THE DEVELOPMENT AND APPLICATION OF A NOVEL METHOD FOR THE QUANTIFICATION OF PREBETA-1 HDL IN PLASMA

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DECLARATION

I certify that none of the work in this thesis has been submitted previously for any degree or diploma at this, or any other university, and that the work described in this thesis, except where duly acknowledged, is my own.

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SUMMARY

The atheroprotective properties of high-density lipoprotein (HDL) are associated with the ability of HDL to initiate reverse cholesterol transport (RCT), a process whereby excess cholesterol is transported from peripheral cells to the liver for excretion (Miller & Miller, 1975). For the past 5 decades HDL quantification has been based on the cholesterol content of HDL (HDL c). While low levels of HDLc are epidemiologically predictive of atherothrombotic cardiovascular events in large populations, this measurement appears to be an unreliable indicator in the precise determination of individual cardiovascular risk (Bruckert & Hansel, 2007). This may relate to the complex biology of HDL, a deeper understanding of which began with the discovery in the Kane laboratory that HDL is not one single lipoprotein entity but consists, of at least 20 individual structurally and functionally distinct heterogeneous, molecular subspecies which are rapidly and continuously interconverted (Kunitake & Kane, 1982).

Prebeta-1 HDL was one of the distinct molecular subspecies of HDL identified in the mid to late eighties (Castro & Fielding, 1988; Kunitake, La Sala & Kane, 1985). It is, now recognized as having a pivotal role in reverse cholesterol transport accounting for up to 90% of cholesterol trafficking from the periphery. Prebeta-1 HDL does so by acting as the quantum particle and primary acceptor of free cholesterol effluxed by the ATP-binding cassette A1 (ABCA1) transporter from arterial macrophages, which are central to the atherosclerotic process (Tall, Yvan-Charvet, Terasaka, Pagler & Wang, 2008). The ability to measure circulating levels of prebeta-1HDL may provide a novel biomarker of cardiovascular risk. The need for a novel, reliable, easy to use method to quantify prebeta-1 HDL in large numbers of patient samples is underscored by the number of technically difficult methods previously described, many of which tend to be slow and semi quantitative, only catering for small numbers of samples at any one time (Nanjee & Brinton, 2000).

The method developed and described in this thesis provides a robust technique for quantifying plasma prebeta-1 HDL, utilizing ultrafiltration of plasma, isotope dilution and an enzyme-linked immunosorbant assay (ELIZA) modified to allow the equal detection of apolipoprotein A-I (apoA-I) in prebeta-1 HDL and in other plasma HDL particle species. This method quantifies the fraction of total apolipoprotein A-I that is present in prebeta-1 HDL particle species.

In brief, using low-velocity centrifugation in ultrafilters with a 100 KDa cut off, it was demonstrated that prebeta-1 HDL, could be separated categorically from larger-diameter HDL particles in plasma and quantified by application of the isotope dilution principle and measurement of Apo A-I by ELIZA. Levels of prebeta-1-HDL are reported as prebeta-1-HDL–A-I.

This method was then utilized to measure prebeta-1 HDL levels: Firstly in 136 normal males and females, which found that, lower levels of prebeta-1 HDL were associated with female gender. Secondly, as exercise is associated with prevention of coronary artery disease, and is thought to be mechanistically related to its HDL raising effect, the effects of acute physical exercise on prebeta-1 HDL was also examined in normolipidemic subjects, demonstrating that acute exercise significantly increased absolute plasma prebeta-1HDL levels. Finally prebeta-1 HDL levels were also measured in large well-characterized clinical cohort of 1255 subjects from the University of California San Francisco Lipid and Cardiovascular Clinics and collaborating Cardiologists. An association was found between, increased absolute levels of prebeta-1 HDL and structural coronary disease and myocardial infarction. This was the first study in which prebeta -1 HDL was identified as a novel and independent predictor of MI above and beyond traditional CHD risk.

There is a continued need to understand, validate, and quantify the diverse roles of HDL subspecies in the atherosclerotic process in order to improve diagnosis, prevention, and treatment of coronary heart disease. The ability to make quantitative measurements of the quantum particle prebeta-1 HDL in the reverse cholesterol pathway, contributes

materially to the armamentarium of cardiovascular risk biomarkers. Measurement of prebeta-1 HDL levels may also assist in both, refining our understanding of the molecular pathophysiology of atherosclerosis, and in the development of pharmacological interventions that will reduce residual risk, thus enhancing the ability of the clinician to optimally manage patients. The innovative features of this novel method of quantifying prebeta-1 HDL described in this thesis are its scalability and its potential to measure large numbers of patient's samples concurrently for clinical purposes.

This research has been published (Guey et al., 2011; Jafari et al., 2003; O'Connor et al., 1997; O'Connor et al., 1998).

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INDEX OF ABBREVIATIONS

ANOVA:	1-way analysis of variance
BSA:	Bovine serum albumin
CHD:	Coronary heart disease
CPM:	Counts per minute
FC:	Free cholesterol
HDL:	High density lipoproteins
HDLc:	High density lipoprotein cholesterol
HL:	Hepatic Lipase
HRP:	Horse radish peroxidase.
LDL :	Low density lipoproteins
VLDL:	Very Low Density Lipoproteins
LCAT :	Lecithin cholesterol acyltransferase
CETP:	Cholesteryl ester transfer protein
FPLC:	Fast protein liquid chromatography
kDA :	Kilodaltons
PLTP:	Phospholipid transfer protein
SR-BI:	Scavenger receptor, type B, one
BMI :	Body mass index
ELISA:	Enzyme-linked immunosorbent assay
ESTRSTAT:	Estrogen-based statistical variable
GENESTR:	Gender- and estrogen-based statistical variable
PBS:	Phosphate-buffered
PCTPREB:	Percent of total apoA-I in prebeta-1 HDL
RPCTPREB:	Square root of PCTPREB A
BSPREB:	Absolute amount apoA-I in prebeta-1 HDL µg/ml
RABSPREB :	Square root of ABSPREB
SED:	Sedentary
TG:	Triglyceride

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Chapter 1

HDL Historical Aspects

1.1 Introduction

Coronary atherosclerosis remains the greatest cause of morbidity and mortality in both men and women in the western world. Refining cardiovascular risk assessment remains challenging and efforts have continued in this area over many decades. Our understanding of HDLc and its complex relationship to atherosclerosis has evolved significantly over recent decades. For many years the concept of HDLc being "good cholesterol" or a strong negative risk factor for cardiovascular disease were based on the early studies of Glomset (Glomset, 1968) , Miller (Miller & Miller, 1975) and the Framingham study in the 1960's and 1970's (Gordon, Castelli, Hjortland, Kannel & Dawber, 1977)which confirmed that HDLc is a strong negative risk marker for cardiovascular disease. Between 1994 and 2001 further studies, including both primary and secondary prevention studies, confirmed the cardiovascular benefits of pharmacologically raising HDL.

Increased understanding of the molecular pathophysiology of atherosclerosis and the identification of several subspecies of HDL and their role in this process has led to identification of new risk markers. The makeup of HDL subspecies is dynamic, as different lipolytic enzymes, lipid transporters and apoprotein exchange mechanisms contribute to the formation and interconversion and remodeling of HDL subspecies (Ji, Wroblewski, Cai, de Beer, Webb & van der Westhuyzen, 2012). Individual HDL subspecies appear to have diverse functions across lipid metabolism, including capacity to mediate cellular efflux of cholesterol by acting as primary acceptors of cholesterol, anti-inflammatory, antithrombotic, host defense and antioxidative functions. Thus total HDLc measurement, while representing global HDL cholesterol content, is too gross a measure to capture the functional variation in individual HDL particles and their association with the risk of cardiovascular disease. There is therefore an increasing need to understand, validate, and quantify the diverse roles and metabolic transitions among individual HDL particles in the atherosclerotic

process, in order to improve diagnosis, prevention, and treatment of cardiovascular disease (Brewer, 2007; Rosenson, 2010). A significant challenge is the lack of robust scalable methods to quantitate HDL subspecies. Prebeta-1 HDL is a 67-kDA species of plasma HDL that contains two copies of apoA-I. It functions in a metabolic cycle of cholesterol retrieval and may be formed during lipolysis in plasma. It has been identified as the quantum particle in reverse cholesterol transport and therefore has a pivotal role in the cholesterol retrieval pathway. The ability to quantify prebeta-1HDL levels may be useful in refining risk stratification for atherosclerotic vascular disease. The main objective of this research was to develop a scalable method for the quantification of prebeta-1 HDL and to use this method to determine the normal levels and also to measure prebeta-1 HDL in various populations.

1.2 HDL and Atherosclerosis

The term "atherosclerosis" was first proposed by the pathologist Felix Marchand in 1904, from the Greek "athero," meaning gruel or paste, and "sclerosis," meaning hardening, to describe the appearance that he observed inside a hardened artery (Li & Fang, 2004).The role of cholesterol, in the pathogenesis of atherosclerosis has been elucidated via experimental, genetic, epidemiologic and therapeutic research and has been described as one of cardiology's great 20th century discoveries (Mehta & Khan, 2002) .

In 1908, Ignatowski described a relationship between cholesterol-rich food and experimental atherosclerosis (Ignatowski 1909). In 1910, Windaus showed that atheromatous lesions contained six times as much free cholesterol and twenty times as much esterified cholesterol as a normal arterial wall (Li & Fang, 2004). Subsequently in 1913, Anichkov fed pure cholesterol to rabbits, which resulted in marked hypercholesterolemia and severe atherosclerosis of the aorta. This was the first experimental production of atherosclerosis and has been reproduced and validated many times in many species over the intervening years (Anitschkow N, 1913). In 1951, Barr's group reported that a minor component of the plasma lipoproteins, now known as high

density lipoprotein or HDL, was paradoxically reduced in patients who had coronary heart disease, in contrast to the bulk of plasma lipoproteins that were notably increased (Barr, Russ & Eder, 1951). In 1964 Glomset and Wright proposed a concept of reverse cholesterol transport, with cholesterol being transported from the periphery to the liver for metabolism and excretion, and suggested the use of HDLc concentration as a biomarker of efflux (Glomset & Wright, 1964).

1.3 Primary and secondary prevention studies raising HDL

Between the early 1960's and 2009 HDL cholesterol was investigated as a risk marker in 68 long-term population-based studies involving more than 300,000 individuals. In multivariate models adjusted for non-lipid and lipid risk factors, raised levels of HDL cholesterol were identified as having an atheroprotective effect (Emerging Risk Factors et al., 2009).

1.3.1 The Framingham Heart Study

The Framingham Heart Study, which followed 2815 men and women aged 49–82 years, reported in 1961, the same inverse correlation between HDL-cholesterol (HDL-C) and coronary risk: the higher the HDL-C, the lower the risk for a coronary event, to the extent that a 1 mg/dl increase in HDL-C corresponded with a 1% decrease in CHD risk (Kannel, Dawber, Kagan, Revotskie & Stokes, 1961). Similarly, in 1983, the Prospective Cardiovascular Munster (PROCAM) study (Kannel, 1983) reported a strong negative linear correlation between the incidence of CHD and HDL-C levels (CHD risk ratio of 4.0 for HDL-C <25 mg/dL versus 1.0 for HDL-C >65 mg/dL, $p < 0.001$) in 4559 male participants aged 40–64 years .

1.3.2 The Helsinki Heart Study

The Helsinki Heart Study was one of the first clinical trials that demonstrated the benefit of raising HDL-C and lowering triglyceride levels in individuals with low baseline HDL-C levels was (Manninen et al., 1992). 4081 men with dyslipidemia were randomized to receive the fibrate drug gemfibrozil or placebo. At 5 years follow-up, gemfibrozil therapy increased HDL-C levels by 11% and reduced total cholesterol (TC), LDL-C, and TG levels by 10%, 11%, and 35% respectively. Gemfibrozil therapy reduced the primary end point of cardiac death or non-fatal MI by 34% (27.3 vs 41.4/1,000, $p < 0.02$) with the greatest reduction found in patients with baseline low HDL-C and high triglycerides (Manttari et al., 1990)(Manttari et al 1990).

1.3.3 The Scandinavian Simvastatin Survival Study Group

The Scandinavian Simvastatin Survival Study Group (4S) was published in 1994. (1994). This was a large, randomized, placebo-controlled trial evaluating simvastatin (20–40 mg/day) in 4444 men and women aged 35–70 years over a median follow-up period of 5.4 years. Simvastatin therapy decreased TC and LDL-C 25% and 35%, respectively and increased HDL-C by 8% compared to placebo. Simvastatin treatment resulted in a 30% relative risk reduction in overall mortality (8.2% vs 11.5%, $p = 0.0003$) and reduced non-fatal MI, ischemic heart disease death, and coronary revascularization (1994).

1.3.4 The Air Force/Texas Coronary Atherosclerosis Prevention Study

In 1998 the Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS) (Downs et al., 1998) confirmed an increased risk associated with low serum levels of HDL-C and the beneficial effects of pharmacotherapy in adults with low HDL-C in primary prevention. This study compared lovastatin versus placebo for the prevention of a first major coronary event in adults with average TC and LDL-C levels, but low baseline levels of HDL-C. Lovastatin decreased both TC and LDL-C levels by 18% and 25%, respectively,

while increasing HDL-C levels by 6%. After more than 5 years of follow-up, the absolute risk in the primary composite end point of fatal or non-fatal MI, unstable angina, or sudden cardiac death was reduced in absolute terms by 2.2% in men and 1.2% in women with a relative risk reduction of 37%. This study was the first primary prevention study to show that individuals with HDL-C <40 mg/dL received the greatest benefit.

1.3.5 The Veterans Affairs High-Density Lipoprotein Cholesterol Intervention Trial

In 2001, patients with manifest CHD were also reported to benefit from pharmacologically raising HDL-C and lowering TG. The Veterans Affairs High-Density Lipoprotein Cholesterol Intervention Trial (VA-HIT) compared treatment with gemfibrozil versus placebo in more than 2500 men with established CHD, average LDL-C levels (<140 mg/dL), and low HDL-C levels (<40 mg/dL). After a mean follow-up of 5 years, gemfibrozil decreased TG levels by 31% and increased HDL-C levels by 6%, while levels of LDL-C remained quantitatively unchanged; there was a relative risk reduction of 22% (17.3% vs 21.7% $p < 0.006$) in CHD death and non-fatal MI in the treatment group. Gemfibrozil therapy was associated with a 24% relative risk reduction in the composite end point of nonfatal MI, stroke, and CHD death ($p < 0.001$) (Robins et al., 2001).

1.4 HDL and it role in reverse cholesterol transport

The atheroprotective properties of HDL are associated with its ability to initiate reverse cholesterol transport, a process whereby excess cholesterol is transported from peripheral cells to the liver for excretion (Miller & Miller, 1975) (Tall, Yvan-Charvet, Terasaka, Pagler & Wang, 2008). However in addition substantial evidence supports many additional atheroprotective pleiotropic effects of HDL including anti-oxidant, anti-thrombotic and anti-inflammatory effects (Bonnefont-Rousselot, Therond, Beaudeux, Peynet, Legrand & Delattre, 1999; Hayek, Oiknine, Dankner, Brook & Aviram, 1995; Mineo, Deguchi,

Griffin & Shaul, 2006; Navab et al., 1996; Watson et al., 1995). The traditional measurement of HDL cholesterol capturing the cholesterol content within all of the HDL particles, while epidemiologically predictive of atherosclerotic cardiovascular events in large populations, appears to be an unreliable indicator in the precise determination of cardiovascular risk (Bruckert & Hansel, 2007).

At a molecular level the underlying exact physiological mechanisms of HDL's atheroprotective effects are not completely understood. This is primarily because HDL is not a single lipoprotein entity as was previously thought, but consists of a spectrum of several complex structurally and functionally heterogeneous, distinct molecular subspecies which are rapidly and continuously interconverted (Kontush A, 2012). These HDL subspecies differ in physical-chemical properties, protein and lipid composition, metabolism, and physiological functions and increasingly, evidence suggests, in pathophysiological significance. It appears that both the quantity and quality of the circulating HDL subspecies may be important to the optimal function and antiatherogenic potential of HDL. Thus HDL subspecies are functionally heterogeneous and it appears that they vary in terms of their ability to protect against coronary heart disease. However many challenges remain with respect to elucidating these functions, as HDL subspecies are difficult to separate and quantify (Asztalos, Tani & Schaefer, 2011)

The challenges surrounding research into HDL biology were underscored by the discovery that preparative ultracentrifugation which isolates HDL as a function of size and density in a high salt solution between 1.063 and 1.21 g/mL.(Havel, Eder & Bragdon, 1955) leads to destruction and loss of a number of molecular species of HDL(Kunitake & Kane, 1982). This discovery drove the development of a nondenaturing minimally perturbing chromatographic strategy of selected-affinity immunosorption by the Kane group, (McVicar, Kunitake, Hamilton & Kane, 1984) which allowed quantitative recovery of HDL particles under minimally denaturing conditions. In addition to a number of other species of HDL, this method facilitated the discovery

by Kane's group of a HDL particle with prebeta mobility (prebeta-1 HDL) that could be isolated in pure form and in bulk by electrophoresis (Kunitake, La Sala & Kane, 1985). Figure 1.1 shows a 2-D western blot of plasma demonstrating multiple HDL subspecies using an anti Apo-AI antibody to detect Apo A-I containing (HDL by definition) lipoproteins.

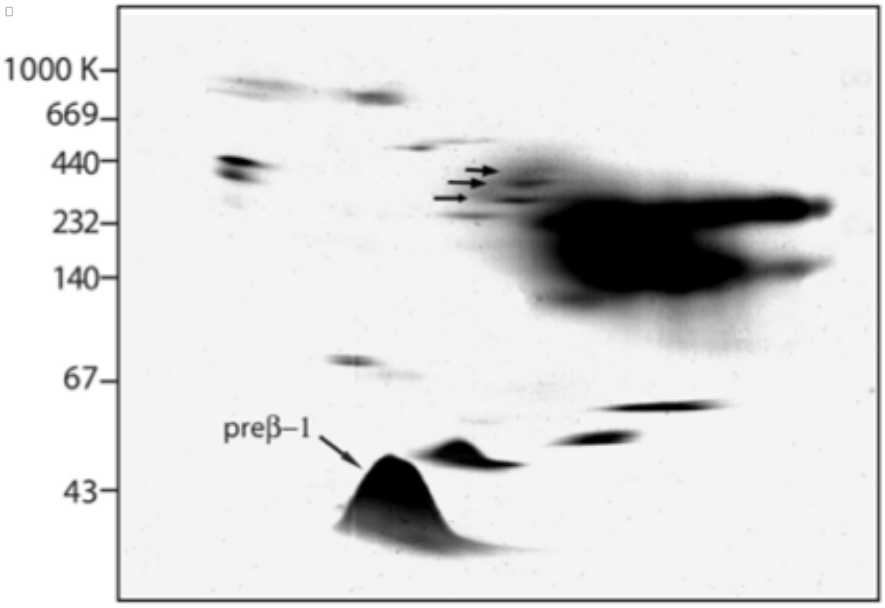

Figure 1.1. ***2D gel electrophoresis of plasma demonstrating multiple HDL subspecies (western blotting using anti-ApoA-I) (Reproduced with permission from Kunitake and Kane).***

Chapter 2

Characteristics of prebeta-1 HDL

2.1 Introduction

Prebeta-1 HDL was so named on the basis of its electrophoretic mobility, and was found to have a particle mass of about 65 kDa. About ninety percent of its mass is exclusively apoA-I protein. The remainder of the mass is comprised of unesterified cholesterol and phospholipid, a small amount of cholesteryl esters, and no triglyceride. (Kunitake, La Sala & Kane, 1985)

The structure of the prebeta-1 HDL particle has been a matter of controversy. It has been proposed by a number of groups that it is actually lipid-free monomeric apoA-I however this has not been confirmed in the Kane laboratory (Ishida B and Kane JP, personal communication). The lack of formation of cross linked dimers after exposure of prebeta-1 HDL to a crosslinking reagent was inferred to indicate that there is a single copy of apoA-I in the particle (Nakamura, Kotite, Gan, Spencer, Fielding & Fielding, 2004), however the conformation of the protein may not be susceptible to crosslinking. Also, the relative proportion of lipid to protein is appropriate for a particle containing two copies of apoA-I. Kane et al have obtained cryoelectronmicroscopic images that are compatible with a twisted ellipse conformation containing two copies of apoA-I in prebeta HDL (Schumaker VN, Phillips ML, and Kane JP personal communication). Many studies have been carried out with reconstituted HDL particles that form when apoA-I is sonicated with an excess of phospholipid. The resulting particles are discoidal. Similar particles are not found in normal plasma, but discoidal forms are demonstrable by electronmicroscopy in LCAT deficient plasma. The apoA-I in the prebeta- 1 HDL particles was found to have less helical conformation than in other HDL species (Kunitake, Chen, Kung, Schilling, Hardman & Kane, 1990). Subsequent studies were in keeping with this finding such as the characterization of a monoclonal antibody that discriminates between the conformation of apoA-I in prebeta-1 HDL and larger HDL particles (Fielding, Kawano, Catapano, Zoppo, Marcovina & Fielding, 1994).

