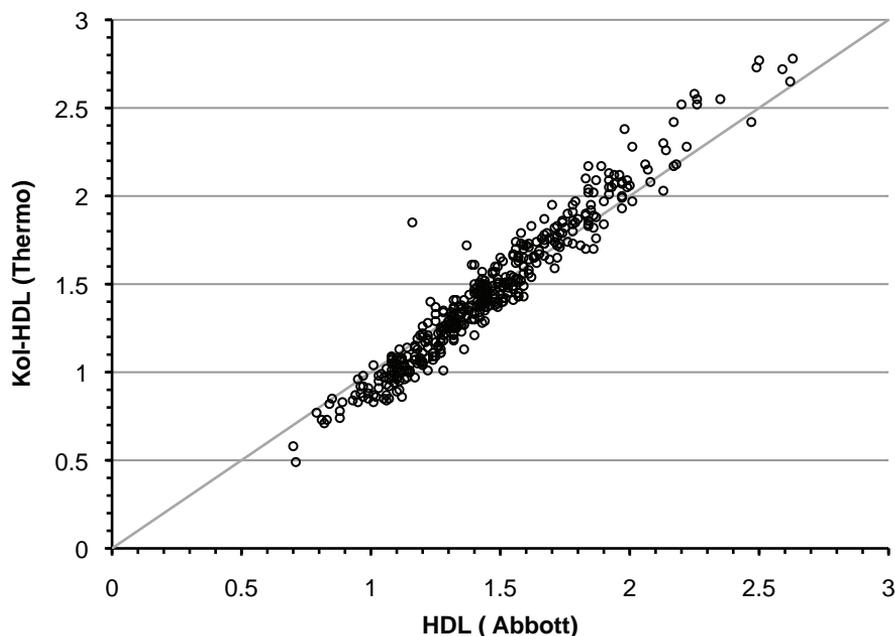


Figure 16. Scatter plot for Abbott and Thermo direct HDL-C methods.

Table 19. Difference and 95% confidence limits between PEG-method (Thermo) and ASD-method (Abbott) at different HDL-C concentrations (mmol/l).

PEG - ASD	HDL-C<1.00	1.00<HDL-C<1.55	HDL-C>1.55
Difference %	-12.0 % ^a	-4.5 % ^a	+9.0 % ^a
95% CI	[-13.5, -10.0]	[-5.5, -4.0]	[7.0, 10.5]

^a Wilcoxon's test, P<0.0001

Table 20. Agreement between two direct HDL-C methods (reagents from Thermo and Abbott) in women. The number shows how many of the samples were classified to the same or different concentration class (n=40).

Women	HDL-C<1.00 (Thermo)	1.00<HDL-C<1.20 (Thermo)
HDL-C<1.00 (Abbott)	2	0
1.00<HDL-C<1.20 (Abbott)	11	27

Table 21. Agreement between two direct HDL-C methods (reagents from Thermo and Abbott) in men. The number shows how many of the samples were classified to the same or different concentration class (n=59).

Men	HDL-C<1.00 (Thermo)	1.00<HDL-C<1.20 (Thermo)
HDL-C<1.00 (Abbott)	21	0
1.00<HDL-C<1.20 (Abbott)	18	20

Kappa value for two different HDL-C methods in women was 0.20 [95% CI -0.04, 0.44] and in men 0.44 [95% CI 0.26, 0.62]. Kappa value 0.20 for women means slight agreement between methods and 0.44 for men shows moderate agreement (Sim and Wright 2005). Kappa values for two different apoA-I methods were better than for HDL-C methods: 0.55 [95% CI 0.31, 0.80] in women and 0.71 [95% CI 0.47, 0.95] in men. Kappa value 0.55 for women shows also moderate agreement between methods, but 0.71 for men means substantial agreement.

Table 22. Agreement between two different apoA-I methods (reagents from Thermo and Abbott) in women. The number shows how many of the samples were classified to the same or different concentration class (n=50).

Women	ApoA-I<1.4 (Thermo)	ApoA-I \geq 1.4 (Thermo)
ApoA-I<1.4 (Abbott)	14	2
ApoA-I>1.4 (Abbott)	7	27

Table 23. Agreement between two different apoA-I methods (reagents from Thermo and Abbott) in men. The number shows how many of the samples were classified to the same or different concentration class (n=56).