This unique conformation of apoA-1 in prebeta-1 HDL may be of key importance for the receptor interactions of prebeta1HDL and this may be the primary basis for the diminished affinity of prebeta-1 HDL for binding to "traditional HDL receptors" in human hepatocytes (Kunitake, Mendel & Hennessy, 1992) and to the scavenger receptor class B type I (SR-BI) receptor (Liadaki et al., 2000). These properties suggested a special role or roles for prebeta-1 HDL in lipid metabolism. The scavenger receptor class B type I (SR-B1) is known to play an important role in meditating the uptake of HDL-derived cholesterol and cholesteryl ester in the liver and steroidogenic tissues, however prebeta- 1 HDL was therefore not involved in this process (Valacchi, Sticozzi, Lim & Pecorelli, 2011).

2.2 Prebeta-1 HDL cycle

A number of important studies in the late 80's and early 90's elucidated the natural life cycle of prebeta-1 HDL in the plasma and gave clues to its function in reverse cholesterol transport. Firstly it was found that prebeta-1 HDL particles disappear when plasma is incubated at 37^0C, adding to the mass of larger a HDL, thus appearing to be converted into the larger HDL species. Secondly an LCAT inhibitor blocked this conversion, indicating that prebeta-1 HDL is a substrate for cholesterol esterification by that enzyme. Thirdly the pre-beta-1 particles were shown to acquire cholesterol from fibroblasts (Castro & Fielding, 1988), and incubation of large alpha HDL particles with CETP released prebeta-1 HDL (Kunitake, Mendel & Hennessy, 1992). Subsequent studies confirmed that prebeta-1 HDL is a substrate for LCAT, and that it is produced during cholesteryl ester transfer by CETP. A role for phospholid transfer protein was also discovered in the formation of pre-beta-1 HDL (Nakamura, Kotite, Gan, Spencer, Fielding & Fielding, 2004)

Prebeta-1 HDL is, now recognized as having a pivotal role in reverse cholesterol transport acting as the quantum particle and primary acceptor of free cholesterol effluxed by the dominant ATP-binding cassette A1 (ABCA1) transporter in arterial macrophages (Castro &

Fielding, 1988; Kunitake, La Sala & Kane, 1985). ABCA1 transporter is a cholesterol efflux regulatory protein, which is a major regulator of cholesterol and phospholipid homeostasis through out the body. It appears that there is a network of ABC transporters involved in cellular lipid homeostasis, directly linked to high density lipoprotein metabolism and atherogenesis (Schmitz & Langmann, 2001). In summary HDL mediated reverse cholesterol transport can be divided into four phases (Figure 2.1)

Phase 1: Transfer of free cholesterol (FC) to prebeta-1 HDL via ABCA1

Pre-β HDL is formed firstly as nascent apolipoprotein A1 by either de novo secretion from hepatocytes or the intestinal mucosa, direct dissociation from chylomicrons and very low density lipoprotein (VLDL) mediated by lipoprotein lipase (LL), it is also a substrate and a product in the interconversion of HDL species (Kwiterovich, 1998). Once generated, pre-β HDL receives free cholesterol and phospholipid from peripheral cells (Castro & Fielding, 1988) by associating with the surface protein ABCA1 (Oram & Lawn, 2001) (Wang, Lan, Chen, Matsuura & Tall, 2004) which is expressed by the liver and intestinal mucosa (Adorni et al., 2007)

Phase 2: Esterification of surface-associated FC by the enzyme LCAT

As cholesteryl esters (CE) accumulate, they form a core in the larger spherical alpha-HDL particles, subsuming prebeta-1 HDL (Kunitake, Mendel & Hennessy, 1992). Cholesterol esterification prevents transfer of FC back to the periphery; thereby potentiating further RCT. Mediating this necessary step in HDL maturation is the enzyme LCAT, which is synthesized by the liver. Circulating LCAT esterifies lecithin and FC on both HDL and Apo B containing lipoproteins. The phospholipid component of HDL-C appears to mediate binding to LCAT, while the apolipoprotein component activates the enzyme (Jonas, 2000).

Phase 3: Transfer of FC and TG between HDL-C and Apo B-containing lipoproteins mediated by the enzyme CETP

The enzyme cholesteryl ester transfer protein (CETP) exchanges CE from large spherical alpha-HDL for TG in LDL-C and VLDL facilitating regeneration of prebeta HDL. Newly acquired CE in VLDL and LDL-C is then taken up by the hepatic LDL receptor for excretion as bile (Morton & Greene, 1997).

Phase 4: Uptake by SR-B1 receptor on the liver and catabolism of mature HDL-C into bile or small HDL-C particles by HL

As CE accumulate in its central core, pre-β HDL-C matures into larger spherical alpha- HDL particles These larger molecules undergo hepatic catabolism and excretion in bile.

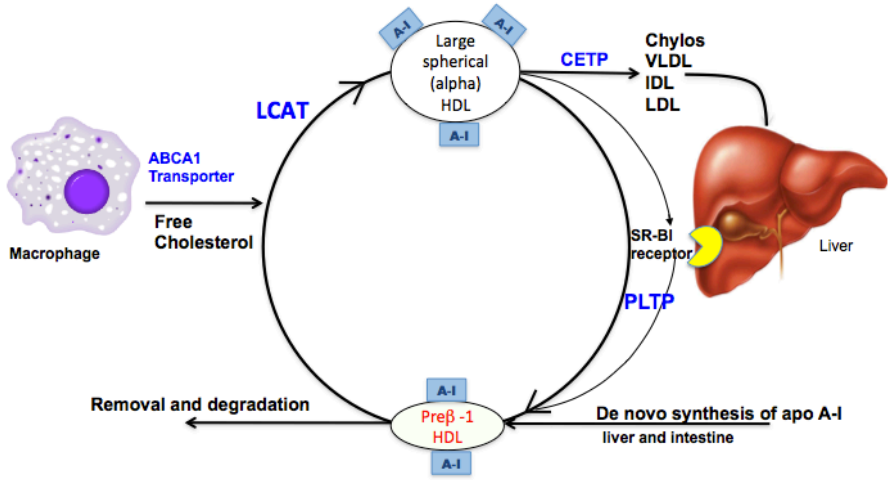

Figure 2.1. ***The prebeta-1 HDL cycle***
The steady-state level of pre beta-1 HDL in plasma reflects opposing rates of formation and removal. It acquires free cholesterol effluxed from the ABCA1 transporter that is then esterified with a free fatty acid derived from lecithin mediated by LCAT. Cholesteryl esters are then incorporated into cores of large-diameter high-density lipoprotein particles (alpha–high-density lipoprotein). Apo A-I is subsumed into those particles. Cholesteryl esters are transferred from alpha -HDL into cores of acceptor lipoproteins by CETP. Most cholesteryl esters are ultimately delivered to the liver in remnant particles and low-density lipoproteins. Cholesteryl esters are also transferred to hepatocytes by SR-BI receptor, liberating prebeta -1 HDL. Concomitant with transfer of cholesteryl esters, pre beta-1 HDL is regenerated. It is also formed de novo during secretion of newly synthesized apoA-I from the liver and intestine and participates in catabolism of apoA-I in the kidney (Guey et al., 2011)

The steady state levels of prebeta-1 HDL must therefore reflect the rate of generation from denovo synthesis, the rate of removal by LCAT esterification of its free cholesterol, its regeneration during alpha HDL catabolism including transfer of cholesterol esters from HDL to acceptor lipoproteins by CETP and the action of PLTP, and loss to the system via ultrafiltration at the glomerulus (figure 2.1). Since prebeta-1 HDL occupies a central role in the reverse cholesterol transport system, an ability to quantify this HDL sub species may refine the assessment the clinical effects of different agents that modulate its function and metabolism, but as a measurable biomarker it may also allow a more efficient prediction of cardiovascular risk enhancing the ability of clinicians to optimally manage patients.

2.3 Justification for Research described in this thesis

Since prebeta-1HDL is known to be the quantum particle in RCT, insight into changes in prebeta-1 HDL levels in health and disease states may provide a greater understanding of its relationship to vascular health and may provide some insight into the mechanisms associated with the strong inverse association between total HDL cholesterol and cardiovascular disease. The ability to measure prebeta-1HDL levels may provide a novel biomarker of cardiovascular risk and may help to identify new therapeutic targets.

2.4 Limitations of methods previously described for measurement of prebeta-1 HDL

The requirement for a novel reliable easy to use method to quantify prebeta-1 HDL directly from plasma is underscored by the number of previously described methods, which are slow, technically difficult and generally semi quantitative, catering for small numbers of samples at any one time. In addition some of these methods fail to fully separate the quantum prebeta-1 HDL subspecies from other HDL subspecies with prebeta electrophoretic mobility.

Prebeta HDL levels have been measured using autoradiography (Ishida, Frolich & Fielding, 1987) (Francone, Fielding & Fielding, 1990), chemiluminescence (O'Kane, Wisdom, McEneny, McFerran & Trimble,

1992), and 2-D electrophoresis with phosphorimaging of anti-apoA-I Western blots from two-dimensional agarose/native gradient polyacrylamide gel electrophoretic separations (Asztalos, Sloop, Wong & Roheim, 1993). These methods tend to be semiquantitative because of intrinsic nonlinearities in solid-phase capture efficiency and immunologic staining intensity of the apoA-I moiety in lipid-poor, prebeta-1 HDL compared with lipid-rich, large HDL (Lefevre, Goudey-Lefevre & Roheim, 1987). Prebeta-1 HDL has also been measured in whole plasma using agarose electrophoresis followed by immunofixation (Giunta, Maddalena-Peruzzi, Gaudio & Semprini, 1992; Semprini, Maddalena-Feruzzi & Giunta, 1992), immunoprecipitation (Daerr, Minzlaff & Greten, 1986; Kunitake, La Sala & Kane, 1985), Western blotting (Miida et al., 1996) and radial immunodiffusion (Borresen & Berg, 1980) A monoclonal antibody capable of selectively binding pre-β_1 LpA-I, has been described (Miyazaki, Kobayashi, Fukamachi, Miida, Bujo & Saito, 2000) and developed into a commercial ELISA kit (Daiichi Pure Chemicals, Inc).

Chapter 3

Development of Ultrafiltration-Isotope Dilution method for Quantification of prebeta-1HDL

3.1 Introduction

This chapter describes the development of a novel simple, highly reproducible scalable method for quantification of native prebeta-1 HDL from plasma by combining plasma ultrafiltration with analysis using the isotope dilution principle and ELIZA. All studies involving human subjects requiring any or all of medical history, physical examination, collection of fasting blood samples, were approved by the Committee on Human Research of University of California San Francisco and written informed consent was obtained from all subjects for participation in this research. The Pepperdine University Institutional Review Board approved the study on the effects of acute exercise in normolipidemic subjects, and written informed consent was obtained from all subjects.

3.2 METHOD OUTLINE

The development of this technique required:

3.2.1 Collection of plasma

3.2.2 Isolation and purification of prebeta-1 HDL for use as a tracer

3.2.2.1 Immunoaffinity Columns to isolate apoA-1 containing Lipoproteins

3.2.2.2 Starch Electrophoresis to separated prebeta-1HDL from alpha HDL

3.2.2.3 Assessment of prebeta-1 HDL purity

3.2.2.3.1 Nondenaturing gradient gel electrophoresis

3.2.2.3.2 SDS polyacrylamide gradient gels

3.2.2.3.3 FPLC

3.2.2.3.4 Agarose gel electrophoresis/Immunoelectrophoresis

3.2.3 Radiolabeling of tracer prebeta-1 HDL

3.2.3.1 Assessment of purity of tracer prebeta-1 HDL

3.2.3.2 Integrity of the prebeta-1 HDL tracer

3.2.3.3 Storage of radiolabeled tracer prebets-1 HDL

3.2.4 Ultrafiltration of apoA-1 lipoproteins isolated by selected affinity immunosorption

3.2.4.1 Ultrafiltration of peaks from starch block

3.2.4.1 Assessment of protein retention by ultrafilter

3.2.5 Quantification of prebeta-1 HDL in plasma by ultrafiltration of plasma samples and application of isotope dilution principle

3.2.6.1 Specific activity of Apo A-I in ultrafiltrate

3.2.6.2 Immunoassay Apo A-I in plasma and ultrafiltrate

3.2.6.3 Assessment of plasma specimen stability

3.2.6.4 Fresh and Frozen Plasma samples quantification of prebeta-1HDL

3.2.6.5 Interassay coefficient of variation for determination of prebeta-1 HDL

3.2.6.6 Calculation of total mass of prebeta-1HDL in plasma samples

3.2.6.7 Assessment of plasma specimen stability

3.2.6.8 Fresh v's frozen plasma assessment

3.3 Quantification of prebeta-1 HDL l in 86 normolipidemic subjects

3.2 MATERIALS AND METHODS

3.2.1 Collection of plasma

Blood samples are treated as follows, for both isolation

of pure prebeta-1 HDL for preparation of the tracer and also for subject samples for measurement of prebeta-1 HDL.

Freshly drawn blood is immediately mixed with the following preservatives: 0.08% (w/v) EDTA; 0.1% (w/v) sodium azide; 50 μg/ml benzamidine; 300 μg/ml ε-amino caproic acid; and 10 μg/ ml gentamycin sulfate (all final concentrations). After mixing gently, the tubes are immediately chilled on ice to arrest the activity of LCAT and, hence, stop conversion of prebeta-1 HDL to alpha HDL species (Kunitake, 1987).

Plasma is separated by centrifugation at 1000*g* for 20 min at 0– 4^{0}C. Until the analyses are completed, the plasma is kept at 0–4^{0}C or is frozen at -70 to -80^{0}C. Total cholesterol and triglyceride levels in plasma are determined by standard chemical techniques (Kunitake, La Sala & Kane, 1985), total HDL cholesterol is measured by precipitation of apoB-containing lipoproteins with dextran sulfate (Bachorik, 1986), and total apoA-1 is measured by enzyme-linked immunosorbent assay (ELISA) as described below.

3.2.2 Isolation and purification of prebeta-1 HDL for use as tracer

3.2.2.1 Immunoaffinity Column to isolate apoA-1 containing Lipoproteins

Plasma is prepared as described above. Apo A-I-containing lipoproteins are isolated from plasma by minimally perturbing selected affinity immunosorption. This technique uses a subpopulation of monospecific antibodies directed against apoA-1, preselected for dissociation under mild conditions of elution, as the solid phase as described previously (McVicar, Kunitake, Hamilton & Kane, 1984). Briefly, plasma is passed through a selected affinity anti-apoA-1 column,

operated under a nitrogen atmosphere in the cold room. Apo A-I-containing lipoproteins bind to the anti A-I column and are subsequently eluted with 3 M acetic acid, (fig x) and the eluate is neutralized immediately to pH 7.0 with 3 M Trisma base. Residual trace albumin and immunoglobulins are removed by passage over anti-albumin and anti-IgG immunosorption columns, respectively.

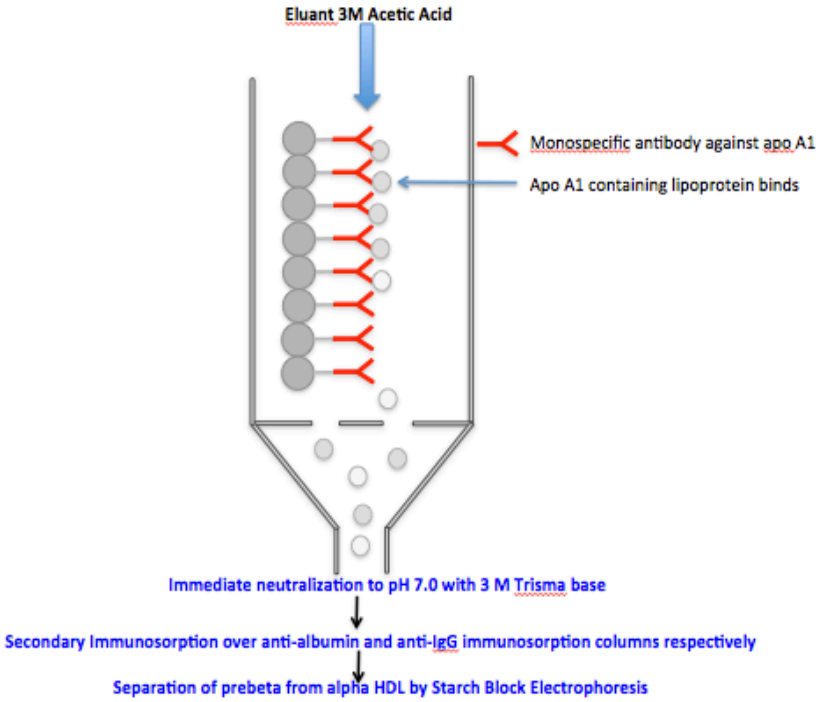

Figure 3.1 Immunoaffinity anti-apoA-1 column with bound A-I containing lipoproteins being eluted with 3M acetic acid

3.2.2.2 Starch electrophoresis to separate prebeta-1HDL from alpha HDL

The prebeta lipoproteins are then separated from other HDL by electrophoresis of the Immunoaffinity isolated A-I containing lipoproteins in 1x 40x10-cm starch blocks in 0.05 M sodium barbital buffer, pH 8.4 (Kunitake, La Sala & Kane, 1985). Following electrophoresis, beginning at the origin, 1-cm slices of starch are extracted in 0.15 M NaCl

with 0.05 M phosphate containing 0.08% EDTA at pH 7.4 and the apoA-1 content of each fraction is detected by rate immunonephelometry on a Beckman ICS Analyser II immunochemistry system (Kunitake, La Sala & Kane, 1985). The high-molecular-weight components, prebeta-2 and pre- beta-3 HDL (Castro & Fielding, 1988) , are excluded by pooling only the eluates from the cathodic upswing of the prebeta HDL peak to its apex. The prebeta-1 HDL eluate is concentrated to between 0.2 and 0.5 mg/ml by diafiltration in a Micro-ProDiCon vacuum dialysis concentrator (Spectrum).

3.2.2.3 Assessment of prebeta-1 HDL purity

The purity of isolated prebeta-1HDL is assessed by four methods:

3.2.2.3.1 Nondenaturing gradient gel electrophoresis: Electrophoresis of 10 – 20 µg of protein (amount determined by Bronstead Lowry method (Lowry, Rosebrough, Farr & Randall, 1951)) in nondenaturing 3 – 34% polyacrylamide gradients to determine that the apparent molecular weight is 67kDa.

3.3.3.3.2 SDS polyacrylamide gradient gels: Electrophoresis in 5 – 25% SDS polyacrylamide gradient gels, immunoblotted with anti- apoA-1 antibodies and stained with Coomassie blue to ensure that the accompanying apolipoprotein is apoA-1.

3.2.2.3.2 FPLC: The concentrated eluate is also examined by FPLC in tandem columns of Superose 12 gels, (Pharmacia) in 0.15 M NaCl containing 5 mM Tris at pH 7.5, run at between 0.4 and 0.8 ml/min on a Beckman 346 FPLC system with a 114M solvent delivery module and a Model 165 variable-wavelength detector, to detect possible contamination with components of higher molecular weight. Protein elution is monitored continually at 280 nm.