Men	ApoA-I<1.2 (Thermo)	ApoA-I \geq 1.2 (Thermo)
ApoA-I<1.2 (Abbott)	8	1
ApoA-I>1.2 (Abbott)	4	43

6 Discussion

The interpretation of trends in serum cholesterol, triglycerides, and HDL-C concentrations in population-based health surveys is demanding because of the possible systematic error in laboratory measurements between the survey years. Different factors affecting the systematic errors and interpretations of the trends were analysed during four decades of FINRISK population studies. Because a full 12-hour fasting is often difficult to organize in population studies, factors for transforming non-fasting triglyceride concentrations to fasting values were calculated in different subgroups. The differences in prevalences of high LDL-C and metabolic syndrome were also estimated using non-fasting and true-fasting triglyceride concentrations. The introduction of new reference values for apoA-I, apoB, and apoB/apoA-I ratio among Finns should encourage clinicians to use apoA-I and apoB measurements for CVD risk assessment, especially in certain risk groups.

6.1 Changes in cholesterol, triglycerides, and HDL-C methods

Cholesterol

Already sixty years ago the causality of cholesterol in the etiology of atherosclerosis was discovered and accurate methods for measuring serum cholesterol in clinical laboratories were needed (Abell et al. 1952). Abell et al. developed a simplified method for measuring cholesterol, which was based on the liberation of cholesterol from serum lipoproteins by alcoholic potassium hydroxide. After saponification of cholesterol esters to free cholesterol and extraction into the petrol ether, cholesterol concentration was measured with the Liebermann-Burchard color reagent (Kenny 1952). During two decades this was the “golden” method to measure serum cholesterol in clinical laboratories and after minor modifications it is still the CDC’s reference method.

The most significant change in the era of cholesterol measurements has been the replacement of the Liebermann-Burchard method by enzymatic methods. This change occurred in TLAB in the end of 1970s and yielded on average a 2.3% decrease in cholesterol results. Since then the cholesterol assay method has remained the same, changes have been made only through reagent suppliers, instruments, and calibrators.

Triglycerides

There have been only minor changes in triglyceride measurements after substituting enzymatic methods for chemical methods. Because triglycerides are measured as a glycerol concentration, increased concentrations of free glycerol in specimen may

cause erroneous results in both chemical and enzymatic methods. For example, recent exercise, liver disease, diabetes mellitus, or stress can increase endogenous glycerol concentration and cause overestimation of triglycerides (Stein and Myers 1995).

HDL-C

When the protective role of HDLs in the progress of atherosclerosis was accepted in 1970s, more convenient methods than ultracentrifugation to measure HDL-C were developed. Because the high-density lipoproteins are a highly heterogenous group of lipoproteins, it has been demanding to develop specific and accurate methods to quantify the cholesterol content in HDL particles. The main principle has been to separate apolipoprotein B-containing lipoproteins from the remaining high-density lipoproteins. The CDC's reference method is based on the removal of VLDL particles by centrifugation followed by the precipitation of non-HDL particles by heparin with manganese. This precipitation method, however, has caused problems, because all heparin preparations have not been equally efficient in precipitating non-HDL particles (Lopes-Virella et al. 1977). A major problem with precipitation methods has been a high triglyceride concentration, which has interfered with the proper precipitation of non-HDL lipoproteins (Warnick 2001 et al. 2001). In addition, the dextran sulphate with magnesium as a precipitation reagent has turned out to be more reliable with enzymatic assays (Rifai et al. 2004).

The next innovation in HDL-C measurements has been the development of homogeneous, direct methods without the need for precipitation step in 1990s. These methods use different approaches to separate lipoprotein fraction from each other: particle size, charge, or density. One of the first innovations was the use of PEG-modified enzymes with sulphated α -cyclodextrin (Sugiuchi et al. 1995). Sulphated α -cyclodextrin together with Mg^{2+} selectively blocked but did not precipitate chylomicrons and VLDL particles. Cholesterol esterase and cholesterol oxidase modified with PEG showed selective catalytic activity towards different lipoproteins: HDL-C had the highest reactivity and LDL-C the lowest reactivity towards these enzymes. The PEG-method includes the apoE-containing HDL particles, instead of the PTA-method, which precipitates these particles (Warnick et al. 2001). ApoE-containing particles comprise on average 10 % of the total HDL population, and the same difference has been observed also in the method comparisons between PTA and dextran sulphate/heparin precipitation methods. In the method comparison we observed a 4 % increase in HDL-C concentrations with the PEG-method compared to the dextran sulphate precipitation method. Homogeneous methods have demonstrated better agreement with CDC's reference method than previous precipitation methods, but discrepancies still exist.

6.2 Systematic error in cholesterol, triglycerides, and HDL-C measurements

6.2.1 External quality assessment

Actions to improve the accuracy of laboratory measurements started in early 1970s in Finland (Grönroos et al. 1984). The target values of EQA programs are usually based either on the average values of participants' results or on the values measured by a reference method. The Finnish Labquality organization has offered EQA programs to clinical laboratories since 1971. TLAB has maintained EQA data from Labquality programs since 1978, WHO Lipid Reference Program 1978 – 1997, and CDC Lipid Standardization Program since 2001. For cholesterol and triglyceride measurements all these three programs have produced quite similar estimations for systematic error, but HDL-C measurements have turned out to be more complicated.

TLAB has been able to maintain the systematic error for total cholesterol and triglycerides nearly within $\pm 3\%$ and $\pm 5\%$, respectively, during the past four decades. Labquality's EQA samples have demonstrated more variations in reference materials (lyophilized human or animal sera, fresh human sera), which may have resulted in larger systematic errors in these EQA samples. Lyophilization of serum samples can change the composition of lipoprotein particles so different enzymes reach cholesterol inside lipoprotein particles slower than in fresh serum samples (Kroll and Chesler 1990). The reaction rates in different methods may vary causing method-dependent biases compared to the reference method. The comparison of fresh-frozen and lyophilized reference materials have been shown to result in 6% lower results with lyophilized sera compared to fresh frozen sera (Thienpont et al. 2003).

The assigned values of the calibrators are the most important factor affecting the systematic error of an analytical method. The different composition between lyophilized and fresh reference materials is a main cause for matrix effects, which have increased the preparation of commutable fresh or frozen reference materials (Cramb et al. 2008). The calibrator for lipid measurement should be fresh sera to avoid the matrix effect caused by lyophilized calibrators. In the end of 1980s CDC created the Cholesterol Reference Method Laboratory Network (CRMLN) to improve the traceability of commercial cholesterol assays to the reference method (Myers et al. 2000). The calibrators should show commutability with patient samples to avoid matrix effects and erroneous results in EQA programs and also in clinical practice (Warnick and Wood 1995).

The precipitation method for analysing HDL-C concentrations showed an average -5% bias compared to the CDC's reference method during the entire period 1980 – 1997. Because precipitation methods are based on different principles than direct methods to separate HDL from the other apoB-containing lipoproteins, method-dependent biases exist compared to the reference method. The experiences

concerning our own national EQA have shown a great variation in results between different manufacturers' direct HDL-C assays. Because this has occurred also with fresh serum samples, discrepancies are not related to matrix effect or non-commutable samples. The solution by the national EQA to this problem was to separate different manufacturers' assays to own result groups to achieve bias criterium. However, this practice obscures the differences between HDL-C methods and leads to an erroneous impression of the accuracy, which ultimately affects the risk scoring by the clinicians.

6.2.2 Systematic error and interpretations of population trends

Population trends have been measured since the 1970s in Finland first in cholesterol and triglyceride concentrations and later in HDL-C (Vartiainen et al. 2010). The same laboratory at the National Institute for Health and Welfare (former the National Public Health Institute) has been responsible for the laboratory measurements in FINRISK population studies since 1970s. During this time the mean cholesterol concentration in the Finnish population has decreased from nearly 7 mmol/l close to 5 mmol/l. At the same time HDL-C concentrations have remained quite stable, on average 1.3 mmol/l in men and 1.6 mmol/l in women.

According to our EQA data since 1982, the cholesterol bias has remained ± 3 % compared to the CDC's reference method. The systematic error has displayed only a minor effect when interpreting population trends. However, for example in 2002 the original data compared to the previous survey five years earlier, showed an 1.8 % increase for cholesterol in men and no change in women, but after bias-correction no change in men and -3.3 % change in women was found. The systematic error with an uncertainty (95% CI) has to be within quite narrow limits to make it possible to detect minor changes on a population level.

Salomaa et al reported in 1991 that the HDL-C concentrations of the population had possibly increased from 1982 to 1987 (Salomaa et al. 1991). However, they did not take into account the systematic error in the laboratory measurements. Our data demonstrated in fact a -9 % bias in 1982 HDL-C concentrations compared to an almost zero bias in 1987. The original HDL-C concentrations from FINRISK studies 1982 – 2012 showed a slightly positive trend, but after bias-correction this trend disappeared.