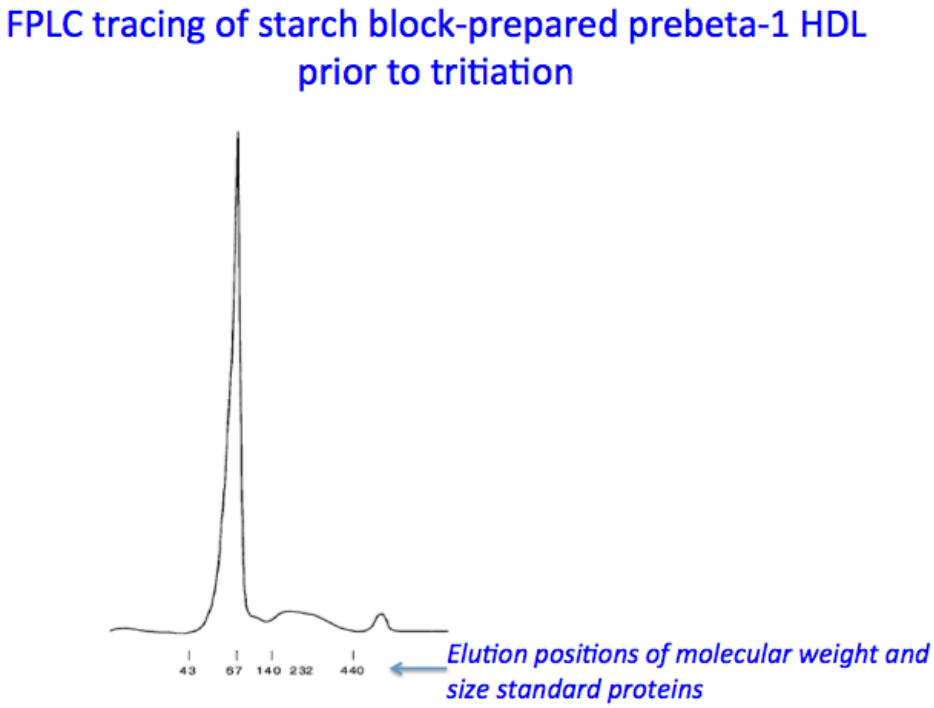

Figure 3.2 FPLC tracing of starch block prepared prebeta-1 HDL showing single prebeta-1 HDL peak

3.2.2.3.4

Agarose gel electrophoresis/Immunoelectrophoresis

Prebeta mobility is verified by vertical agarose gel electrophoresis (Asztalos, Sloop, Wong & Roheim, 1993). Initially this was also demonstrated by immunoelectrophoresis (Kunitake, La Sala & Kane, 1985) in 1% agarose using monospecific goat antiserum directed against apoA-1.

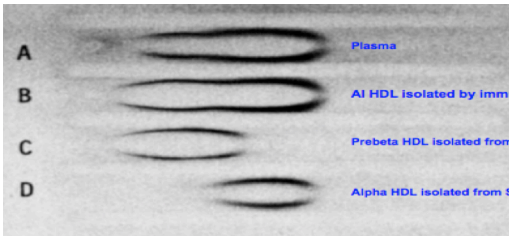

Figure 3.3 Agarose gel immunoelectrophoresis comparing (A) plasma, (B) A1HDL isolated by immunosorbtion, (C) prebeta-1 HDL and (D) alpha HDL both isolated via starchblock electrophoresis of A1 HDL isolated by immunosorption

The content of apoA-1 in the eluate is determined by ELISA as described below.

3.2.3 Radiolabeling of tracer prebeta-1 HDL

The purified pre- beta-1 HDL is labeled by the formation of a tritiated adduct using *N*-succinimidyl [2,3-3H] propionate [sp act 100 Ci/mM (Amersham)] at 1–4^{0}C for 6 h in PBS (Fink & Gainer, 1980) The lipoprotein (0.2–0.5 mg) is labeled in a volume of 0.25 to 0.5 ml. Label that is not covalently bound is removed by diafiltration at 0^{0}C against four changes of 4 liters each of PBS containing EDTA and azide as above for 36 h. Specific activity of the tracer lipoprotein is measured by ELISA for measuring apoA-1 concentration and by measurement of radiolabel by scintillation counting in a Beckman LS 7000 liquid scintillation counter using Cytoscint scintillation cocktail (ICN Bio- medicals). A specific activity (the amount of radiolabeled mass in a sample) of 8000 – 9000 cpm/mg of A-I apoprotein is optimal for analysis by the isotope dilution method.

3.2.3.1 Assessment of purity of tracer prebeta-1 HDL

Following labeling, prebeta mobility of the tracer is verified by vertical agarose gel electrophoresis as described above. The appropriate molecular weight (67 kDa) is confirmed by electrophoresis in 3–34% nondenaturing polyacrylamide gradient gel electrophoresis, as above. Verification that label is only present in apoA-1 is established by SDS–gel electrophoresis as above using autoradiography. The extent of lipid labeling of the pre- beta A-I tracer is measured following extraction of lipid by the method of Folch *et al. (Folch, Lees & Sloane Stanley, 1957)*.

3.2.3.2 Integrity of the prebeta-1 HDL tracer

The integrity of the prebeta HDL tracer was also tested. On FPLC the purified fraction was shown to comprise a major peak with an elution volume corresponding to 67 kDa (Figure 3.4).

Figure 3.4 FPLC tracing of starch-block-prepared prebeta-1 HDL prior to tritiation. *Elution positions of molecular weigh and size standards are shown.*

The radiolabel had prebeta electrophoretic mobility in immunoelectrophoresis (Figure 3.5), and the apparent molecular weight was 67 kDa by nondenaturing gradient gel electrophoresis (data not shown).

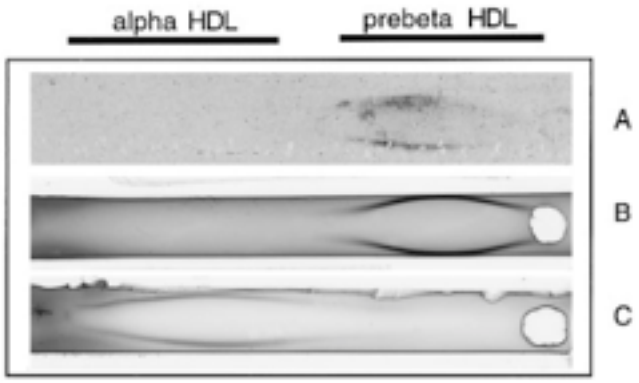

Figure 3.5 Immunoelectrophoretogram. A: Tritiated prebeta-1 HDL tracer. B: Cold prebeta-1 HDL prior to tritiation. C: Alpha HDL isolated from starch block. Apo A-I was immunolocalized using monospecific goat anti-human apoA-1 antiserum.

Finally, protein electrophoresis in SDS showed the entire label to be present in intact apoA-1 (data not shown). The extent of lipid labeling of the prebeta A-I tracer measured following lipid extraction in chloroform–methanol was less than 1%.

3.2.3.3 Storage of radiolabeled tracer prebeta-1HDL

Labeled tracer prebeta-1 HDL is then stored in aliquots of 50 µl at -80^{0}C each, when thawed and diluted, contains sufficient material for analysis of 45 plasma samples in duplicate. Ultrafiltration behavior of frozen aliquots of labeled prebeta-1 HDL tracer remains unchanged over a period of at least 6 months.

3.2.4 Ultrafiltration of Apo A-I lipoproteins isolated by selected affinity immunosorption

When the entire apoA-1 lipoprotein fraction isolated by selected affinity immunosorption was subjected to ultrafiltration, the ultrafiltrate was found to contain only pre- beta-1 HDL by FPLC analysis (Fig. 3.6A) and by nondenaturing gradient gel electrophoresis (data not shown). In addition, when plasma was subjected to ultrafiltration, the filtrate migrated with prebeta mobility on an immunoelectrophoresis gel (Fig. 3.6B) and vertical agarose gel electrophoresis (Fig. 3.6C), indicating the selective filtration of prebeta-1 HDL. In addition, purified pre-beta-1 HDL was subjected to ultrafiltration and ultrafiltered a second time, demonstrating that its filtration fraction was constant. Apo A-I was immunolocalised by western blotting using monospecific goat anti human apoA-1 antiserum.

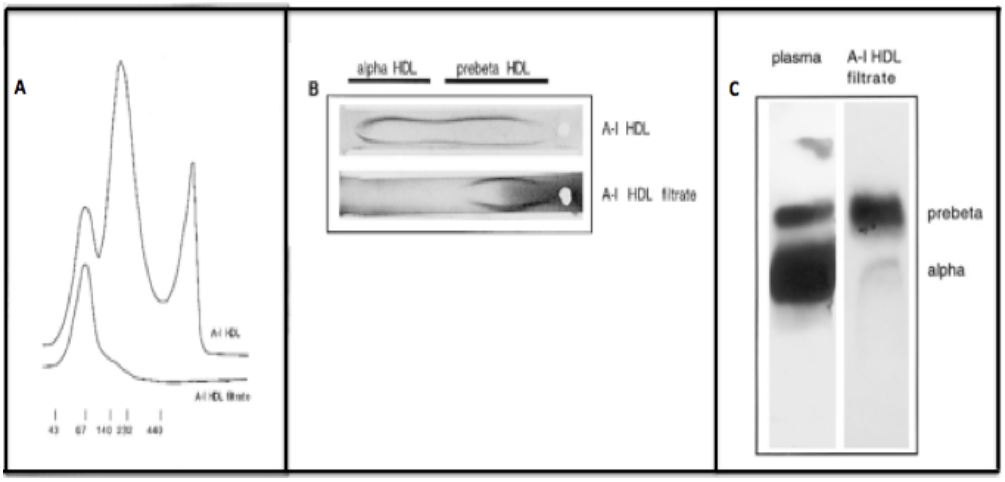

Figure 3.6 Isolation of prebeta-1 HDL using ultrafiltration.
(A) Comparison of FPLC tracings of Apo A-I containing lipoproteins isolated by immunosorption (upper tracing) and ultrafiltration (lower tracing).
(B) Comparison of immunoelectrophoresis of Apo A-I containing lipoproteins isolated by immunosorption and ultrafiltrate of latter. (C) Vertical agarose electrophoresis of plasma and ultrafiltrate of Apo A-I containing lipoproteins isolated by immunosorption. Apo A-I was immunolocalised by western blotting using monospecific goat anti human apoA-1 antiserum.

3.2.4.1 Ultrafiltration of peaks from starch block

When the alpha-migrating peak from starch block was subjected to ultrafiltration, apoA-1 was undetectable in the ultrafiltrate. When prebeta-1 HDL, recovered from the ultrafiltrate of the total apoA-1 lipoprotein fraction, was subjected to a second ultrafiltration, it retained the electrophoretic characteristics of freshly isolated pre- beta-1 HDL. These observations indicate that the ultrafilter retains all of the HDL species larger than pre- beta-1 HDL and that prebeta-1 HDL is not formed passively from larger alpha-migrating HDL in the absence of acceptor lipoproteins and cholesteryl ester transfer (Fielding & Fielding, 1995; Hennessy, Kunitake & Kane, 1993). By maintaining the sample temperature at 1–4^{0}C at all times the activity of LCAT is effectively blocked, thus preventing the conversion of prebeta-1 HDL to alpha HDL species.

3.2.4.2 Assessment of protein retention by ultrafilter

As expected in ultrafiltration, based on the geometry of the ultrafilter, there was partial retention of the 67- kDa prebeta-1HDL particle, similar to the behavior of albumin. The observed concentration ratio between the applied solution and filtrate was the same for tritium-labeled tracer prebeta-1 HDL added to plasma as for the tracer when applied in aqueous buffer (0.43 +/- 0.06, n = 4). This observation indicates that the presence of plasma proteins does not significantly affect ultrafiltration of pre-beta-1 HDL and that the lipoprotein has very limited physical interaction with the tube or filter material. The isotope dilution principle inherently corrects for all such effects.

Ultrafiltered tracer prebeta lipoprotein retained the electrophoretic properties of the tracer before ultrafiltration and, when subjected to a second ultrafiltration, exhibited the same filtration fraction as the original tracer prebeta-1 HDL. The

filtration fraction for prebeta-1 HDL remained constant over a range of 10 to 300 µg/ml. The detection of incrementally added unlabeled prebeta-1 HDL in plasma samples was linear, with a correlation coefficient close to unity (r=0.99) (Figure 3.7).

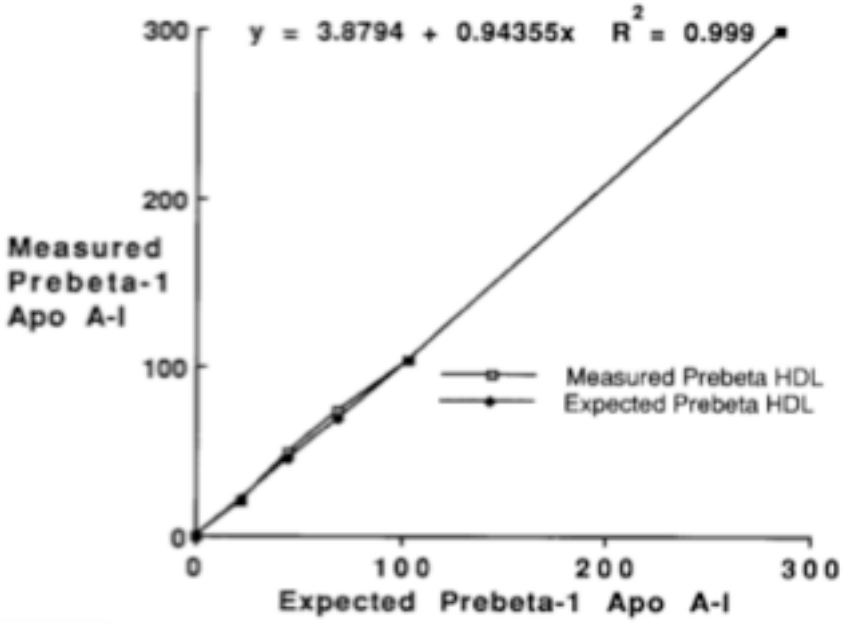

Figure 3.7 Detection of prebeta-1 HDL added to plasma. *Increasing amounts of unlabeled prebeta-1 HDL (35-105 microgram apoA-1/ml) were added in duplicate to standard plasma samples and ultrafiltered. The recovery of prebeta-1 HDL is shown and the regression equation is representative of the results of four similar experiments.*

3.2.6 Quantification of prebeta-1 HDL in plasma by ultrafiltration of plasma samples and application of isotope dilution principle

Plasma samples were spiked with known prebeta-HDL tracer counts ultrafiltered and specific activity of apoA-1 in ultrafiltrate determined as follows:

Frozen plasma samples are centrifuged after thawing for 10,000*g*.min to remove any particulate material. This does not affect the measured prebeta HDL content, but ensures uniform ultrafiltration. An appropriate volume of tritium-labeled prebeta-1 HDL tracer, optimally 50 µl of specific activity 8 – 9 x 10^3 cpm/ µg of

A-I apoprotein, is added to 1.0-ml samples of fresh or thawed plasma by a robotic pipetter (Beckman Biomek 1000 automated laboratory workstation) with five programmed mixing cycles. Alternatively, manual mixing may be employed. A 400- µl aliquot of the labeled plasma is transferred to each of two centrifugal ultrafilters (Microcon 100, Amicon Corp., Cambridge, MA figure 3.8) and centrifuged at 1 – 4^{0}C at 300*g* in a refrigerated microcentrifuge rotor in which 18 tubes rest at a 30^0 angle (Eppendorf Model 5402).

Figure 3.8 Microcon 100, Amicon Corp., Cambridge, MA

After 45 min no more than 50 µl will have been filtered. Adjustment of centrifugation time can be made such that no more than this quantity is ultrafiltered. This is important to provide uniform ultrafiltering of all samples.

3.2.6.1 Specific activity of Apo A-I in ultrafiltrate

Specific activity (the amount of radiolabeled mass: counts per minute/mg of Apo A-I), in the ultrafiltrate is calculated, by measurement of radiolabel activity using scintillation counting in a Beckman LS 7000 liquid scintillation counter using Cytoscint scintillation cocktail (ICN Bio- medicals) and ELISA (immunoassay of apoA-1 in plasma and ultrafiltrate described in section 7 below) which measures apoA-1 concentration, and is expressed as cpm/mg of apoA-1.)

3.2.6.2 Immunoassay of Apo A-I in plasma and ultrafiltrate

The method for ELISA has been described previously (Kunitake, 1996). Some modifications were introduced to simplify the assay while still retaining the same response to apoA-1 in prebeta-1 HDL as in larger HDL complexes.

- Standards and samples were initially diluted 50-fold with PBS, pH 7.5, containing 5 mM sodium dodecyl sulfate and 0.13% Thesit (polyoxyethylene 9-lauryl ether, Sigma, St. Louis, MO) and are incubated at 1 – 4^0C for 75 min to induce uniform exposure of apoA-I epitopes.

- Ninety-six-well plates were coated with 50 µl per well of the capture antibody, goat anti-apoA-1 antibodies, diluted to 1 µg/ml IgG in PBS, and incubated for 75 min at 37^0C.

- The plates are then washed three times with ELISA wash buffer [PBS, pH 7.5, 0.05% Tween 20, and 0.5% bovine serum albumin (BSA) (Sigma, RIA grade)] and blocked with 3% BSA in PBS, pH 7.5, for 1 h at 37^0C with shaking. This is followed by six washes with the wash buffer.

- Detergent-treated samples are added to the plates in a volume of 50 µl per well for 1 h at 37^0C. Plasma samples are applied in two dilutions: 1/60,000 and 1/120,000 in duplicate, and ultrafiltrate samples in three dilutions: 1/400, 1/1200, and 1/3600.

- After incubation for 1 h at 37^{0}C, the plates are washed six times with wash buffer. Bound antigen is detected by addition of the detection antibody, 50 µl (1.3 mg/ml IgG) of an affinity-purified monospecific goat polyclonal antibody to apoA-1, conjugated to horseradish peroxidase (HRP).

- Specifically bound HRP is quantitated colorimetrically with 50 µl per well of ABTS peroxidase substrate-one component (Kirkegaard & Perry, Gaithersburg, MD). The reaction is stopped with 50 µl per well of 1% SDS.

- The optical density of the reaction product is measured at 405 nm using an automated microtiter plate reader (Molecular Devices, Mountain View, CA).

- The standard curve is generated with dilutions of purified human apoA-1. It extends from 0 to 50 mg/ml and is linear over the range of 6 to 25 ng/ml. A frozen plasma standard and a frozen standard ultrafiltrate are included in each plate for quality control.

- Linearity of detection of prebeta-1 HDL was tested by addition of increasing amounts of unlabeled purified prebeta-1 HDL to plasma samples and measuring the incremental prebeta-1 HDL detected by the isotope dilution technique.

3.2.6.3 Assessment of plasma specimen stability

Plasma was obtained from 10 normal subjects and split into two aliquots, one of which was stored at 4^{0}C and the other at -80^{0}C for 24 h. Plasma prebeta-1 HDL was measured by the isotopic dilution method. Stability over time was also assessed for up to 6 months of storage at -80^{0}C.

3.2.6.4 Fresh and Frozen Plasma samples: quantification of prebeta-1HDL

Prebeta-1 HDL levels obtained from fresh and stored frozen plasma samples were not statistically different.

3.2.6.5 Interassay coefficient of variation for determination of prebeta-1 HDL

The interassay coefficient of variation for determination of prebeta-1 HDL was 7% for the plasma standard and 6.6% for the ultrafiltrate standard for 21 sequential assays. Intra-assay coefficient of variation for duplicate assays was 7.5%.