The standardization of methods and pre-analytical factors is important when comparing the prevalences of hypercholesterolemia and other dyslipidemias between different countries (Tolonen et al. 2005). In spite of the fairly good precision of cholesterol measurements, considerable systematic errors may exist between the laboratories. The laboratory measurements for Euroaspire I and II studies showed a 13 % difference between the cholesterol concentrations in two laboratories (EUROASPIRE I and II Group and European Action on Secondary Prevention by Intervention to Reduce Events 2001). The Diabetes Intervention

Study (DAIS) implemented a quality control program for the core laboratories, one in Canada and another in Finland (McGuinness et al. 2000). The Canadian Reference Laboratory assisted in the standardization and EQA samples for the core laboratories. This clinical trial succeeded well in maintaining the lipid and lipoprotein analyses within the allowable total error limits. Systematic and random errors in the laboratory measurements were identified and improved by the aid of the EQA program.

6.3 Transforming non-fasting triglyceride concentrations to fasting values

The overnight fasting is recommended to proper comparability of triglyceride concentrations, but in the population studies this demand is difficult to carry out. Triglyceride concentrations in serum increase after a meal and then start to decrease as the result of lipolysis by lipoprotein lipase. VLDL particles contain the most of the serum triglycerides in fasting condition. Triglyceride-rich lipoprotein remnants are considered to have a role in the progress of atherosclerotic plaques, because they are small enough to penetrate the arterial wall (Chapman et al. 2011).

The comparison of triglyceride concentrations in population studies is difficult, because of the different fasting period. This situation has also affected the calculations of LDL-C by Friedewald formula and the prevalences of metabolic syndrome. Our data from FINRISK studies 1992, 1997, and 2002 showed that triglyceride concentrations reached the plateau after seven hours fasting in women and after eight hours in men. We calculated conversion factors for transforming non-fasting triglyceride concentrations to the fasting values. The conversion factors were slightly diverse in different subgroups: the factor was -2.3 % per hour in low triglyceride concentrations compared to the -6.5 % in subjects whose BMI was $>35 \text{ kg/m}^2$. There was a consistent difference of 0.4 mmol/l in triglyceride concentrations between men and women. This sex difference should be taken into account when defining reference intervals and target values in the national guidelines.

The prevalences of high LDL-C and metabolic syndrome were calculated with the original non-fasting triglyceride values, with corrected values, and with true-fasting values. Non-fasting triglyceride concentrations resulted in a 5.1 % misclassification of patients having LDL-C higher than 3.00 mmol/l. Although triglycerides compose only a minor factor, when calculating LDL-C concentration by Friedewald formula, too high triglyceride concentration has an effect to the final LDL-C concentration. After correction the proportion of misclassified subjects decreased to 1.6 % compared to the true-fasting subjects. In the population level this means that some tens of thousands more subjects have increased risk for CVD. The prevalences for metabolic syndrome proved to be more comparable with true-fasting subjects after corrected the non-fasting triglyceride concentrations to true-fasting values: the difference between prevalences decreased from 4.2 % to 2.1 %. Using

the true-fasting triglyceride concentrations instead of non-fasting ones is physiologically relevant, when comparing prevalences for metabolic syndrome.

6.4 Reference intervals for apoA-I, apoB, and apo B/apoA-I ratio

Reference intervals for apoA-I, apoB and apoB/apoA-I ratio were calculated from the FINRISK 2007 study. The population sample was stratified according to age and sex, making it suitable for determining reference intervals. The healthy reference group was selected by excluding subjects who had obesity, self-reported CVD, hypertension, or diabetes. Women had higher apoA-I concentration than men in all age groups, but women gained men's apoB levels in the oldest age group of 65-75. The former reference intervals from the Finnish population were from 1990s (Leino et al. 1995) demonstrating lower apoA-I and higher apoB concentrations than our data. However, sex- and age-specific differences were quite similar in both studies.

A Swedish and a US population sample (NHANES III) showed on average 11 % lower mean values for apoA-I and 40 % higher for apoB when compared to our Finnish apoA-I and apoB values (Jungner et al. 1998, Bachorik et al. 1997). The subjects for the Swedish study were collected from the general healthcare without any exclusion of subjects, which may have affected the apoA-I and apoB concentrations. The subjects in NHANES III were a noninstitutionalized civilian population. In the Framingham Offspring Study the mean values for apoA-I (Contois et al. 1996a) and apoB (Contois et al. 1996b) in both sexes were similar with the Swedish study. The cut-off value of 1.2 g/l for low apoA-I was selected from the Framingham Offspring Study according to the 25th percentile value, which coincided with the HDL-C concentration 0.9 mmol/l in men. For women the same 25th percentile showed an apoA-I concentration of 1.34 g/l. However, the same cut-off value of 1.2 g/l for apoA-I was suggested for both men and women. Based on data in this thesis, cut-off values 1.2g/l for men and 1.4 g/l for women were selected. Because of the evident sex-difference between apoA-I concentrations, women having more than 10 % higher concentrations, the same cut-off values for both sexes underestimate the risk in women.