3.2.6.6 Calculation of total mass of prebeta-1 HDL in plasma samples

To calculate the total mass of prebeta-1 HDL per plasma sample, the isotope dilution equation was employed (figure 3.9).

$$\frac{\text{Counts applied to plasma sample}}{\text{Specific activity of the ultrafiltrate}} = \text{Mass of prebeta-1 HDL in plasma}$$

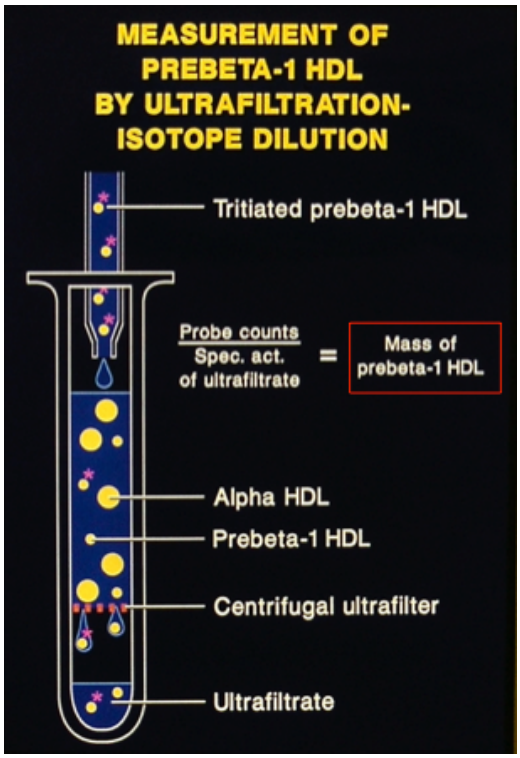

Figure 3.9 Process and principles of measurement of prebeta-1HDL by ultrafiltration/isotope dilution technique.

3.3 Quantification of prebeta-1 HDL in 86 normolipidemic subjects

Fasting blood samples were drawn from 86 normolipidemic subjects' 46 females and 40 males. Samples were handled as outlined above in 3.2.1. Total triglyceride and cholesterol contents were measured in all samples by automated fluorescence analysis (Hoffman-LaRoche, Inc., Nutley, NJ). HDL was measured by precipitation with heparin and manganese (Warnick, Benderson & Albers, 1982) in 44 samples. For those samples the LDL cholesterol was calculated by the Friedewald formula (Friedewald, Levy & Fredrickson, 1972). Subjects with cholesterol levels over 230 mg/dl or triglyceride levels over 170 mg/dl were excluded. Prebeta-1 HDL levels were measured by isotopic dilution ultrafiltration method as described in 3.2.4. All measurements were made in duplicate. Paired values that did not agree within 10 microgram were repeated. Values were rejected if final duplicates did not agree within 10 microgram. Prebeta-1 HDL levels were expressed as absolute levels of prebeta-1 HDL apoA-I and as a percent of total plasma apoA-I, as seen in Table 3.1.

Total Chol (mg/dL) Mean ± SD	LDL Chol (mg/dL) Mean ± SD	HDL Chol (mg/dL) Mean ± SD	Trig (mg/dL)	Plasma ApoA1 (mg/dL)	Prebeta HDL (µg/ml)	Prebeta HDL % plasma A1	Age (years)
173 ± 27	118 ± 28	52 ± 10	71 ± 21	1.13 ± 0.22	74 ± 43	6.6 ± 3.5	34 ± 9

Table 3.1 Plasma Levels of Lipids, Lipoproteins, Prebeta1-HDL and Apolipoprotein A-I in 86 Normal Subjects

3.3.1 Distribution of prebeta-1HDL levels in 86-normolipidemic subjects

Figure 3.10 shows the distribution of prebeta HDL levels in the entire group of normal individuals. Although not statistically significant in this study, there was a trend towards higher levels of prebeta HDL in males than in females.

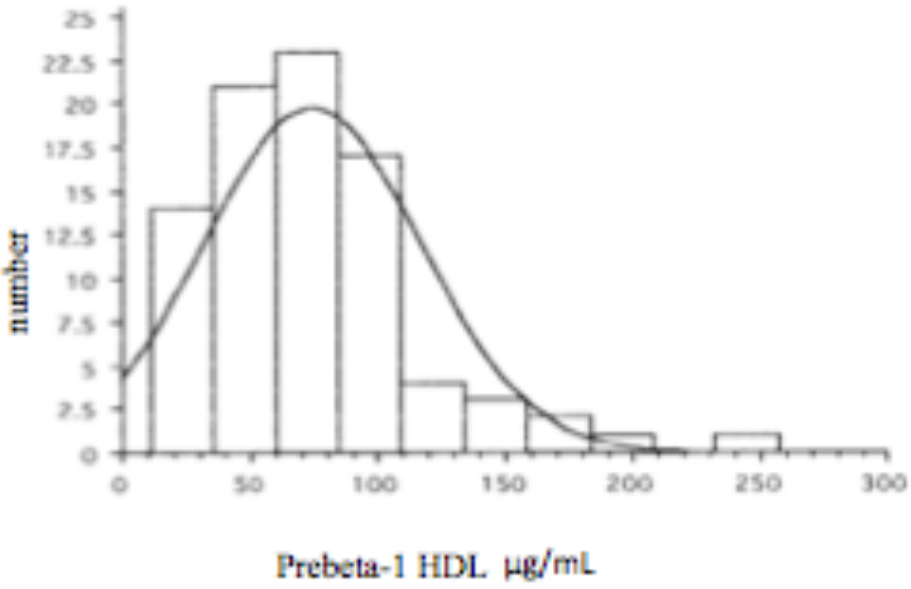

Figure 3.10. Histogram of distribution of prebeta-1HDL levels in normal volunteers n=86

Chapter 4

Application of Ultrafiltration-Isotopic Dilution technique to Measurement of Prebeta-1 HDL in plasma

4.1 Introduction

This chapter describes three studies, which apply the ultrafiltration-isotope dilution method described in chapter 3 for quantification of prebeta-1 HDL in the three populations defined in the table below. The results of the studies are shown in chapter 5 and the discussion in chapter 6.

Study	Study name	Reference
1.	Measurement of prebeta-1 HDL in plasma of normolipidemic subjects: influences of plasma lipoproteins, age, and gender.	(O'Connor et al., 1998)
2.	The Effects of Physical Exercise on Plasma Prebeta-1 High-Density Lipoprotein.	(Jafari et al., 2003) (Erratum in Metabolism.2003Oct;270(19): 4039)
3.	Relation of Increased Prebeta-1 High-Density Lipoprotein Levels to Risk of Coronary Heart Disease.	(Guey et al., 2011)

Table 4.1. Summary of study populations in which prebeta-1 HDL levels were measured using the ultrafiltration-isotope dilution method

All studies involving human subjects requiring any or all of: medical history, physical examination, collection of fasting blood samples, were approved by the Committee on Human Research of University of California San Francisco and written informed consent was obtained from all subjects. The Pepperdine University Institutional Review Board approved the study examining the effects of acute exercise in normolipidemic subjects and written informed consent was obtained from all subjects. Expert advice was sought regarding statistical analysis.

4.2 Study 1: Measurement of prebeta-1 HDL in plasma of normolipidemic subjects: influences of plasma lipoproteins, age, and gender (O'Connor et al., 1998).

4.2.1 Subjects

This study included 136 healthy subjects 90 female (F), 46 male (M). Total cholesterol and triglyceride levels were below 240 mg/dl and 180 mg/dl, respectively, in all subjects. The ages ranged from 16 to 66, with a mean of 37.9 years. Sixteen of the women were postmenopausal of

which four were receiving exogenous estrogens and seventy-four were pre-menopausal of which four were receiving exogenous estrogens. None of the 136 subjects was receiving medications known to affect lipids, other than estrogens. Accurate data for calculation of BMI were available on 92 subjects. BMI ranged from 17.9 to 41.6, mean 22.6 with four values exceeded a BMI of 30.

4.2.2 Lipoprotein measurements

Blood was drawn, after a minimum 10 h fast, into tubes containing sodium EDTA, 1.7-mg/ml final concentration. The blood was chilled immediately on ice to inhibit LCAT activity (Kunitake, 1987) and was kept at 0°C during the separation of plasma and during storage up to 24 hours before lipoprotein measurements were made.

Individual lipoprotein fractions were separated by sequential ultracentrifugation in 92 of the 136 samples and triglyceride and cholesterol contents of these lipoprotein fractions were measured (Havel, Eder & Bragdon, 1955). Total triglyceride and cholesterol contents were measured in all samples by automated fluorescence analysis (Hoffman-LaRoche, Inc., Nutley, NJ). HDL was measured by precipitation with heparin and manganese (Warnick, Benderson & Albers, 1982) in 44 samples. For those samples the LDL cholesterol was calculated by the Friedewald formula (Friedewald, Levy & Fredrickson, 1972). Where LDL cholesterol and HDL cholesterol measurements were available, VLDL cholesterol was approximated as the difference between total cholesterol levels and the sum of HDL cholesterol and LDL cholesterol levels. Total apoA-1 was measured by ELISA and prebeta-1 HDL was measured by the ultrafiltration-isotope dilution method as described in chapter 3 (3.2.5) (O'Connor et al., 1997).

4.2.3 Statistical Analysis

The SAS (1990) system for statistical analysis was used, principally procedure REG for multiple linear regression models. On candidate sets of predictors, subset regression models were ranked by the Cp criterion. Models having the minimal value of AICC (the bias-corrected

Akaike information criterion (TSAI, 1989)) were selected. Partial regression plots, residual plots, and Cook's distance were examined to check analysis assumptions and detect outliers. Power transformations of response variables were examined using the methods of Box and Cox (Box G. E. P., 1964). Covariate-adjusted means for levels of categorical factors were generated using procedure GLM (least-squares means).

4.3 Study 2: The Effects of Physical Exercise on Plasma Prebeta-1 High-Density Lipoprotein (Jafari et al., 2003).

4.3.1 Subjects

This study included nineteen nonsmoking men and women volunteers in self-reported good health who were not taking medications that would effect their plasma lipids (including lipid-lowering agents and oral contraceptives). They maintained their usual diets, and were able to complete the required exercise protocol. The study was carried out in collaboration with colleagues from the Department of Medicine, University of California, Irvine, Irvine, CA; UCLA School of Medicine and VA Greater Los Angeles Healthcare System, Los Angeles, CA; Department of Sports Medicine, Pepperdine University, Malibu, CA.

4.3.2 Protocol

Subjects refrained from participation in physical exercise and consumption of alcohol for 3 days prior to the testing session. They reported following a 12-hour overnight fast to the exercise laboratory. A history and physical examination were performed to confirm each subject's health status and pre-exercise laboratory collection, which included the following measurements:

(1) Fasting plasma lipids, lipoproteins, Apo A-I, prebeta-1 HDL
(2) Percent body fat
(3) Dietary fat and cholesterol intake

Subjects were classified as exercise-trained (ET) if they reported

regular participation in physical exercise of at least 45 minutes of vigorous, aerobic physical exercise training 3 times per week, or sedentary (SED) if they reported no regular physical exercise behavior. Subjects then underwent a bout of protracted physical exercise consisting of maximal cardiopulmonary exercise (CPX) stress testing followed by a 4-km run-jog (total exercise time, 45 to 60 minutes). Following the exercise bout, blood samples were taken, and handled as previously described, for measurements of fasting plasma lipids, lipoproteins, Apo A-I, and prebeta-1 HDL were repeated.

4.3.3 Procedures

4.3.3.1 Body composition

Prior to undergoing cardiopulmonary exercise stress testing, body composition was determined via skinfold thickness measurements at specific sites (chest, abdomen, and thigh for men; triceps, supra-iliac, and thigh for women) using standard procedures (Jackson AS 1985) Measurements at each site and on each subject were made by an experienced investigator using Harpenden skinfold calipers (British Indicators, Dover, England). Each skinfold site was measured using the thumb and index finger of the left hand to elevate a double fold of skin, with subcutaneous tissue approximately 1 cm proximal to the site being measured. Each skinfold was raised perpendicular to the surface of the body at the measurement site, with the long axis of the skinfold parallel to the natural cleavage lines of the skin. The skinfold was kept elevated until the measurement was completed, and the jaws of the caliper were placed so that the thickness of the skinfold was measured perpendicular to its long axis when the pressure of the caliper was released and the caliper jaws moved toward each other. A reading of the skinfold thickness, in millimeters, was made 2 seconds after the caliper was released at the skinfold site. All sites on each subject were measured once, and then each site was measured a second time. A third skinfold measurement was made if the second

measurement at a particular site exceeded the first by more than 1 mm. The skinfold thickness at a particular site was taken as the average of the 2 closest measurements. Intra-measurer reliability coefficients for experienced investigators using this technique are 0.97 (triceps), 09.6 (chest), 0.98 (abdomen), 0.97 (supra-iliac), and 0.98 (thigh).

4.3.3.2 Cardiopulmonary Exercise (CPX) testing

CPX testing was conducted using a standard Bruce protocol (Bruce, 1974). Heart rate was continuously monitored during CPX testing by telemetry (Polar Vantage XL, Polar USA, Hartford, CT), with heart rates recorded at one-minute intervals for 10 minutes following exercise. Metabolic measurements for determination of maximal aerobic capacity (VO2max) were made using a MedGraphics CardioO2 system (Medical Graphics, St Paul, MN). Expired gas measurements were recorded at 30-second intervals, and VO2max was determined by the following criteria:

(1) <100 mL increases in VO2 in consecutive 30-second sampling periods prior to exhaustion
(2) Respiratory exchange ratio (RER) in excess of 1.10
(3) Attainment of predicted maximal heart rate +/- 15 bpm

Approximately 5 to 10 minutes after the CPX test, when subjects' heart rates had recovered to < 100 beats per minute, they engaged in a run-jog exercise bout covering 4 km on a running track adjacent to the laboratory, at a self-selected pace. This distance was selected such that, when combined with the CPX test, the subjects would expend approximately 300 to 350 kcal in aerobic exercise. Upon completion of the run-jog, the subjects underwent a final blood sampling.

4.3.3.3 Dietary surveys

A 3-day dietary record was dispensed to all subjects within 1 week prior to the run-jog exercise session (Posner et al., 1992) (Crawford, Obarzanek, Morrison & Sabry, 1994). Subjects were instructed to list all daily food and drink intake for 2 days of the week and one day of the weekend. Portion sizes were estimated using household measures, and adherence to typical dietary intake was strongly recommended (Luhrmann, Herbert, Gaster & Neuhauser-Berthold, 1999). Daily caloric intake, dietary cholesterol, and fat were calculated using food composition tables (Grodner TK: Philadelphia, 1996).

4.3.4 Lipoprotein measurements

Two 20-mL fasting blood samples were drawn into tubes containing sodium EDTA, 1.7-mg/ml final concentration from each subject before and immediately after exercise. The blood was chilled immediately on ice to inhibit LCAT activity (Kunitake, 1987) and was kept at 0°C during the separation of plasma and stored at -60°C until assayed. Individual lipoprotein fractions were separated by sequential ultracentrifugation, followed by measurement of the content of cholesterol and triglycerides in each (Havel, Eder & Bragdon, 1955). The content of Apo A-I was measured by enzyme-linked immunoassay (ELISA) and the content of prebeta-1 HDL in plasma was measured using the ultrafiltration-isotope dilution technique described in3.2.4. The relative percentage of plasma Apo A-I present as prebeta-1 HDL and the absolute amount of prebeta-1 HDL in plasma were determined as described earlier (O'Connor et al., 1997).

4.3.5 Statistical Analysis

Data were analyzed using EXCEL software (Microsoft, Seattle, WA) . Means +/- SD were determined for the following entry characteristics: age, percent body fat, VO2max, dietary caloric intake, dietary cholesterol intake, and percentage dietary fat intake. As an internal validity check, 1-way analysis of variance (ANOVA) was conducted between exercise-trained (ET) and sedentary (SED) subjects' VO2max

and pre-exercise plasma levels of plasma HDL-cholesterol, and between male and female subjects' pre-exercise plasma levels of HDL-cholesterol. Paired t-tests were conducted for all subjects (N = 19) between pre- and post-exercise plasma levels of fasting plasma lipids, lipoproteins, Apo A-I, and prebeta-1 HDL.

4.4 Study 3: Relation of Increased Prebeta-1 High-Density Lipoprotein Levels to Risk of Coronary Heart Disease (Guey et al., 2011)

4.4.1 Subjects

Subjects referred because of demonstrable coronary heart disease (CHD) or for risk-factor assessment and management to the University of California–San Francisco Lipid and Cardiology Clinics and to collaborating cardiology practices were recruited for the study. All were clinically stable at time of recruitment. No samples were collected within 2 weeks of a myocardial infarction (MI). The committee on human research of University of California–San Francisco, approved the study and written consent was obtained from all subjects. Because of the potentially confounding effect of lipid-lowering medications, subjects with missing information regarding medication were excluded (n 1,048), leaving 1,255 available for analysis. Subjects were considered to have CHD if any of the following criteria shown in Table 4.2 was fulfilled.

CHD inclusion criteria	Number of subjects
Coronary disease on angiogram	296
MI	213
Coronary revascularization	167
Angioplasty	227
Stent placement	116

Table 4.2: Subjects meeting criteria for diagnosis of CHD

The demographic and clinical characteristics of the subjects included in the study are shown in table 4.3

Demographic and clinical characteristics	Number
Age (years)	50.8 +/-16.9
Men	558 (44.4%)
Body mass index (kg/m$^{2)}$	26.6 +/- 5.4
Caucasian	901 (73.4%)
Asian	192 (15.6%)
Other (African-American, Hispanic, other)	134 (10.9%)
Smoker (Current)	83 (6.6%0
Lipid-lowering medication	193 (15.4%)
Triglycerides (mg/dl)	148 +/- 87.5
LDL (mg/dl)	147.9 +/- 58.6
HDL (mg/dl)	50.8 +/- 18.1
Apo A-1 (mg/ml)	1.17 +/- 0.35
Type 2 diabetes mellitus	105 (8.4%)
Coronary heart disease	454 (36.2%)
Myocardial infarction	213 (17%)

Table 4.3 Demographic and clinical characteristics of subjects.
Data are presented as number of subjects (percentage), mean SD, or median (median absolute deviation). Number of subjects for each demographic and clinical characteristic varies because of missing data

4.4.2 Coronary Imaging

The Cardiologists imaged the 4 principal coronary vessels and their branches after administration of nitroglycerin. Angiographic disease was graded by a "sum score" (sum of percent luminal intrusion of all lesions). A sum score of 60 was the threshold discriminant for diagnosis of CHD. Mean sum score was 240.6. Diagnosis of MI was based on 1 of the following: troponin levels, electrocardiographic evidence, or echocardiographic studies. No samples were collected within 2 weeks after an MI.

4.4.3 Lipoprotein measurements

Lipoproteins were measured after a 10-hour fast on plasma drawn into buffered EDTA and chilled immediately to 0°C to stop esterification of cholesterol by LCAT, preventing conversion of prebeta-1 HDL to larger HDL species (Kunitake, 1987). Lipids were measured on

ultracentrifugal fractions (Pullinger et al., 1995) in 65% of subjects including all subjects with TG > 300 mg/dl. VLDL were separated at density 1.006 g/L. LDL and HDL were separated using heparan sulfate and magnesium chloride. In the remainder, apolipoprotein B–containing lipoproteins were precipitated with dextran sulfate,(Warnick, Benderson & Albers, 1982) and LDL cholesterol was calculated by the Friedewald formula (Friedewald, Levy & Fredrickson, 1972). Subjects with plasma triglycerides 500 mg/dl were excluded to avoid confounding alterations in molecular speciation of HDL. Plasma content of prebeta-1 HDL was measured using the ultrafiltration–isotope dilution technique previously described in chapter 3 (3.2.5) (O'Connor et al., 1997).

4.4.4 Statistical analysis

Descriptive statistics of demographic and clinical characteristics are presented as mean +/- SD or median (median absolute deviation) for continuous variables and as number of subjects (percentage) for categorical variables. Percent prebeta-1 HDL level, defined as the percent total plasma apolipoprotein A-I in pre -1 HDL, was used in all analyses. Percent prebeta-1 HDL was categorized into tertiles and distribution of traditional CHD risk factors compared across tertiles. Associations between prebeta-1 HDL tertiles and CHD risk factors were examined using analysis of variance for continuous variables and chi-square test of independence for categorical variables. To evaluate the association of prebeta-1 HDL with CHD and MI, unadjusted and adjusted odds ratios and their 95% confidence intervals were estimated for prebeta-1 HDL in univariable and multivariable logistic regression models. The latter included conventional non-lipid CHD risk factors (age, gender, race, body mass index, smoking status, type 2 diabetes mellitus, and hypertension), lipid risk factors (HDL, LDL, and triglycerides), apoA-1, and lipid-altering medication usage. Continuous variables with positively skewed distributions (prebeta-1 HDL, body mass index, HDL, and LDL) were log-transformed and standardized. Thus, their odds ratios represent the increased odds of disease prevalence given a 1-SD increment. Age was divided into 5-year intervals such that its odds ratio represents the increased odds of risk

for a 5-year increase. Because of the highly skewed nature of triglycerides, levels were dichotomized at 200 mg/dl in regression models. Interactions between prebeta-1 HDL and gender were also examined. To evaluate the contribution of prebeta-1 HDL to risk models, likelihood-ratio tests were performed comparing risk models with and without the inclusion of prebeta-1 HDL as a predictor. Also, improvements in area under receiver operating characteristic curves were evaluated. Because improvements in area under receiver operating characteristic curves can be insensitive to biomarkers with moderate effect sizes and even established CHD risk factors such as lipids, hypertension, and smoking, (Cook, 2007; Pencina, D'Agostino, D'Agostino & Vasan, 2008) improvement in classification of cases and controls with the addition of prebeta-1 HDL in multivariable models with 2 measurements was evaluated:

(1) Net reclassification index using predefined risk categories of < 6%, 6% to 20% and >20%
(2) Integrated discrimination improvement index.

The net reclassification index classifies subjects into risk categories defined a priori in a model with and without a biomarker and formally tests whether there is a net gain in reclassification because of the biomarker. A gain in reclassification occurs when cases are reclassified into higher-risk categories and controls are reclassified into lower-risk categories. Conversely, a loss in reclassification occurs when cases are reclassified into lower-risk categories and controls are reclassified into higher-risk categories. These gains and losses are subsequently aggregated to assess statistically whether there is a net gain in reclassification because of a biomarker (Pencina, D'Agostino, D'Agostino & Vasan, 2008). Because the net reclassification index is dependent on predefined risk categories, we evaluated improvement in classification with the integrated discrimination improvement index, which does not require predefined risk categories, by quantifying improvement in sensitivity and specificity integrated over all possible thresholds (Pencina, D'Agostino, D'Agostino & Vasan, 2008). Results with a p value <0.05 were regarded as statistically significant. All analyses were performed in R 2.9.0 (MediaFire Wood- lands, Texas).

CHAPTER 5

Results

5. Introduction

This chapter describes the results from the three studies, which apply the ultrafiltration-isotope dilution method described in chapter 3 for quantification of prebeta-1 HDL in the three populations defined in the table below.

Study	Study name	Reference
1.	Measurement of prebeta-1 HDL in plasma of normolipidemic subjects: influences of plasma lipoproteins, age, and gender.	(O'Connor et al., 1998)
2.	The Effects of Physical Exercise on Plasma Prebeta-1 High-Density Lipoprotein.	(Jafari et al., 2003) (Erratum in Metabolism.2003Oct;270(19): 4039)
3.	Relation of Increased Prebeta-1 High-Density Lipoprotein Levels to Risk of Coronary Heart Disease.	(Guey et al., 2011)

Table 5.1. Summary of study populations in which prebeta-1 HDL levels were measured using the ultrafiltration-isotope dilution method

5.1 Study 1: Results for Measurement of prebeta-1 HDL in plasma of normolipidemic subjects: influences of plasma lipoproteins, age, and gender (O'Connor et al., 1998)

Prebeta-1 HDL as percent of total plasma apoA-I ranged from 0.47 to 19.65 % with an average of 6.10%, median 5.39 (10th percentile 1.79 and 90th percentile 10.0) as shown in table 5.2. Absolute levels of prebeta-1 HDL-associated apoA-I ranged from 5 to 257 μg/ml, with an average of 73 μg/ml, median 66 μg/ml (10th percentile 22 and 90th percentile 131 μg/ml). As is evident from the asymmetry with respect to the median, both of these measures had distributions that were substantially skewed toward higher levels (skewness coefficients were 1.06 and 1.31, respectively. Non-normality of distribution was highly significant ($P < 0.0001$ for both measures) (Fig. 5.1 and Fig. 5.2).

Subjects (n)	ApoA1	Prebeta	Abs. Prebeta	HDL Chol	HDL TG	Total TG	Total Chol	LDL Chol	LDL TG	VLDL Chol	VLDL TG
	mg/ml		µg/ml	mg/dl							
All (136)	1.23 ± 0.3	6.1 ± 3.6	73± 44	54 ± 13	17± 7	79± 29	178± 29	109± 25	34± 6	8± 5	36± 20
Women (90)	1.25± 0.31	5.5± 3.3	68± 40	58± 12	19± 6	77± 28	179± 29	107± 25	34± 6	7± 4	31± 18
Men (46)	1.16± 0.27	7.2± 4.0	84± 49	48± 11	11± 3	81± 21	175± 28	112± 24	33± 6	10± 5	44± 21
Post-menopausal women, no estrogen (12)	1.53± 0.37	5.8± 4.4	80± 47	61± 15	18± 6	94± 29	198± 27	124± 23	34± 6	8± 5	35± 18
Menstruating women, no estrogen (70)	1.16± 0.2	5.5± 3.2	63± 37	56± 11	19± 7	71± 27	173± 28	103± 24	32± 5	6± 3	27± 14

Table 5.2 Lipid and Lipoprotein levels for study 2. *Values given as mean +/- SD. ApoA-1, total plasma apoA-1; % prebeta, percent of total apoA-I in prebeta-1HDL; Abs. prebeta, concentration of prebeta-1HDL in plasma*

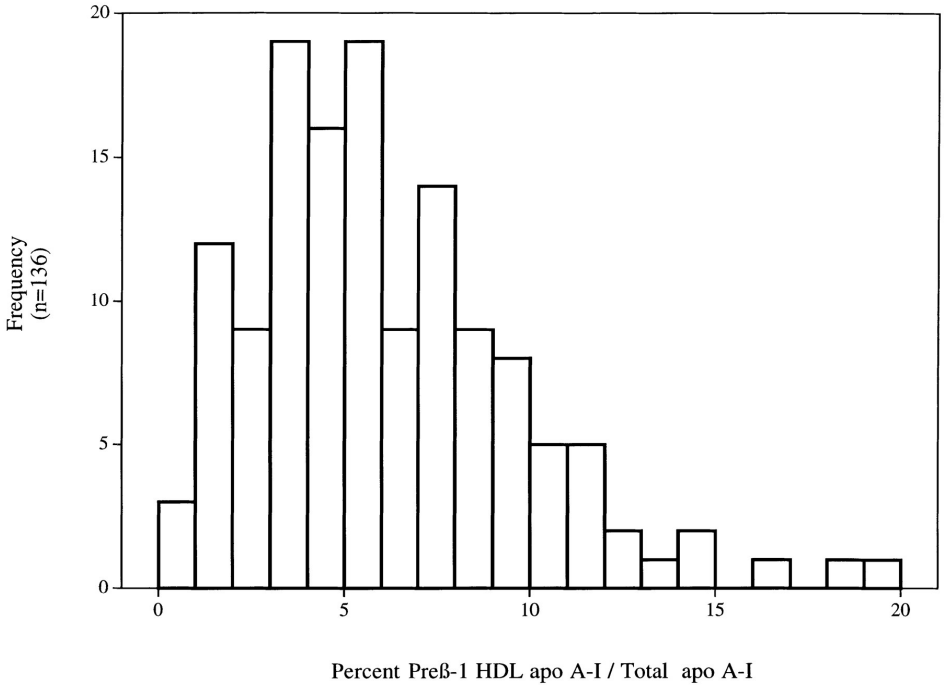

Figure 5.1 Frequency distribution of percent prebeta-1 HDL as percent of total apolipoprotein A-I in plasma samples from normolipidemic subjects.

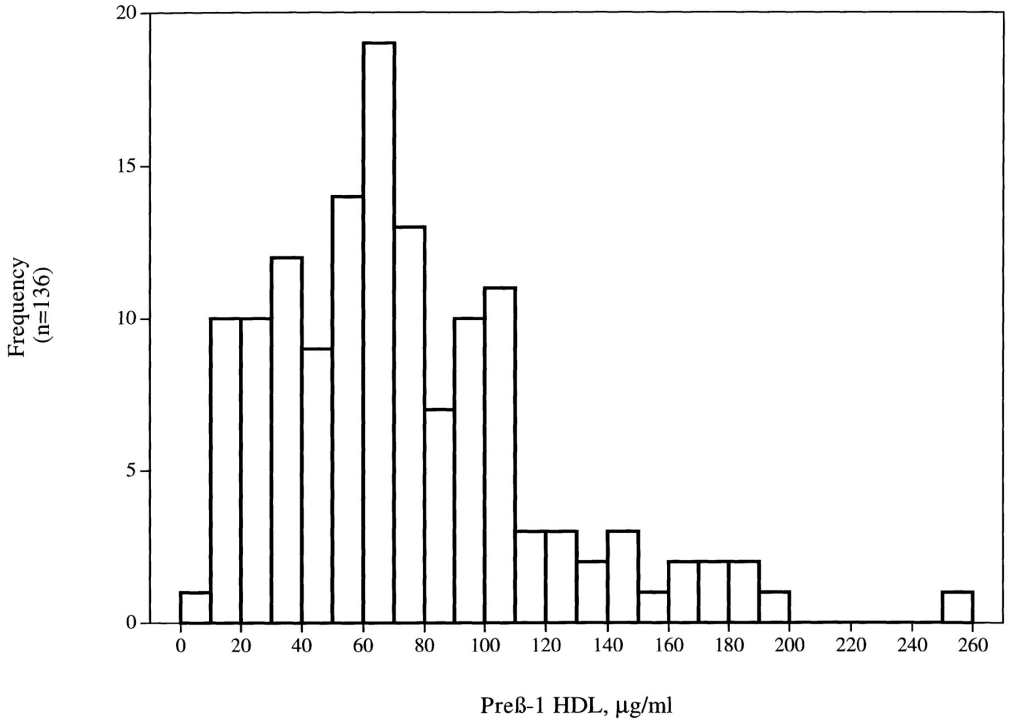

Figure 5.2 Frequency distribution of absolute levels of prebeta-1 HDL in plasma samples from normolipidemic subjects, µg/ml

5.1.1 Preliminary screening

The potential predictors of the percentage of prebeta-1 HDL and absolute prebeta-1 HDL were the absolute level of apoA-I, triglycerides, HDL cholesterol, LDL cholesterol, VLDL cholesterol, BMI, age, and gender. Of these, apoA-I, total cholesterol, HDL cholesterol, and gender (coded as male = 1, female = 2) had Pearson correlations of some significance ($P < 0.1$) with at least one of the response variables (Table 5.3).

Variable	ApoA1	TG	HDLC	LDLC	VLDLC	Age	BMI	Gender
Percentprebeta-1 HDL								
r	0.162	0.069	-0.354	-0.006	0.021	-0.138	0.112	-0.0218
P	0.06	0.424	0.001	0.942	0.812	0.109	0.286	0.011
n	136	136	136	136	136	136	92	136
Absolute prebeta-1 HDL								
r	0.222	0.156	-0.161	0.101	0.063	-0.007	0.129	-0.175
P	0.010	0.070	0.062	0.243	0.469	0.939	0.220	0.041
n	136	136	136	136	136	136	92	136

(Pearsons Correlation Coefficients/Prob>® under Ho: Rho 0/Number of Obs.) ***Table 5.3: Correlations of response variables with potential predictors.*** *TG: triglycerides; HDLC, LDLC and VLDLC: HDL cholesterol, LDL cholesterol and VLDL cholesterol respectively in mg/dL; BMI: body mass index; r: Pearson Correlation Coefficients; p: P value; n, number of subjects*

Two alternatives to the gender categories were considered that utilized information on endogenous or supplemental estrogen. An "estrogen status" variable (ESTRSTAT) had: category 1 for males and postmenopausal females without estrogen supplementation, and category 2 for menstruating females and postmenopausal females with estrogen supplementation.

A second recoding with three categories (GENESTR) separated the females from category 1. Preliminary models also including apoA-I, triglycerides, and HDL cholesterol showed no significant differences for gender or the alternatives ($P > 0.16$), and neither of the latter exhibited substantial superiority to gender. The alternative codings are reconsidered below using final models developed to include gender.

On the subset on which BMI could be computed ($n = 92$), BMI was not significant in preliminary models also including apoA-I, triglycerides, HDL cholesterol, and gender ($P > 0.7$). Further, there were no significant differences in means or medians of the response variables

between the subsets with and without BMI (P > 0.2). Therefore, BMI was not further considered as a predictor.

5.1.2 Final Models

Candidate predictors for final models included:

- ApoA-I
- Triglycerides
- HDL cholesterol
- LDL cholesterol
- VLDL cholesterol
- Age
- Gender

Using the methodology of Box and Cox (Box G. E. P., 1964), power transformations of the response variables in models including all candidate predictors showed the 0.4 power to be optimal, to the nearest 0.1. For convenience, the 0.5 power or square root transformation was examined. Models ranked as first using the Cp criterion also had minimum AICC (the bias-corrected Akaike information criterion) (Hurvich, 1989)) as seen in Table 5.4.

Parameter Estimates for	Parameter Estimates	Standard Error	Prob > T
PCTPREB			
Intercept	11.53	1.279	0.0001
HDL-Chol	-0.1001	0.023	0.0001
Square root of percent prebeta-1 HDL			
Intercept	3.5	0.257	0.0001
HDL-Chol	-0.0209	0.005	0.0001
ABSPRES			
Intercept	0.0803	0.019	0.0001
ApoA-1	0.0700	0.015	0.0001
HDL-Chol	-0.0013	0.000	0.0001
Age	-0.0005	0.000	0.1178
Square root of absolute prebeta-1 HDL			
Intercept	0.2785	0.034	0.0001
ApoA-1	0.1300	0.027	0.0001
HDL-Chol	-0.0025	0.000	0.0001
Age	-0.0010	0.000	0.00825

Table 5.4 Parameter estimates for final model. CTPREB: Percent of A-I in prebeta-1 HDL, ABSPREB: Absolute level of Apo A-1 in prebeta-1 HDL

For PCTPREB (percent prebeta-1 HDL) and RPCT PREB (square root of percent of total apoA-I contained in prebeta-1 HDL) the selected models included only one predictor: HDL cholesterol.

For ABSPREB (absolute prebeta-1 HDL), the selected predictors were apoA-I, HDL cholesterol, and age, while for RAB SPREB (square root of the absolute level of prebeta-1 HDL); the same predictors were included as well as gender.

For each of these four response variables, models with and without gender but otherwise including the same predictors were ranked 1st or

2nd. Thus, while gender was not a significant predictor in any of these models ($0.15 < P < 0.23$), models including gender were the best or next to best that could be produced according to the selection criterion used.

The models excluding gender are summarized in Table 3. These show that HDL cholesterol was negatively related to the response in all instances ($P < 0.0001$), while age was at most a weakly significant negative predictor ($P = 0.12$ for ABSPREB, $P = 0.082$ for RABSPREB). The same findings hold for models including gender, with only minor changes in significance levels. In all instances, the estimated effect of being female was negative.

For the final models for the untransformed response variables, the three recordings of gender and estrogen status were reexamined. Table 4 presents adjusted means for gender and ESTRSTAT. Adjusted means for GENESTR suggest that postmenopausal women without estrogen (category 3) are more similar to males (category 1) in these responses than they are to women with estrogen (category 2). Also, the differences between categories 1 (males) and 2 are similar to the differences between males and all females. Results for ESTRSTAT were similar to those for gender.

Variable	LSMEAN	Std Err		P Value	
Gender	PCTPREB				
Males	6.678	0.524		0.22	
Females	5.869	0.371			
	ABSPRES				
Males	0.081	0.006		0.15	
Females	0.069	0.004			
ESTRSTAT	PCTPRES				
Cat.1	6.626	0.456		0.17	
Cat.2	5.781	0.396			
	ABSPRES				
Cat.1	0.080	0.006		0.11	
Cat.2	0.068	0.005			
GENESTR	PCTPRES		1	2	3
Cat.1 (males)	6.674	0.525		0.18	0.85
Cat.2	5.778	0.398	0.19		0.51
Cat.3	6.463	0.993	0.85	0.52	
	ABSPRES				
Cat.1 (males)	0.081	0.006		0.12	0.88
Cat.2	0.068	0.005	0.12		0.48
Cat.3	0.078	0.014	0.88	0.48	

Table 5.5 ***Adjusted means of response variables by gender, ESTRSTAT, and GENESTR.*** *ESTRSTAT = estrogen-based statistical variable; GENESTR = gender- and estrogen-based statistical variable; PCTPREB = percent of total apoA-I in prebeta-1 HDL; ABSPREB = absolute amount of apoA-I in prebeta-I HDL.*

All values are least squares means (LSMEAN).

Category 1 (Cat. 1) includes males and postmenopausal females without supplemental estrogen. Category 2 (Cat. 2) includes premenopausal females and postmenopausal females treated with estrogen. Category 3 (Cat. 3) includes postmenopausal females without estrogen.

To more fully examine effects of plasma triglyceride level, that predictor was added to final models for the untransformed response

variables. In models without gender, triglycerides had a slope of 0.00516 (P = 0.96) for PCTPREB and a slope of 0.000046 (P = 0.75) for ABSPREB. Results in models including gender were quite similar.

5.2 Study 2: The Effects of Physical Exercise on Plasma Prebeta-1 High-Density Lipoprotein. (Jafari et al., 2003) (Erratum in Metabolism. 2003 Oct; 270(19): 4039).

5.2.1 Subjects

All subjects completed the exercise protocol. Group characteristics are shown in Table 5.6 It is notable that subjects no. 2 and 18 reported zero cholesterol and very low fat diets. These subjects were young women who where using commercial very-low-fat meal replacements for weight management. Most subjects (N =13) did not consume alcohol and the remainder consumed fewer than 5 units of alcohol per week. Subjects refrained from consuming alcohol during the 3 days preceding the exercise session. The comparisons made between exercise trained (ET) and sedentary (SED) subjects as an internal validity check showed that VO2max was greater in ET than in SED subjects (52.8 +/- 6.8 v 45.0 +/- 7.3 mL O2/kg/min, P =. 026), and HDL-cholesterol levels were greater in ET than in SED subjects (54.8 +/- 11.2 v 43.3 +/- 8.7 mg/dL (P =_ .024).

Subject	Age (yr)	Sex	Height (cm)	Weight (kg)	BMI	% Body Fat	Dietary Calories (kcal/d)	Dietary C (mg/d)	Dietary Fat	VO_2 peak	HDL-C	Exercise
1	20	F	175	71.623.9	23.9	11.0	3221	132	21.5	55.0	44	PT
2	19	F	168	62.6	22.3	23.8	599	0	6.0	50.3	53	PT
3	21	M	170	74.4	25.7	5.3	1881	252	12.2	47.8	41	PT
4	21	M	162	43.1	16.6	4.7	1019	112	24.6	62.2	66	PT
5	22	F	183	79.8	24.2	17.2	1475	158	31.5	49.7	45	PT
6	21	F	183	79.4	24.1	13.6	2415	50	32.9	52.6	67	PT
7	20	M	183	72.6	22.0	5.2	1702	123	19.3	64.8	74	PT
8	21	F	173	72.6	24.2	18.3	2212	122	34.8	41.2	59	PT
9	29	M	178	83.9	26.2	15.3	1916	359	39.2	51.9	51	PT
10	31	M	196	78.7	20.7	10.5	1459	127	32.3	52.7	48	PT
11	41	M	185	74.6	21.9	13.8	2118	215	38.0	40.7	35	SED
12	21	F	163	60.8	22.5	17.2	2315	28	20.3	36.2	50	SED
13	21	M	164	54.4	20.1	7.0	1342	36	5.6	56.9	44	SED
14	24	M	191	73.9	20.5	5.2	1633	176	35.0	47.8	45	SED
15	28	M	162	44.9	17.3	14.5	1081	364	48.9	35.7	32	SED
16	33	M	170	62.1	21.4	21.0	2018	321	34.8	40.0	35	SED
17	21	M	171	68	23.4	11.6	1337	192	20.4	49.9	40	SED
18	21	F	173	77.6	25.9	13.6	1446	0	25.8	51.7	58	SED
19	31	M	168	64.1	22.3	17.9	1614	281	39.7	45.7	51	SED
Mean±SD	24.0 ±5.8		174.6±9.9	68.4±11.5	22.4±2.6	13.0±5.7	1733±592	160.4±114.9	28.0±10.8	49.1±7.9	49.3±xxx x	

NOTE. Dietary fat in % fat of total daily calories; $VO_{2\ peak}$ in mL O_2/kg/min.
Abbreviations: C, cholesterol, PT, physical exercise-trained; SED, sedentary.