The cut-off value for apoB was selected to be equivalent to the LDL-C cut-off values. In the Framingham Offspring Study the high risk cut-off value for LDL-C was 1.60 g/l (4.1 mmol/l), which was the 75th percentile in men. The corresponding apoB concentration was 1.2 g/l. In Finland the cut-off value for LDL-C is 3.0 mmol/l, which was close to the 50th percentile in the FINRISK 2007 study (Graham et al. 2007). According to this data, the same cut-off value of 0.9 g/l was chosen for apoB in men and in women

The apoB/apoA-I ratio estimates the balance between atherogenic and antiatherogenic particles in the circulation. This ratio has turned out to be a better risk indicator for CVD than traditional cholesterol, LDL-C, HDL-C, and lipid ratios

(Walldius and Jungner 2006). The data from the AMORIS study and the INTERHEART study resulted in an increased risk for CVD with an apoB/apoA-I ratio of 0.6 for women and 0.7 for men (Walldius et al. 2001, Yusuf et al. 2004). In our data these apoB/apoA-I ratios coincided with the 75th percentile.

6.5 Method comparisons of two homogeneous HDL-C methods and two apoA-I methods

Since homogeneous HDL-C methods had been introduced to the clinical practice in 1990s, their specificity regarding abnormal lipoproteins has been challenged. Recently Miller et al. reported that homogeneous HDL-C and LDL-C measurements are nonspecific, when analysing samples from patients with CVD or other dyslipidemias (Miller et al. 2010). Our experiences from EQA programs have indicated that different manufacturers' assay systems occasionally produce enormously different HDL-C concentrations. To find out the actual situation in the clinical practice, we chose two methods demonstrating the largest discordance in the Finnish EQA program: PEG-method and ASD-method. This study was carried out in the occupational health care with fresh serum samples.

The results demonstrated the same phenomenon, which had been seen with some EQA samples: PEG-method produced on average 12 % lower results when HDL-C concentrations were <1.0 mmol/l and 9 % higher results when HDL-C was >1.55 mmol/l when compared to ASD-method. This concentration-dependent difference is almost impossible to correct with calibration, because the difference varies from negative to positive with increasing HDL-C concentration. The cut-off value for low HDL-C used in Finland is 1.0 mmol/l, so depending on the method used in the laboratory, patients may either be in the increased risk or low risk for CVD. According to Kappa statistic, agreement between these two homogeneous methods was only slight in women near the HDL-C cut-off value of 1.0 mmol/l. ApoA-I concentrations were also measured from the subjects and they showed better agreement in both sexes compared to the HDL-C measurements.

The basic principle in measuring cholesterol content in different lipoprotein particles has been to separate apoB-containing, non-HDL lipoproteins from the high-density lipoproteins (Warnick et al. 2001). The new innovation of using sulphated α -cyclo dextrin together with Mg^{2+} was discovered to selectively block but not precipitate chylomicrons and VLDL particles (Sugiuchi et al. 1995). This method used also PEG-modified enzymes, whose selectivity toward HDL-C was increased leaving LDL-C non-reacting. The increased β -VLDL in type III hyperlipoproteinemia overestimates HDL-C concentrations in PEG-method, because the PEG-modified enzymes apparently react with the cholesterol in VLDL particles (Lackner and Schmitz 1998). The other article reported in contrast that underestimation of HDL-C concentrations with PEG-method may occur when analysing samples from type III hyperlipoproteinemic patients (Roberts et al. 2000).

The mechanism behind the selectivity of PEG-modified enzymes is not clear, but the enzymes might recognise differences in lipoprotein particle density, net charge, or size (Sugiuchi et al. 1995). Increased number of sd-LDL particles in hyperbetalipoproteinemia may be one of the explanations to these discrepancies, because the change of particle size in turn can change the net charge of particles more negative (Prassl and Laggner 2008). The ASD-method is based on accelerating the reaction of cholesterol oxidase with cholesterol in non-HDL particles. The main principles to separate particles by this method have not been published in details, however, the bias between CDC's reference method has been narrower than with PEG-method in our laboratory.

Data in this thesis showed that differences between the HDL-C methods existed also when analysing apparently healthy subjects, so it is possible to misclassify people in the risk assessment for CVD in clinical practice. These differences in HDL-C measurements affect also the calculated non-HDL-C and LDL-C by the Friedewald formula. One suggested solution to resolve this problem has been to proceed to apolipoprotein measurements, which are better standardized than direct HDL-C and LDL-C methods and in addition, do not need fasting samples (Contois et al. 2011, Sniderman et al. 2011).