Table 5.6 Subject entry Characteristics for study 2 (Mean ± SD) (N = 19). Pre-exercise plasma HDL-cholesterol levels were not significantly different between male (n =12, 48.0 +/-13.3 mg/dL) and female (n = 7, 51.1 +/- 9.4 mg/dL) subjects. Table 2 summarizes mean _+/- SD values of fasting lipids, lipoproteins, Apo A-I, and prebeta HDL before and after exercise.

Table 5.7. Effects of Physical Excerise on Fasting Levels of Plasma Lipids, Lipoproteins, Apo A-1, and Prebeta HDL.

Total cholesterol (mg/dL)	
Pre-	183.1 ± 42.7
Post-	177.8 ± 38.7
LDL-cholesterol (mg/dL)	
Pre-	112.9 ± 37.8
Post-	104.6 ± 31.2
Triglycerides (mg/dL)	
Pre-	95.7 ± 36.8
Post-	92.4 ± 36.5
HDL-cholesterol (mg/dL)	
Pre-	49.3 ± 11.8
Post-	55.2 ± 21.8
HDL-triglycerides* (mg/dL)	
Pre-	23.3 ± 10.8
Post-	12.5 ± 5.6
Apo A-1 (mg/mL)^	
Pre-	1.36 ± 0.19
Post-	1.31 ± 0.19
Absolute prebeta^{+} (μg/mL)^	
Pre-	100 ± 60
Post-	130 ± 70
% prebeta	
Pre-	7.9 ± 3.82
Post-	8.88 ± 4.08

*Pre-v. post-exercise, P = 0.012
$^{+}$Pre-v. post-exercise, P= 0.039

Table 5.7 Effects of Physical Excerise on Fasting Levels of Plasma Lipids, Lipoproteins, Apo A-1, and Prebeta HDL.

Paired t-tests conducted for all subjects (N =19) between pre- and post-exercise plasma levels of fasting lipids, lipoproteins, Apo A-I, and prebeta-1 HDL showed a significant decrease in plasma HDL-triglyceride levels (23.3 +/-10.8 to 12.5 +/- 5.6 mg/dL, P _ .012) and an increase in absolute prebeta-1 HDL (100 +/- 60 to 130 +/- 70 P = .039) after exercise.

Table 5.8 shows individual subjects' plasma prebeta-1 HDL levels before and after exercise. The response was heterogeneous but favored increased plasma prebeta-1 HDL levels (n = 11). Plasma prebeta-1 HDL levels were unchanged in 6 subjects and decreased slightly in 3 subjects.

Subject No.	Before Excercise	After Excercise
1	80	60
2	120	120
3	50	50
4	110	120
5	170	170
6	30	30
7	130	150
8	160	160
9	100	110
10	80	280
11	40	50
12	70	100
13	260	240
14	90	130
15	110	190
16	90	180
17	120	170
18	120	110
19	30	30
Mean ± SD	100 ± 50	130 ± 70

Table 5.8 Individual subjects' plasma prebeta-1 HDL Values (ug/mL) before and after exercise

5.3. Study 3: Relation of Increased Prebeta-1 High-Density Lipoprotein Levels to Risk of Coronary Heart Disease

5.3.1. Subjects

Characteristics of the 1,255 subjects according to prebeta-1 HDL tertiles are presented in Table 5.9.

Variable	Total	Prebeta-1 HDL Tertiles		
	(n = 1255)	<4.171 (n = 418)	4.171-8.838 (n = 418)	>8.383 (n = 419)
Age (years)	50.8 ± 16.9	52.2 ± 17.1	51.2 ± 17.0	48.9 ± 16.6
Men	558 (44.4%)	155 (37.1%)	201 (48.1%)	202 (48.2%)
BMI (kg/m^2)	26.6 ± 5.4	25.2 ± 5.0	27.2 ± 5.8	27.3 ± 5.1
Caucasian	901 (73.4%)	287 (70.0%)	299 (73.6%)	315 (76.7%)
Asian	192 (15.6%)	84 (20.5%)	57 (14.0%)	51 (12.4%)
Other (African-American, Hispanic, other)	134 (10.9%)	39 (9.5%)	50 (12.3%)	45 (10.9%)
Hypertension	422 (33.7%)	123 (29.5%)	138 (33.0%)	161 (38.4%)
Smoker (current)	83 (6.6%)	16 (3.8%)	25 (6.0%)	42 (10.0%)
Lipid-lowering medication	193 (15.4%)	68 (16.3%)	61 (14.6%)	64 (15.3%)
Triglycerides (mg/dL)	148 (87.5)	109 (56.3)	151 (77.1)	199 (147.8)
LDL cholesterol (mg/dL)	147.9 ± 58.6	146.5 ± 53.3	148.7 ± 56.2	148.5 ± 65.8
HDL cholesterol (mg/dL)	50.8 ± 18.1	58.4 ± 19.8	48.4 ± 16.8	45.5 ± 14.7
Apo A-1 (mg/ml)	1.17 ± 0.35	1.22 ± 0.35	1.15 ± 0.38	1.14 ± 0.31
Type 2 DM	105 (8.4%)	32 (7.7%)	28 (6.7%)	45 (10.7%)
Coronary heart disease	454 (36.2%)	126 (30.1%)	158 (37.8%)	170 (40.6%)
Myocardial infarction	213 (17.0%)	56 (13.4%)	68 (16.3%)	89 (21.2%)

Distributions of demographic and clinical charisteristics are shown across tertiles of prebeta-1 HDL, defined as percentage Apo A-1 present in prebeta-1 HDL. Data are presented as number of subjects (percentage), mean ± SD, or median (mean deviation). Number of subjects for each demographic and clinical characteristic varies because of missing data.

Table 5.9 Demographic and clinical characteristics according to tertiles of prebeta-1 HDL.

Onset of clinical CHD occurred at a relatively young age in affected subjects (mean age 60.2 years). Most were of European ancestry, followed by Asians, and the rest were distributed in African- Americans and Hispanics. Few subjects were receiving lipid-lowering medications. Because virtually all samples were drawn at initial visits and many subjects were asymptomatic, lipid-lowering drug therapy had not been initiated by referring physicians.

5.3.2 Lipid Lowering drugs

Of the 16% taking lipid-lowering drugs at entry, 64% had been prescribed statin, 8% niacin, and the remainder a combination of statin with resin or fibrate. It is unlikely that lipid-lowering medications had a significant impact on the finding of this study because there was no significant difference in levels of percent prebeta-1 HDL in subjects taking versus not taking those medications (mean ± SD, no lipid medication 7.9% ± 6.5 vs lipid medication 7.7% ± 6.4, p =0.73). Mean ± SD prebeta-1 HDL was 7.87% ± 6.45 of plasma apolipoprotein A-I in the entire cohort.

Prebeta-1 HDL levels were associated with all demographic and clinical characteristics with the exception of LDL cholesterol, diabetes mellitus, and lipid-lowering medication. Subjects with increased prebeta-1 HDL were slightly younger and had more adverse clinical characteristics. They had higher body mass indexes and triglyceride levels, lower HDL cholesterol and apolipoprotein A-1 levels, and were more likely to be men, current smokers, and hypertensive. The negative correlation between prebeta-1 HDL and HDL cholesterol was more pronounced in subjects without CHD ($r = -0.34$, $p<0.001$) than in subjects with CHD ($r= -0.16$, $p <0.001$). Prebeta-1 HDL differed among races with Asians having significantly lower levels than others.

5.3.3 Menopause

Although menopausal status was not obtained, prebeta-1 HDL levels were examined in women> 55 years old (who are most likely postmenopausal, n= 212) and women< 35 years old (who are most likely premenopausal, n=132). Percent prebeta-1 HDL levels were similar between these groups (mean ± SD, premenopausal 6.84% ± 6.07 vs postmenopausal 6.92% ± 5.91, p= 0.78).

5.3.4 Association with Myocardial infarction

Unadjusted and adjusted associations of prebeta-1 HDL with CHD and MI are presented in Table 5.10. Prebeta-1 HDL levels were significantly and positively associated with increased risk of CHD and MI even after adjustment for conventional risk factors. Likelihood-ratio tests showed that prebeta-1 HDL contributed significantly to unadjusted and adjusted models. Estimates for all covariates entered in multivariable risk models are shown in the supplementary material. Results were similar when analysis was limited to the subgroup of subjects who were not taking lipid-lowering medication.

	CHD		MI	
	OR (95% CI)	p-value	OR (95% CI)	p-value
Unadjusted				
Prebeta-1 HDL tertiles				
<4.171	1.00	-	1.00	-
4.171-8.838	1.41 (1.06-1.88)	0.02	1.25 (0.85-1.84)	0.2
>8.838	1.58 (1.19-2.11)	0.002	1.74 (1.21-2.51)	0.003
Likelihood-ratio test	21.4	<0.001	18.4	<0.001
Adjusted				
Prebeta-1 HDL tertiles				
<4.171	1.00	-	1.00	-
4.171-8.838	1.34 (0.92-1.95)	0.1	1.17 (0.75-1.83)	0.5
>8.838	1.65 (1.11-2.46)	0.01	1.94 (1.23-3.05)	0.004
Likelihood-ratio test	6.1	0.047	10.0	0.007

Unadjusted and adjusted odds ratios and their 95% confidence intervals are shown for tertiles of prebeta-1 HDL, defined as percent total Apo A-1 present in plasma. Adjusted odds ratios adjust for age, gender, BMI, race, hypertension, current smoking status, type 2 diabetes mellitus, HDL cholesterol, LDL cholesterol, triglycerides, Apo A-1, and usage of lipid-lowering medication in multivariable logistic regression models. Likelihood-ratio test were performed comparing nested models with and without inclusion of prebeta- HDL tertiles in logistic regression models. CI = confidence interval; OR = odds ratio

Table 5.10. Associations of prebeta-1 HDL tertile with coronary heart disease and myocardial infarction.

5.3.5 Effect of adding prebeta-1HDL lipoprotein tertiles to risk-prediction models

Table 5.11 presents 3 statistical measurements that were used to evaluate the utility of adding prebeta-1 HDL as an additional predictor in conventional risk factor models. Inclusion of prebeta-1 HDL in multivariable models led to trivial increments in area under receiver operating characteristic curves for CHD and MI. However, changes in area under receiver operating characteristic curves are known to be insensitive to biomarkers of moderate effect (Cook, 2007; Pencina, D'Agostino, D'Agostino & Vasan, 2008) including prebeta-1 HDL in the risk model for CHD resulted in a significant improvement in classification as indicated by the net reclassification index; however, the integrated discrimination improvement index estimate was non-significant, suggesting that reclassification improvement was not robust to different definitions of risk categories. In contrast, incorporation of prebeta-1 HDL into the risk model for MI led to a significant improvement in the net reclassification index and integrated discrimination improvement index.

	AUC		NRI		IDI	
	Change (95% CI)	p value	Estimate (95% CI)	p value	Estimate (95% CI)	p value
Coronary heart disease	0.001 (-0.002 to 0.004)	0.5	0.15 (0.04-0.28)	0.01	0.003 (-0.001 to 0.007)	0.2
Myocardial infarction	0.006 (-0.003 to 0.015)	0.2	0.21 (0.05-0.36)	0.008	0.010 (0.002-0.018)	0.02

The increment because of inclusion of prebeta-1 HDL tertiles in risk models compared to conventional risk factors alone is shown for various calibration statistics. Areas under reciever operating characteristic curves in risk models that exlude prebeta-1 HDL are 0.85 for coronary heart disease and 0.78 for myocardial infarction.
AUC = area under reciever operationg characteristic curve; IDI integrated discrimination improvement index; NRI = net reclassifcation improvement index

Table 5.11. Effect of adding prebeta-1 HDL lipoprotein tertiles to risk-prediction models

CHAPTER 6

Discussion

6.1 The Isotopic Dilution-Ultrafiltration Method for measurement of prebeta-1 HDL

The requirement for a novel reliable easy to use method to quantify prebeta-1 HDL directly from plasma is underscored by the number of previously described methods, which are slow, technically difficult and generally semi quantitative, catering for small numbers of samples at any one time. In addition some of these methods fail to fully separate the quantum prebeta-1 HDL subspecies from other HDL subspecies with prebeta electrophoretic mobility. There are a number of advantages to the use of the isotope dilution technique in combination with plasma ultrafiltration for quantification of plasma prebeta-1HDL including:

- Low detection limit
- High accuracy
- Insensitivity to interfering factors

George de Hevesy was first to describe Isotope dilution analysis, which he used to estimate the rate of turnover of water in the human body using deuterium oxide as a biological tracer (Hevesy, 1934). The isotope dilution technique is a radiochemical analytical technique, used to quantify the amount of a given substance in a mixture where it is not possible to easily separate the substance from the mixture for quantification. Using this technique quantification of an unlabeled compound, by dilution with a labeled form of the pure compound, can be employed. Quantification of the amount of a given compound (in this case prebeta-1 HDL) in a complex mixture (plasma) is possible using isotope dilution for two reasons. Firstly part of the compound with added tracer may be isolated in a pure form (pure prebeta-1 HDL can be isolated by Immunoaffinity chromatography and starch electrophoresis). Secondly the specific activity of the compound may be determined both in the tracer amount and in the mixture. This is possible because ultrafiltration separates plasma prebeta-1 HDL and ELIZA is used to measure its apoA-1 content.

Isotope dilution analysis was therefore appropriate for the quantification of plasma prebeta-1 as both of these requirements were met. A known amount of tritiated pure prebeta-1 HDL was added to plasma, which contained an unknown quantity of, prebeta-1 HDL. Ultrafiltration of this mixture was carried out using a chosen ultrafilter, which discriminates categorically between prebeta-1 HDL and other much larger HDL species as was shown in the experiments describe in chapter 3. Thus prebeta-1 HDL is the only apolipoprotein A-1 containing lipoprotein, which passes through the filter into the ultrafiltrate. Apo A-1 levels in plasma and ultrafiltrate were measured by ELIZA, as described. The mass of prebeta-1 HDL could therefore be calculated according to the isotope dilution formula:

$$\frac{\textbf{Counts applied to plasma sample}}{\textbf{Specific activity of the ultrafiltrate}} = \textbf{Mass of prebeta-1 HDL in plasma}$$

Thus, ultrafiltration effectively samples the prebeta 1-HDL pool in plasma and the isotope dilution principle allows for calculation of the original concentration applied to the ultrafilter because, the behavior of the tracer (tritiated prebeta-1 HDL) is assumed to be identical to that of the analyte (plasma prebeta-1 HDL).

This is a novel approach to the accurate assessment of prebeta-1 HDL levels in plasma. Previously measurements of prebeta-1 HDL in the $d >$ 1.21 g/ cm^3 ultracentrifugal fraction included apoA-I that may be stripped from other HDL species as a result of exposure to the high fluid pressures encountered at 100,000*g* during centrifugation (Kunitake & Kane, 1982). Strategies directed at measurement of apoA-I in the prebeta electrophoretic zone by any of the non-gradient one-dimensional techniques will also include the high-molecular- weight HDL components prebeta-2 and prebeta-3 HDL (Fielding & Fielding, 1995) because the methods lack a discriminant based on particle mass. Methods using two-dimensional separations (Asztalos, Sloop, Wong & Roheim, 1993) eliminate the latter problem but suffer from two limitations. The first is the need to transfer all or a constant fraction of

the lipoprotein from gel to membrane for quantitation. More important practically is the need to run an individual two-dimensional gel or even duplicate gels for each sample, which is very time consuming and impossible to scale for large numbers of clinical samples.

Physically, the fraction filtered would not be expected to approach the concentration in the solution applied if the Stokes radius of the particle is close to the pore diameter. However, the ratio filtered would be expected to be constant over a considerable range of concentrations found in plasma because of the low absolute levels present. Thus, ultrafiltration effectively samples the prebeta 1-HDL pool in plasma and the isotope dilution principle allows for calculation of the original concentration applied to the ultrafilter because, in this approach, the behavior of the tracer is assumed to be identical to that of the analyte. In this case we have validated the latter by the behavior of the tracer with respect to its apparent Stokes radius as measured by gradient gel electrophoresis and its electrophoretic mobility.

An important observation with respect to the behavior of HDL particles is that with eightfold dilution, changes in physical behavior appear (Asztalos & Roheim, 1995) As plasma is diluted, lipoprotein species with pre-alpha mobility appear. Changes in the content of prebeta-1 HDL occur and monomeric apoA-I becomes significant. It is possible that this reflects changes that HDL particles undergo in peripheral lymph fluid where dilution approaches eightfold. Because of this phenomenon the ultrafiltration – isotope dilution technique was designed to operate with undiluted plasma.

The results show that about 7% of the apoA-I in plasma is present in the form of prebeta-1 HDL in normolipidemic fasting humans. The turnover of prebeta-1 HDL is likely to be rapid, because it participates in the prebeta-1 HDL cycle in which it is transformed into larger species of HDL and then regenerated (Hennessy, Kunitake & Kane, 1993) and it may also be generated by the lipolysis of triglyceride-rich lipoproteins,. Thus, the levels measured in plasma represent values represent a "snapshot" of a dynamic process at a point in time.

6.2 Prebeta-1 HDL in normolipidemic subjects; influences of plasma lipoproteins, age and gender

With respect to the measurement of prebeta-1 HDL in plasma of normolipidemic individuals, the influences of plasma lipoproteins, age, and gender were examined. In the 136-normolipidemic individuals examined (46 M, 90 F), the mean absolute concentration of prebeta-1 HDL as apolipoprotein A-I was 68 ± 40 μg/ml for women, and 84-± 49 m/ml for men. Prebeta-1 HDL represented 5.5 ± 3.3% of total apolipoprotein A-I in women, and 7.2 ± 4.0% in men. The distributions of both absolute and percent prebeta-1 HDL are highly asymmetric, with skew toward higher values. However, the skew appears not to be attributable to either plasma cholesterol or triglyceride levels which are also skewed in population samples. The percent prebeta-1 HDL was negatively correlated with HDL cholesterol levels ($P < 0.0001$), whereas absolute levels of prebeta-1 HDL were positively correlated with apolipoprotein A-I and negatively correlated with HDL cholesterol (P, for both, < 0.0001). Multiple linear regression analysis revealed effects of age and gender, but no association with lipoprotein fractions other than HDL. Lower levels of prebeta-1 HDL were associated with female gender in all models. One of the most prominent predictors (negative) of percent prebeta-1 HDL and absolute prebeta-1 HDL levels was found to be HDL cholesterol. This is consistent with the model described, in which the level of prebeta-1 HDL would be expected to be relatively low when there is a high content of cholesteryl esters in alpha HDL particles. On the other hand, high levels of apoA-I are correlated positively with absolute levels of prebeta-1 HDL, suggesting that increasing the availability of apoA-I may favor the maintenance of a higher steady state level of prebeta-1 HDL, even in the presence of increased levels of alpha HDL particles. This possibility is supported by the observation that expression of a human apoA-I transgene results in increased levels of prebeta HDL in rabbits (Duverger et al., 1996). It is interesting that both the absolute levels of prebeta-1 HDL and the percent of total apoA-I found in the prebeta-1 fraction distribute with significant skew toward higher levels, similar to total cholesterol levels, LDL cholesterol levels, and total triglycerides in the general population.

The skew is apparently not attributable to the levels of triglycerides or cholesterol per se, however, because of the lack of association between these variables observed in our statistical models.

In the normolipidemic subjects, no association was found between either of the prebeta-1 HDL values and body mass index. The lack of significantly obese subjects in this cohort limits the interpretation of this finding. It is possible that alterations in lipoprotein metabolism associated with significant obesity may well affect one or more of the determinants of levels of prebeta-1 HDL in plasma. The mechanisms underlying the negative trend detected with age are not readily apparent. This correlation was observed almost entirely with percent prebeta-1 HDL.

Both percent prebeta-1 HDL and absolute prebeta-1 HDL were significantly correlated with gender in bivariate relationships. In both single and multiple predictor models female gender was associated with lower values. Though the numbers of subjects limit the strength of the observation, the finding that adjusted mean levels for postmenopausal women without estrogen supplementation more closely resemble those of males suggest that the effect is mediated by ovarian steroid hormones. This could reflect the effect of estrogen in down-regulating the SR-BI receptor (Landschulz, Pathak, Rigotti, Krieger & Hobbs, 1996). If the SR-BI receptor contributes significantly to the removal of cholesteryl esters from HDL, its down-regulation by estrogen could increase the plasma content of HDL cholesteryl esters and hence the content of alpha HDL particles in plasma. The latter is associated negatively with plasma levels of prebeta-1 HDL in this study. Indeed, down-regulation of the SR-BI receptor could be a major determinant of the differences in HDL cholesterol levels between men and menstruating women.

Another mechanism that could underlie the lower levels of prebeta-1 HDL in women is their relatively lower levels of hepatic lipase activity (Brinton, Eisenberg & Breslow, 1989). Hepatic lipase has been demonstrated to promote the formation of prebeta HDL in vitro

(Barrans et al., 1994). Also, prebeta HDL appears to be formed during the perfusion of liver with HDL_2 (Barrans et al., 1994). Decreased activity of either SR-BI or hepatic lipase could contribute to the observed increase in mean diameters of HDL particles among women (Li et al., 1996).

Clearly, relative changes in the activities of LCAT, CETP, PLTP, and also lipoprotein lipase and hepatic lipase could individually alter the steady state levels of prebeta-1 HDL in plasma. Modulating effects of apolipoproteins such as apoA-II on the LCAT reaction (Francone, Gong, Ng, Fielding & Rubin, 1995) and other processes (Zhong, Goldberg, Bruce, Rubin, Breslow & Tall, 1994) and upon the molecular speciation of HDL may also have significant impact on prebeta-1 HDL levels and upon the rate of reverse cholesterol transport.

6.3 The Effects of Physical Exercise on Plasma Prebeta-1 High-Density Lipoprotein

The impact of physical exercise on HDL metabolism is recognized as a major mechanism of coronary artery disease (CAD) risk reduction. Prebeta-1 HDL sub particle species play a pivotal role in initiating reverse cholesterol transport. Gupta et al. (Gupta, Ross, Myers & Kashyap, 1993) have shown that the net mass of free cholesterol transport out of cultured human fibroblasts into athlete's serum was greater than that of sedentary controls. This indicates that physical exercise may promote RCT by increasing cholesterol efflux from peripheral tissues. Because specific subfractions of HDL, such as the prebeta-1 HDL fraction (Fielding & Fielding, 1995) may be especially efficient at mediating cholesterol removal from peripheral cells, physical exercise would be anticipated to increase plasma prebeta- 1 HDL levels.

The evaluation of prebeta-1 HDL metabolism may therefore be of value in identifying mechanisms by which physical exercise-induced effects on HDL particles exert a protective action against coronary artery disease. The purpose of this exercise study was to evaluate the effect of

a single bout of protracted aerobic exercise on plasma prebeta-1 HDL levels. The findings confirm the expected result that young adults' level of regular habitual exercise is related to increased VO2max and increased plasma HDL-cholesterol levels with significant difference seen between the exercise trained subjects and their sedentary counterparts. Plasma prebeta-1 HDL levels increased and plasma HDL-triglyceride levels were lowered following physical exercise, which may be of relevance with respect to effects of acute exercise on reverse cholesterol transport and may therefore have implications with respect to the metabolic role of exercise in coronary artery disease risk reduction. The study was conducted in young, healthy adults, and hence the findings may not extend to older persons. Acute exercise has generally been shown to increase plasma HDL-cholesterol levels by 4% to 43%.(Thompson, Crouse, Goodpaster, Kelley, Moyna & Pescatello, 2001) The lack of acute exercise-induced increase in plasma HDL-cholesterol noted in this study may reflect a "masking effect" of exercise-induced offsetting reciprocal changes in plasma HDL2-cholesterol (increased) and HDL3-cholesterol (decreased) sub particle levels. (Nye, Carlson, Kirstein & Rossner, 1981). This study did not measure HDL sub particle composition. Physical exercise is recognized to have a triglyceride lowering effect (Leaf, Parker & Schaad, 1997) that is prominent following acute and chronic exercise in hypertriglyceridemics (Lampman et al., 1980). The lack of an acute exercise response on total plasma triglyceride lowering may be reconciled by the relatively low pre-exercise triglyceride levels among these subjects as exercise-induced triglyceride lowering occurs more prominently among those with increasing triglyceride levels (Crouse et al., 1995).

As described earlier prebeta-1 HDL behaves as the quantum HDL particle in the initial phase of the pathway for cholesterol retrieval from peripheral cells to the liver (von Eckardstein, Nofer & Assmann, 2001) It is the first acceptor of unesterified cholesterol, becoming a substrate for LCAT. As the cholesterol is esterified, the prebeta particles are ultimately subsumed into larger, spherical alpha HDL particles (Phillips, Gillotte, Haynes, Johnson, Lund-Katz & Rothblat, 1998). As

cholesteryl esters are then transferred, under the influence of cholesteryl ester transfer protein (CETP), to acceptor lipoproteins that contain the B apolipoproteins, prebeta-1 HDL particles are regenerated. Thus, the circulating level of prebeta-1 HDL reflects the relative activities of LCAT and CETP, in the cyclic process described. Increased LCAT activity increases alpha HDL formation and has been shown to be greater in athletes than sedentary counterparts (Gupta, Ross, Myers & Kashyap, 1993) (Tsopanakis, Kotsarellis & Tsopanakis, 1988) (Lopez, Vial, Balart & Arroyave, 1974) and is increased immediately after acute exercise (Dufaux, Order, Muller & Hollmann, 1986) (Frey, Baumstark, Berg & Keul, 1991). Decreased CETP activity increases alpha HDL levels by increasing cholesterol ester content, and concomitantly reduces HDL-triglyceride content by triglyceride transfer to non-HDL lipoprotein species. On the other hand, increased CEPT activity could facilitate alpha HDL turnover and promote prebeta HDL formation. In some studies CETP activity is greater among athletes than sedentary individuals, while it has also been shown to decrease following physical exercise training (Seip et al., 1993) Following acute exercise CEPT activity is shown to decrease (Foger et al., 1994) or be unchanged (Grandjean, Crouse & Rohack, 2000). This study shows that acute exercise increases prebeta-1HDL without increasing plasma Apo A1 levels. This is consistent with studies showing that prebeta-1 HDL is derived from alpha HDL particles rather from de novo Apo A-I synthesis (Hennessy, Kunitake & Kane, 1993). Prebeta HDL particles are generated from alpha HDL during cholesterol clearance by several pathways, including:

(1) CETP-mediated exchange of cholesterol esters from HDL to the very–low-density lipoprotein (VLDL)/low-density lipoprotein (LDL) particle series which then provides cholesterol removal by the LDL receptor pathway (von Eckardstein, Nofer & Assmann, 2001),(Willnow, Nykjaer & Herz, 1999).

(2) Direct HDL-mediated receptor pathways that include: firstly cubulin (traditionally known as the receptor for intrinsic

> factor-B12 complexes present in the intestinal and kidney epithelium) (Acton, Rigotti, Landschulz, Xu, Hobbs & Krieger, 1996) (Hammad et al., 1999; Kozyraki et al., 1999) that mediates HDL holoparticle endocytosis, and secondly the scavenger receptor, type BI (SR-BI) that binds HDL and selectively mediates the uptake or removal of HDL cholesterol esters without the uptake/degradation of HDL–Apo A-I.(Acton, Rigotti, Landschulz, Xu, Hobbs & Krieger, 1996),(Krieger, 2001)

The SR-BI receptor appears to act as a "docking" site where cholesterol esters can be removed and subsequently the HDL particle (depleted of cholesterol esters) "undocks" and recirculates to pick up more cholesterol esters from peripheral tissues. Exercise-induced modulation of these HDL catalytic pathways could potentially play an important role in regulating plasma prebeta-1 HDL levels. Hepatic lipase has been shown to play a major role in HDL catabolism by hydrolyzing both triglycerides and phospholipids within HDL particles (Rader & Ikewaki, 1996).(Santamarina-Fojo, Haudenschild & Amar, 1998). LP A-I particles appear to be the most efficient in facilitating cholesterol ester uptake by SR-BI receptors: Hep G2 cell uptake of [3H] cholesterol ester is approximately 75% greater from LP A-I than LP A-I _ A-II particles (Sakai, Kamanna & Kashyap, 2001). Niacin has been shown to selectively inhibit plasma LP A-I removal without affecting cholesterol ester uptake (Jin, Kamanna & Kashyap, 1997), which may promote prebeta-1 HDL regeneration. It is conceivable that physical exercise could exert a similar effect to increase plasma LP A-I levels by reducing LP A-I removal. Indeed Herbert et al (Herbert, Bernier, Cullinane, Edelstein, Kantor & Thompson, 1984) found that the mean biological half-life of HDL proteins was longer in runners (6.2 days) than in sedentary controls (3.8 days). Tracer studies of radioiodinated autologous HDL demonstrated that runners did not produce more HDL protein but rather catabolized less. These studies were subsequently corroborated by the same group (Thompson et al., 1988) who showed that participation in 8 to 11 months of aerobic based prolonged exercise training increased the biological half-life of Apo A-I by 10% (P =0.07) by decreasing the fractional catabolic rate by a corresponding

amount and not affecting Apo A-I synthetic rate. Some (Couillard et al., 2001) (Kiens et al., 1980),(Thompson et al., 1997) but not other (Thompson et al., 1988) (Wood et al., 1988), studies indicate that physical exercise training raises plasma Apo A-I levels. No difference was observed in the current study in plasma Apo A-I levels between pre and post acute exercise. These findings are consistent with the possibility that physical exercise promotes alpha HDL recycling and regeneration of HDL.

It is likely that intravascular lipolysis also contributes to the levels of prebeta-1 HDL in plasma. Lipoprotein lipases are a family of tissue-specific hydrolytic enzymes that are rate-limiting for the removal of circulating lipoprotein triglycerides and have been implicated in atherogenesis (Eckel,1989) (Goldberg,1996). As lipoprotein lipase–mediated lipolysis proceeds, phospholipid, and some small molecular weight apolipoproteins move from the surface monolayers of triglyceride-rich lipoproteins to HDL. Exercise induced lipoprotein lipase activity may account for exercise related reductions in plasma HDL-triglyceride levels (Kantor, Cullinane, Herbert & Thompson, 1984).(Sady, Thompson, Cullinane, Kantor, Domagala & Herbert, 1986).

Adipose lipoprotein lipase activity, involved in triglyceride storage of acyl groups derived primarily from the hydrolysis of plasma lipoproteins, increases with physical exercise training (Taskinen & Nikkila, 1980) Acute exercise also increases lipoprotein lipase activity and fat tolerance (Kantor, Cullinane, Herbert & Thompson, 1984; Sady, Thompson, Cullinane, Kantor, Domagala & Herbert, 1986). This has been most widely shown in fit individuals performing at high levels of protracted physical exertion, but also has been shown to occur in sedentary individuals exercising for as little as 1 hour at 80% of maximal heart rate (Kantor, Cullinane, Sady, Herbert & Thompson, 1987). These observations lead to the hypothesis that exercise acutely depletes intramuscular triglycerides, which was confirmed by Rico-Sanz et al. (Rico-Sanz et al., 2000). This stimulates the synthesis or translocation of lipoprotein lipase, which hydrolyzes triglycerides from lower-density lipoproteins with transfer of the excess surface

cholesterol to the HDL particle (Thompson, Cullinane, Henderson & Herbert, 1980).

Skeletal muscle lipoprotein lipase also exerts exercise-related effects on plasma lipids and lipoproteins (Thompson, 1990). Exercise raises skeletal lipoprotein lipase activity in the capillary beds of skeletal muscles where fatty acids are hydrolyzed from circulating lipoproteins for energy utilization. This could influence plasma HDL-triglyceride metabolism as we have previously shown acute exercise to reduce plasma HDL-triglycerides by 30% in hypertriglyceridemics (Leaf DA, 1988).

In conclusion, both acute physical exercise and regular physical exercise training are recognized for their effects on HDL metabolism that can mitigate coronary artery disease risk. The findings in this study indicate that the effect of acute physical exercise on plasma HDL particles involves increasing prebeta-1 HDL particles, which may be a transient effect reflecting increased flux of cholesterol through the reverse cholesterol transport pathway. Additional studies are warranted to further evaluate the role of exercise-related effects upon plasma prebeta 1 HDL relating to coronary artery disease risk reduction.

6.4 The associations between prebeta-1 HDL, CHD and MI

The aims of this study were firstly to test previous associations between prebeta-1 HDL and CHD and secondly to investigate whether prebeta-1 HDL levels are also associated with risk of myocardial infarction (MI). The study was carried out in a large well-characterized clinical cohort and confirmed the association between increased levels of prebeta-1 HDL and risk of CHD even after adjustment for traditional risk factors (Asztalos et al., 2005; Asztalos, Collins, Horvath, Bloomfield, Robins & Schaefer, 2008; Asztalos et al., 2004; Lamon-Fava et al., 2008; Miida et al., 1996; Sethi et al., 2010).

This study also reveals a novel association between increased levels of prebeta-1 HDL and risk of MI. Higher levels are predictive of MI above and beyond conventional risk factors. The magnitude of effect was comparable to effect sizes observed for factors such as lipids and systolic blood pressure.

Inclusion of prebeta-1 HDL in traditional risk-factor models significantly improved classification of subjects for MI and modestly improved classification for CHD, suggesting that prebeta-1 HDL has more discriminatory power for MI than for CHD in the population studied. Because progression of coronary atherosclerosis to infarction depends on factors contributing to instability of plaque, this observation may have mechanistic importance.

Consistent with our finding, that levels of prebeta-1 HDL are higher in subjects with CHD, increased levels were correlated with more adverse characteristics such as higher body mass index, hypertension, and smoking. Prebeta-1 HDL was correlated with all lipid risk factors except LDL. It was positively correlated with levels of triglycerides and negatively with levels of HDL cholesterol.

A number of studies to date have examined prebeta-1 HDL as a predictor of CHD (Asztalos et al., 2005; Asztalos, Collins, Horvath, Bloomfield, Robins & Schaefer, 2008; Asztalos et al., 2004; Lamon-Fava et al., 2008; Miida et al., 1996; Sethi et al., 2010). These have reported a positive association both in men (Asztalos et al., 2005; Asztalos et al., 2004), and women (Lamon-Fava et al., 2008).

The association between prebeta-1 HDL and CHD was robust to the adjustment of established CHD risk factors in contrast to previous reports (Asztalos et al., 2005; Asztalos et al., 2004). These discrepancies may be attributed to differences in sample size, prevalence of CHD in study cohorts, and covariates included in the multivariable model. Because this study included subjects from lipid and cardiology clinics, prevalence of CHD was increased and therefore increased the power to detect an association of prebeta-1 HDL with CHD.

Measurement of prebeta-1 HDL in this study used a different analytic approach, as described earlier, ultrafiltration and isotope dilution (O'Connor et al., 1997), from methods in previous studies. However, because this method is based on quantitative analysis of purified prebeta-1 HDL added to plasma, yielding a linear response, it is a quantitative measurement. Several methods have been applied to assess prebeta-1 HDL such as one and two-dimensional gel electrophoresis. However, these methods are not based on purified prebeta-1 HDL as a standard and involve differences in antibody affinity among HDL species, the reported prebeta-1 HDL values cannot be directly compared to those in this study. Size-exclusion chromatography can separate prebeta-1 HDL particles that share characteristics of those separated by ultrafiltration including particle mass and percent composition of HDL (Nanjee & Brinton, 2000). The physical identity of the prebeta-1 HDL species across different methods and concordance between the association of levels of prebeta-1 HDL with CHD by ultrafiltration and western blotting are noteworthy (Asztalos et al., 2005; Asztalos, Collins, Horvath, Bloomfield, Robins & Schaefer, 2008; Asztalos et al., 2004; Lamon-Fava et al., 2008; Miida et al., 1996; Sethi et al., 2010).

The ability to make quantitative measurements of individual molecular species of HDL is critical to a mechanistic understanding of the atheroprotective functions of HDL. These include antioxidant and anti-inflammatory activities (Navab, Gharavi & Watson, 2008). The best understood process is reverse cholesterol transport. At least 4 mechanisms for efflux from macrophages are recognized: the ABCA1 and ABCG1 transporters, the scavenger receptor class B type I receptor, and passive diffusion. Sviridov et al. (Sviridov, Miyazaki, Theodore, Hoang, Fukamachi & Nestel, 2002) recognized that plasma depleted of prebeta-1 HDL could still support cholesterol efflux from macrophages. Later, de la Llera-Moya et al. (de la Llera-Moya, Drazul-Schrader, Asztalos, Cuchel, Rader & Rothblat, 2010) studied the relative contributions of the pathways using a selective inhibition protocol. They concluded that the ABCA1 mechanism predominates, with the ABCG1 pathway contributing about 20% and the scavenger receptor

class B type I receptor very little (de la Llera-Moya, Drazul-Schrader, Asztalos, Cuchel, Rader & Rothblat, 2010). In vitro studies with human macrophages have demonstrated that prebeta-1 HDL is the primary acceptor in ABCA1-mediated efflux. In plasma from normal subjects, rate of cholesterol efflux from the ABCA1 transporter has been shown to be positively related to the level of prebeta-1 HDL.(de la Llera-Moya, Drazul-Schrader, Asztalos, Cuchel, Rader & Rothblat, 2010). Esterification of cholesterol by lecithin–cholesterol acyl transferase (LCAT) depletes the level of prebeta-1 HDL. Cholesteryl ester transfer protein (CETP), phospholipid transfer protein, and scavenger receptor class B type I (SRB1) activities can produce prebeta-1 HDL (Francone, Royer & Haghpassand, 1996; Jiang et al., 1996; Kunitake, Mendel & Hennessy, 1992; Lee, Metso, Jauhiainen & Kovanen, 2003). The findings of high levels of prebeta-1 HDL in subjects with CHD points to some defect in the efflux mechanism or subsequent metabolic processes in reverse cholesterol transport. If efflux by ABCA1 or LCAT activity were impaired, an accumulation of unrepleted acceptor prebeta-1 HDL would be expected. This is consistent with the observation on 2-dimensional gel analysis that lower levels of large-diameter particles are associated with increased risk of CHD. The finding that the rate of efflux of cholesterol through the ABCA1 pathway in vitro can differ significantly between subjects with identical levels of HDL cholesterol (de la Llera-Moya, Drazul-Schrader, Asztalos, Cuchel, Rader & Rothblat, 2010) and the association of impaired acceptor properties of HDL with CHD suggest that the acceptor properties of HDL present an additional variable determinant of CHD risk (Khera et al., 2011). Because measurement of the level of prebeta-1 HDL is easier to perform and is scalable for large numbers of samples, as compared to the determination of kinetics of efflux, it may become a clinically valuable indicator of risk. High levels could reflect a high chemical potential for the acquisition of cholesterol and efficient transfer of cholesteryl esters from HDL to acceptor lipoproteins. However, high levels would also occur if cholesterol esterification by LCAT was impaired. Similar levels should also result from active intravascular lipolysis. In addition various mechanisms could cause functional inactivation of prebeta-1 HDL and impaired cholesterol retrieval, which could be reflected in

raised prebeta-1 HDL levels. Extracellular modifications which could prevent prebeta-1HDL's interaction with the ABCA-1 receptor and its further metabolism in the prebeta HDL cycle described earlier *(Kunitake, Mendel & Hennessy, 1992),* (Hennessy, Kunitake & Kane, 1993). Proteolytic inactivation of prebeta-1 HDL, secondary to various proteases, such as the mast cell-derived neutral protease chymase present in the arterial intima has been described as a potential mechanism that impairs the efficiency of HDL to promote cholesterol efflux from macrophage foam cells. Enzymatic oxidation, lipolysis and proteolysis, and non-enzymatic glycosylation are among the HDL modifications that adversely affect HDL functionality (Lee-Rueckert & Kovanen, 2011). In contrast, low levels could signify less efficient transfer of cholesterol esters. Thus, the level of prebeta-1 HDL in plasma will affect the relative rates of cholesterol esterification (LCAT), ester transfer (CETP), and perhaps other processes involved in the retrieval pathway. Measurement of prebeta-1 HDL levels in plasma would also provide an empirical approach to determining the relationship of plasma levels of prebeta-1 HDL to the risk of coronary and peripheral vascular disease.