6.6 Lipids, lipoproteins, and apoA-I and apoB in different pathophysiological conditions

The changes in lifestyle have increased the prevalences in obesity, MetS and type 2 diabetes, which demonstrate different lipoprotein distributions from traditional cardiovascular diseases. Total cholesterol, triglycerides, and HDL-C measurements and calculated LDL-C are not enough anymore for accurate CVD risk assessment. Direct HDL-C and LDL-C measurements are not the best methods to estimate the numbers of HDL and LDL particles in serum, because of different cholesterol content in lipoprotein subpopulations. Occurrence of sd-LDL particles in obesity, MetS, and type 2 diabetes results in normal or even low LDL-C concentrations that may lead to misclassifications of these subjects.

In this thesis the healthy reference group from FINRISK 2007 study was compared with the different disease groups from the same study. Men with CVD, hypertension, diabetes, or obesity had lower total and LDL cholesterol compared to the reference group, and the mean apoB concentration was increased only in obese subjects. Women with CVD and diabetes had also lower total and LDL cholesterol compared to the reference group, but contrary to men, they had simultaneously higher apoB concentrations. In fact, women had higher apoB concentrations in all disease groups compared to the healthy reference group.

The nutritional recommendations for cardiovascular disease, type 2 diabetes, and MetS have emphasized reducing fat, especially saturated fat, in the diet (Catapano et al. 2011). However, a low-fat diet together with a high amount of carbohydrates

increases the atherogenic dyslipidemia: increased triglycerides, decreased HDL-C, and increased apoB-containing lipoproteins, especially sd-LDL (Parks and Hellerstein 2000, Siri-Tarino et al. 2010). High sugar consumption also may have association to type 2 diabetes and other metabolic disorders (Sonestedt et al. 2012). In addition to excess amount of free fatty acids, excess carbohydrates, especially fructose, increase the VLDL production in the liver (Zammit et al. 2001). On the other hand, increasing the amount of polyunsaturated fatty acids (PUFA) in the diet is associated with increased susceptibility of LDL particles to oxidation, which makes them more atherogenic (Reaven and Witztum 1996). Sd-LDL particles penetrate more easily the arterial wall and are oxidized as well as aggregated, which increase the formation of cholesterol-containing macrophages in the subendothelial space (Rizzo and Berneis 2007). Carbohydrate restriction in the diet has been shown to improve the atherogenic lipoprotein profile and symptoms of MetS and diabetes (Volek and Feinman 2005, Volek et al. 2008, Feinman and Volek 2008).

The amount of low-density lipoprotein particles in the blood circulation instead of their cholesterol content is suggested to be more relevant to the risk evaluation for CVD in many studies (Sniderman et al. 2011). The total number of atherogenic particles includes chylomicron remnants, VLDL, VLDL remnants, IDL, LDL, and Lp(a), which all contain apoB. Sniderman et al. reported recently results from a meta-analysis, which indicated that apoB was superior to non-HDL-C and LDL-C as a predictor of cardiovascular risk (Sniderman et al. 2011). According to their calculations the use of apoB as a risk marker could prevent 800 000 more events than the use of LDL-C in the US population during a 10-year period.

Some studies have shown that apoB or apoB/apoA-I ratio are better in the CVD risk assessment (Walldius et al. 2001, Yusuf et al. 2004), but there are also studies, for example the EPIC-Norfolk study, where no difference compared to the traditional lipids was observed (van der Steeg et al. 2007). However, the cohort of EPIC-Norfolk study was comprised of apparently healthy people and only few diabetic subjects. The data from the Women's Health Study and Framingham Offspring Study found also no evidence for replacing standard lipid measurements with apolipoprotein measurements in the primary risk screening (Ridker et al. 2005, Ingelsson et al. 2007).

A case-control study among myocardial infarction (MI) patients reported that apolipoprotein ratios were more informative regarding the MI risk than LDL-C or HDL-C, but direct measurements of specific lipoprotein subpopulations may be even more informative (Parish et al. 2009). The Emerging Risk Factors Collaboration concluded that the risk assessment of vascular diseases could be simplified by measuring total cholesterol and HDL-C or apoA-I and apoB without fasting and avoiding thus possible interferences from triglycerides (Emerging Risk Factors Collaboration et al. 2009).

6.7 Laboratory diagnostics of dyslipidemia

NCEP ATP established in 1988 that LDL-C was the principle target in the treatment of CVD. Since then homogeneous methods first to measure HDL-C and later LDL-C were developed. The secondary treatment goal for subjects with hypertriglyceridemia has been non-HDL-C. Total cholesterol and triglyceride measurements have been reasonably accurate during the decades, but the separation of different lipoprotein fractions has been challenging. Homogeneous HDL-C and LDL-C measurements separate lipoprotein fractions based on divergent mechanisms, which are not clear even to the reagent developers themselves (Sugiuchi et al. 1995). ApoA-I and apoB as the main proteins in HDL and LDL particles would be the obvious solution to this problem, but many arguments against them have been expressed (Boekholdt et al. 2012).

One of the counterclaims has been that the National Cholesterol Education Program Adult Treatment Panel is using the word “cholesterol” in the title of the guidelines. However, the European guidelines are titled as “cardiovascular disease prevention in clinical practice” and in Finland the guideline is for “treatment of dyslipidemia”. Because cholesterol in the circulation is mainly transported in the LDL particles, measurement of total cholesterol has been an apparently good estimate of atherogenic particles and the risk for CVD in the population level (Vartiainen et al. 2000). Measurement of serum total cholesterol, however, does not tell how cholesterol is divided between different lipoprotein fractions: atherogenic and antiatherogenic. The next effort to improve risk assessment for CVD has been to measure the cholesterol content in HDL and LDL particles, but these measurements have been proven to be nonspecific especially for atypical lipoprotein particles during different dyslipidemic states (Miller et al. 2010).

The major question has arisen: what is the best marker for increased risk for atherosclerosis: LDL-C, non-HDL-C, or apoB? LDL-C measures the amount of cholesterol in low-density lipoprotein particles, non-HDL-C consists of the cholesterol in all potentially atherogenic particles and apoB is an estimate of the number of atherogenic particles (Grundy 2002, Sniderman 2002, Contois et al. 2009). Direct LDL-C methods should ideally separate the relevant LDL particles for the cholesterol measurement and if calculated by the Friedewald formula, HDL-C methods should measure the cholesterol only from the circulating HDL particles. This could have been successful once lipoprotein particles comprised homogeneous population of particles with equal characteristics like size and charge. However, the size, charge, lipid and apolipoprotein compositions of lipoproteins vary according to the metabolic status (Rizzo and Berneis 2007). Non-HDL-C has been suggested to be an estimate of apoB particles especially in hypertriglyceridemia. However, non-HDL-C and apoB are not equal and can lead to different CVD risk assessment (Sniderman et al. 2010).

The recommendations for the diagnosis and treatment of dyslipidemia and prevention of CVD by the Canadian Cardiovascular Society are one of the first guidelines to include apoB to the risk assessment (McPherson et al. 2006). The American Diabetes Association and the American College of Cardiology Foundation suggested using apoB measurements for patients with cardiometabolic risk during statin therapy in addition to LDL-C and non-HDL-C measurements (Brunzell et al. 2008). The American Association for Clinical Chemistry has given a position statement to implement apoB measurement together with LDL-C to the NCEP ATP III guidelines in the US (Contois et al. 2009).

The flow chart in Figure 17 demonstrates the proposed laboratory measurements to diagnose dyslipidemia. Because patients with obesity, MetS, and type 2 diabetes may have an increased number of atherogenic particles in the circulation, the apoA-I and apoB measurements should be included to the basic screening tests for dyslipidemia (Marcovina and Packard 2006). Also if the triglyceride concentration is >1.7 mmol/l or HDL-C concentration is <1.0 mmol/l in men or <1.2 mmol/l in women, apoA-I and apoB concentrations should be measured after the screening tests: total cholesterol, HDL-C, triglycerides and LDL-C.

Table 24 shows the suggested therapeutic target goals for low risk subjects. For higher risk patients the target goals for LDL-C and apoB should be lower. The NCEP ATP III guidelines have suggested the target LDL-C concentration as low as 1.8 mmol/l for the highest risk patients (Grundy et al. 2004), which is equivalent with an apoB concentration of 0.6 g/l. Because non-HDL-C is based on the HDL-C measurement, the differences between direct HDL-C methods may have an impact on the accuracy of non-HDL-C concentration and as a consequence to the risk assessment. In this context the direct measurement of apoB is a superior estimate of the atherogenic particles in the circulation (Barter et al. 2006, Sniderman et al. 2011). HDL-C and apoA-I concentrations are higher in women than in men, thus this sex-difference should be taken into account also in target goals for dyslipidemia. As with apoB, also apoA-I is a better estimate for antiatherogenic HDL particles than the HDL-C concentration, which may vary according to the method. ApoA-I and apoB measurements do not need fasting before blood sampling, and non-fasting triglycerides could be an even better measurement for the CVD risk assessment (Nordestgaard et al. 2007, Langsted et al. 2008).

In the future, the analyses of different HDL and LDL subpopulations might produce even more specific information of these particles' atherogenic or antiatherogenic properties. However, substituting apoA-I and apoB measurements for direct HDL-C and LDL-C methods could be a proper step forward to more accurate CVD risk assessment.

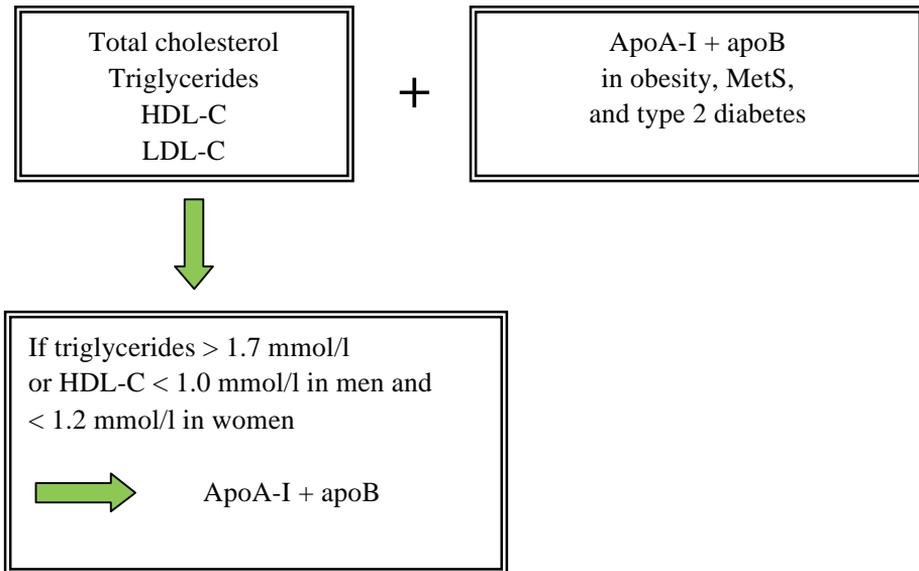


Figure 17. Proposed chart of laboratory diagnostics for dyslipidemia based on the data in this thesis.

Table 24. Suggested treatment goals for lipids, lipoproteins, and apolipoproteins for low risk subjects based on the European guidelines (Graham et al. 2007) and data in this thesis.

Measurement	Men	Women
Total cholesterol, mmol/l	<5.0	<5.0
LDL-C, mmol/l	<3.0	<3.0
Triglycerides, mmol/l	<1.7	<1.7
HDL-C, mmol/l	>1.0	>1.2
ApoA-I, g/l	>1.2	>1.4
ApoB, g/l	<0.9	<0.9
ApoB/apoA-I	<0.7	<0.6

7 Conclusions

Most of the serum cholesterol is transported as a cargo inside low-density lipoproteins. Because proportion of low-density lipoproteins is about 70 % of circulating lipoproteins, total cholesterol and LDL-C measurements have been good estimates for the amount of atherogenic particles in the circulation. However, LDL particle number is more critical in the genesis of atherosclerosis than their cholesterol content. Obesity, MetS, and type 2 diabetes appear commonly with normal or even decreased total cholesterol and LDL-C, but increased numbers of triglyceride-rich lipoproteins and sd-LDL, and decreased HDL-C. ApoB incorporates all atherogenic lipoproteins from chylomicron remnants to LDL particles, which all can participate in the genesis and progression of atherosclerosis. Cholesterol-lowering should not be the main aim of CVD prevention anymore, but lowering the number of atherogenic particles as well as increasing those HDL subpopulations that really display protection.

Major conclusions of this thesis are as follows:

- Participating in the external quality assessment programs with target values measured by reference methods is essential, when interpreting the effects of the systematic errors on the population trends.
- Transforming non-fasting triglyceride concentrations to fasting values has minor, although significant effect on the prevalences in high LDL-C and MetS.
- Two homogeneous HDL-C methods demonstrated concentration-dependent differences in HDL-C concentrations, which may cause misclassification of subjects in the risk assessment of cardiovascular disease.
- ApoB measurements produce more specific information for the risk evaluation of CVD, especially in obesity, MetS, and type 2 diabetes as compared to total cholesterol and LDL-C measurements.

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