The strengths of this study are in its large sample and detailed clinical phenotypes. Nevertheless, there are also several limitations to the study. The cohort is not representative of a general population because it was recruited from lipid and cardiology clinics. However, all associations for established risk factors were in the same direction as those reported in general population studies, excepting male gender. Also, all analyses were cross sectional because the study lacked longitudinal information. Further research regarding the prospective discriminatory and reclassification power of prebeta-1 HDL with respect to CHD and MI is warranted to fully evaluate it as a biomarker and to study effects of lifestyle, medications, and other factors. Our analyses focused on prebeta-1 HDL and did not include other HDL subpopulations that are highly correlated with each other. In conclusion, the results confirm previous associations between prebeta-1 HDL and CHD in a large well-characterized clinical cohort. Also, this is the first study in which prebeta-1 HDL was identified as a

novel and independent predictor of MI above and beyond traditional CHD risk factors.

6.5 Conclusions and future directions

The measurement of plasma prebeta-1 HDL may be of value in assessing cardiovascular risk and may also provide new mechanistic insights into the process of atherosclerosis. The ability to quantify prebeta-1 HDL levels in plasma provides a functional measurement of value in the interpretation of the complex processes involved in HDL metabolism and the reverse cholesterol transport process. Future research further characterizing prebeta-1 HDL utilizing new modes of inquiry including lipidomics and proteomics with mass spectroscopy, may allow a greater understanding of other potential roles for prebeta-1 HDL. Further investigation elucidating the structural and functional relationships between prebeta-1 HDL and reverse cholesterol transport may facilitate the identification of new drug targets for reversal of atherosclerotic cardiovascular disease.

Publications:

O'Connor PM, Naya-Vigne JM, Duchateau PN, Ishida BY, Mazur M, Schoenhaus SA*, et al.* (1997). Measurement of prebeta-1 HDL in human plasma by an ultrafiltration-isotope dilution technique. Analytical biochemistry 251**:** 234-240.

O'Connor PM, Zysow BR, Schoenhaus SA, Ishida BY, Kunitake ST, Naya-Vigne JM*, et al.* (1998). Prebeta-1 HDL in plasma of normolipidemic individuals: influences of plasma lipoproteins, age, and gender. Journal of lipid research 39**:** 670-678.

Kunitake ST, O'Connor, P., and Naya-Vigne, J. (1996). Methods in Enzymology (Bradley, G, and Segrest, Eds) Academic Press,SanDiego**:** 260-267.

Guey LT, Pullinger CR, Ishida BY, O'Connor PM, Zellner C, Francone OL*, et al.* (2011). Relation of increased prebeta-1 high-density lipoprotein levels to risk of coronary heart disease. The American journal of cardiology 108**:** 360-366.

Jafari M, Leaf DA, Macrae H, Kasem J, **O'Conner P**, Pullinger C*, et al.* (2003). The effects of physical exercise on plasma prebeta-1 high-density lipoprotein. Metabolism 52**:** 437-442.

References

Erratum. Metabolism - Clinical and Experimental 52**:** 1372.

Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). (1994) Lancet 344**:** 1383-1389.

Acton S, Rigotti A, Landschulz KT, Xu S, Hobbs HH, & Krieger M (1996). Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. Science 271**:** 518-520.

Adorni MP, Zimetti F, Billheimer JT, Wang N, Rader DJ, Phillips MC*, et al.* (2007). The roles of different pathways in the release of cholesterol from macrophages. Journal of lipid research 48**:** 2453-2462.

Anitschkow N CS (1913). Ueber experimentelle Cholesterinsteatose und ihre Bedeutung fuer die Entstehung einiger pathologischer Prozesse. Zentrbl Allg Pathol Pathol Anat 24**:** 1-9.

Asztalos BF, Collins D, Cupples LA, Demissie S, Horvath KV, Bloomfield HE*, et al.* (2005). Value of high-density lipoprotein (HDL) subpopulations in predicting recurrent cardiovascular events in the Veterans Affairs HDL Intervention Trial. Arterioscler Thromb Vasc Biol 25**:** 2185-2191.

Asztalos BF, Collins D, Horvath KV, Bloomfield HE, Robins SJ, & Schaefer EJ (2008). Relation of gemfibrozil treatment and high-density lipoprotein subpopulation profile with cardiovascular events in the Veterans Affairs High-Density Lipoprotein Intervention Trial. Metabolism 57**:** 77-83.

Asztalos BF, Cupples LA, Demissie S, Horvath KV, Cox CE, Batista MC*, et al.* (2004). High-density lipoprotein subpopulation profile and coronary heart disease prevalence in male participants of the Framingham Offspring Study. Arterioscler Thromb Vasc Biol 24**:** 2181-2187.

Asztalos BF, & Roheim PS (1995). Presence and formation of 'free apolipoprotein A-I-like' particles in human plasma. Arterioscler Thromb Vasc Biol 15**:** 1419-1423.

Asztalos BF, Sloop CH, Wong L, & Roheim PS (1993). Two-dimensional electrophoresis of plasma lipoproteins: recognition of new apo A-I-containing subpopulations. Biochimica et biophysica acta 1169**:** 291-300.

Asztalos BF, Tani M, & Schaefer EJ (2011). Metabolic and functional relevance of HDL subspecies. Current opinion in lipidology 22**:** 176-185.

Bachorik P, and Albers, J. (1986).
Methods in Enzymology , Academic Press, San Diego 78 – 100.

Barr DP, Russ EM, & Eder HA (1951). Protein-lipid relationships in human plasma. II. In atherosclerosis and related conditions. The American journal of medicine 11**:** 480-493.

Barrans A, Collet X, Barbaras R, Jaspard B, Manent J, Vieu C*, et al.* (1994). Hepatic lipase induces the formation of pre-beta 1 high density lipoprotein (HDL) from triacylglycerol-rich HDL2. A study comparing liver perfusion to in vitro incubation with lipases. The Journal of biological chemistry 269**:** 11572-11577.

Bonnefont-Rousselot D, Therond P, Beaudeux JL, Peynet J, Legrand A, & Delattre J (1999). High density lipoproteins (HDL) and the oxidative hypothesis of atherosclerosis. Clinical chemistry and laboratory medicine : CCLM / FESCC 37**:** 939-948.

Borresen AL, & Berg K (1980). Presence of "free" apoA-I in serum: implications for immunological quantification of HDL and its apoproteins. Artery 7**:** 139-160.

Box G. E. P. CDR (1964). An analysis of transformations (with discussion). J R Statist Soc A143**:** 383-430.

Brewer HB, Jr. (2007). HDL metabolism and the role of HDL in the treatment of high-risk patients with cardiovascular disease. Current cardiology reports 9**:** 486-492.

Brinton EA, Eisenberg S, & Breslow JL (1989). Elevated high density lipoprotein cholesterol levels correlate with decreased apolipoprotein A-I and A-II fractional catabolic rate in women. The Journal of clinical investigation 84**:** 262-269.

Bruce RA (1974). Methods of exercise testing. Step test, bicycle, treadmill, isometrics. The American journal of cardiology 33**:** 715-720.

Bruckert E, & Hansel B (2007). HDL-c is a powerful lipid predictor of cardiovascular diseases. International journal of clinical practice 61**:** 1905-1913.

Castro GR, & Fielding CJ (1988). Early incorporation of cell-derived cholesterol into pre-beta-migrating high-density lipoprotein. Biochemistry 27**:** 25-29.

Cook NR (2007). Use and misuse of the receiver operating characteristic curve in risk prediction. Circulation 115**:** 928-935.

Couillard C, Despres JP, Lamarche B, Bergeron J, Gagnon J, Leon AS*, et al.* (2001). Effects of endurance exercise training on plasma HDL cholesterol levels depend on levels of triglycerides: evidence from men of the Health, Risk Factors, Exercise Training and Genetics (HERITAGE) Family Study. Arterioscler Thromb Vasc Biol 21**:** 1226-1232.

Crawford PB, Obarzanek E, Morrison J, & Sabry ZI (1994). Comparative advantage of 3-day food records over 24-hour recall and 5-day food frequency validated by observation of 9- and 10-year-old girls. Journal of the American Dietetic Association 94**:** 626-630.

Crouse SF, O'Brien BC, Rohack JJ, Lowe RC, Green JS, Tolson H*, et al.* (1995). Changes in serum lipids and apolipoproteins after exercise in men with high cholesterol: influence of intensity. Journal of applied physiology 79**:** 279-286.

Daerr WH, Minzlaff U, & Greten H (1986). Quantitative determination of apolipoprotein A-I in high-density lipoproteins and 'free' apolipoprotein A-I by two-dimensional agarose gel lipoprotein-'rocket' immunoelectrophoresis of human serum. Biochimica et biophysica acta 879**:** 134-139.

de la Llera-Moya M, Drazul-Schrader D, Asztalos BF, Cuchel M, Rader DJ, & Rothblat GH (2010). The ability to promote efflux via ABCA1 determines the capacity of serum specimens with similar high-density lipoprotein cholesterol to remove cholesterol from macrophages. Arterioscler Thromb Vasc Biol 30**:** 796-801.

Downs JR, Clearfield M, Weis S, Whitney E, Shapiro DR, Beere PA*, et al.* (1998). Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS. Air Force/Texas Coronary Atherosclerosis Prevention Study. JAMA : the journal of the American Medical Association 279**:** 1615-1622.

Dufaux B, Order U, Muller R, & Hollmann W (1986). Delayed effects of prolonged exercise on serum lipoproteins. Metabolism 35**:** 105-109.

Duverger N, Viglietta C, Berthou L, Emmanuel F, Tailleux A, Parmentier-Nihoul L*, et al.* (1996). Transgenic rabbits expressing human apolipoprotein A-I in the liver. Arterioscler Thromb Vasc Biol 16**:** 1424-1429.

Eckel RH (1989). Lipoprotein lipase. A multifunctional enzyme relevant to common metabolic diseases. The New England journal of medicine 320**:** 1060-1068.

Emerging Risk Factors C, Di Angelantonio E, Sarwar N, Perry P, Kaptoge S, Ray KK*, et al.* (2009). Major lipids, apolipoproteins, and risk of vascular disease. JAMA : the journal of the American Medical Association 302**:** 1993-2000.

Fielding CJ, & Fielding PE (1995). Molecular physiology of reverse cholesterol transport. Journal of lipid research 36**:** 211-228.

Fielding PE, Kawano M, Catapano AL, Zoppo A, Marcovina S, & Fielding CJ (1994). Unique epitope of apolipoprotein A-I expressed in pre-beta-1 high-density lipoprotein and its role in the catalyzed efflux of cellular cholesterol. Biochemistry 33**:** 6981-6985.

Fink DJ, & Gainer H (1980). Retrograde axonal transport of endogenous proteins in sciatic nerve demonstrated by covalent labeling in vivo. Science 208**:** 303-305.

Foger B, Wohlfarter T, Ritsch A, Lechleitner M, Miller CH, Dienstl A*, et al.* (1994). Kinetics of lipids, apolipoproteins, and cholesteryl ester transfer protein in plasma after a bicycle marathon. Metabolism 43**:** 633-639.

Folch J, Lees M, & Sloane Stanley GH (1957). A simple method for the isolation and purification of total lipides from animal tissues. The Journal of biological chemistry 226**:** 497-509.

Francone OL, Fielding CJ, & Fielding PE (1990). Distribution of cell-derived cholesterol among plasma lipoproteins: a comparison of three techniques. Journal of lipid research 31**:** 2195-2200.

Francone OL, Gong EL, Ng DS, Fielding CJ, & Rubin EM (1995). Expression of human lecithin-cholesterol acyltransferase in transgenic mice. Effect of human apolipoprotein AI and human apolipoprotein all on plasma lipoprotein cholesterol metabolism. The Journal of clinical investigation 96**:** 1440-1448.

Francone OL, Royer L, & Haghpassand M (1996). Increased prebeta-HDL levels, cholesterol efflux, and LCAT-mediated esterification in mice expressing the human cholesteryl ester transfer protein (CETP) and human apolipoprotein A-I (apoA-I) transgenes. Journal of lipid research 37**:** 1268-1277.

Frey I, Baumstark MW, Berg A, & Keul J (1991). Influence of acute maximal exercise on lecithin:cholesterol acyltransferase activity in healthy adults of differing aerobic performance. European journal of applied physiology and occupational physiology 62**:** 31-35.

Friedewald WT, Levy RI, & Fredrickson DS (1972). Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clinical chemistry 18**:** 499-502.

Giunta S, Maddalena-Peruzzi A, Gaudio MR, & Semprini A (1992). New in vitro findings on the "free" form of apolipoprotein A-1. Annals of the New York Academy of Sciences 673**:** 342-349.

Glomset JA (1968). The plasma lecithins:cholesterol acyltransferase reaction. Journal of lipid research 9**:** 155-167.

Glomset JA, & Wright JL (1964). Some Properties of a Cholesterol Esterifying Enzyme in Human Plasma. Biochimica et biophysica acta 89**:** 266-276.

Goldberg IJ (1996). Lipoprotein lipase and lipolysis: central roles in lipoprotein metabolism and atherogenesis. Journal of lipid research 37**:** 693-707.

Gordon T, Castelli WP, Hjortland MC, Kannel WB, & Dawber TR (1977). High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. The American journal of medicine 62**:** 707-714.

Grandjean PW, Crouse SF, & Rohack JJ (2000). Influence of cholesterol status on blood lipid and lipoprotein enzyme responses to aerobic exercise. Journal of applied physiology 89**:** 472-480.

Grodner TK: .. Philadelphia P, Mosby-Yearbook, (1996). In Foundation and Clinical Applications of Nutrition:
A Nursing Approach (ed 4). pp 638-726.

Guey LT, Pullinger CR, Ishida BY, O'Connor PM, Zellner C, Francone OL*, et al.* (2011). Relation of increased prebeta-1 high-density lipoprotein levels to risk of coronary heart disease. The American journal of cardiology 108**:** 360-366.

Gupta AK, Ross EA, Myers JN, & Kashyap ML (1993). Increased reverse cholesterol transport in athletes. Metabolism 42**:** 684-690.

Hammad SM, Stefansson S, Twal WO, Drake CJ, Fleming P, Remaley A*, et al.* (1999). Cubilin, the endocytic receptor for intrinsic factor-vitamin B(12) complex, mediates high-density lipoprotein holoparticle endocytosis. Proceedings of the National Academy of Sciences of the United States of America 96**:** 10158-10163.

Havel RJ, Eder HA, & Bragdon JH (1955). The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. The Journal of clinical investigation 34**:** 1345-1353.

Hayek T, Oiknine J, Dankner G, Brook JG, & Aviram M (1995). HDL apolipoprotein A-I attenuates oxidative modification of low density lipoprotein: studies in transgenic mice. European journal of clinical chemistry and clinical biochemistry : journal of the Forum of European Clinical Chemistry Societies 33**:** 721-725.

Hennessy LK, Kunitake ST, & Kane JP (1993). Apolipoprotein A-I-containing lipoproteins, with or without apolipoprotein A-II, as progenitors of pre-beta high-density lipoprotein particles. Biochemistry 32**:** 5759-5765.

Herbert PN, Bernier DN, Cullinane EM, Edelstein L, Kantor MA, & Thompson PD (1984). High-density lipoprotein metabolism in runners and sedentary men. JAMA : the journal of the American Medical Association 252**:** 1034-1037.

Hevesy GaH, E. (1934). Elimination of water from the human body. . Nature 134 134**:** 879.

Hurvich CM, and C-L. Tasi. (1989). Regression and time series model selection in small samples. Biometrika 76**:** 297-307.

Ignatowski A (1909). Ueber die Wirkung der tierschen Einweisse auf der Aorta. Virchow's Arch Pathol Anat 1909**:** 198:248.

Ishida BY, Frolich J, & Fielding CJ (1987). Prebeta-migrating high density lipoprotein: quantitation in normal and hyperlipidemic plasma by solid phase radioimmunoassay following electrophoretic transfer. Journal of lipid research 28**:** 778-786.

Jackson AS PM (1985). Practical assessment of body composition Phys Sportsmed 13**:** 76-90.

Jafari M, Leaf DA, Macrae H, Kasem J, O'Conner P, Pullinger C*, et al.* (2003). The effects of physical exercise on plasma prebeta-1 high-density lipoprotein. Metabolism 52**:** 437-442.

Ji A, Wroblewski JM, Cai L, de Beer MC, Webb NR, & van der Westhuyzen DR (2012). Nascent HDL formation in hepatocytes and role of ABCA1, ABCG1, and SR-BI. Journal of lipid research 53**:** 446-455.

Jiang X, Francone OL, Bruce C, Milne R, Mar J, Walsh A*, et al.* (1996). Increased prebeta-high density lipoprotein, apolipoprotein AI, and phospholipid in mice expressing the human phospholipid transfer protein and human apolipoprotein AI transgenes. The Journal of clinical investigation 98**:** 2373-2380.

Jin FY, Kamanna VS, & Kashyap ML (1997). Niacin decreases removal of high-density lipoprotein apolipoprotein A-I but not cholesterol ester by Hep G2 cells. Implication for reverse cholesterol transport. Arterioscler Thromb Vasc Biol 17**:** 2020-2028.

Jonas A (2000). Lecithin cholesterol acyltransferase. Biochimica et biophysica acta 1529**:** 245-256.

Kannel WB (1983). High-density lipoproteins: epidemiologic profile and risks of coronary artery disease. The American journal of cardiology 52**:** 9B-12B.

Kannel WB, Dawber TR, Kagan A, Revotskie N, & Stokes J, 3rd (1961). Factors of risk in the development of coronary heart disease--six year follow-up experience. The Framingham Study. Annals of internal medicine 55**:** 33-50.

Kantor MA, Cullinane EM, Herbert PN, & Thompson PD (1984). Acute increase in lipoprotein lipase following prolonged exercise. Metabolism 33**:** 454-457.

Kantor MA, Cullinane EM, Sady SP, Herbert PN, & Thompson PD (1987). Exercise acutely increases high density lipoprotein-cholesterol and lipoprotein lipase activity in trained and untrained men. Metabolism 36**:** 188-192.

Khera AV, Cuchel M, de la Llera-Moya M, Rodrigues A, Burke MF, Jafri K*, et al.* (2011). Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. The New England journal of medicine 364**:** 127-135.

Kiens B, Jorgensen I, Lewis S, Jensen G, Lithell H, Vessby B*, et al.* (1980). Increased plasma HDL-cholesterol and apo A-1 in sedentary middle-aged men after physical conditioning. European journal of clinical investigation 10**:** 203-209.

Kontush A CMJ (2012). High-density lipoproteins: structure, metabolism, function, and therapeutics. .

Kozyraki R, Fyfe J, Kristiansen M, Gerdes C, Jacobsen C, Cui S*, et al.* (1999). The intrinsic factor-vitamin B12 receptor, cubilin, is a high-affinity apolipoprotein A-I receptor facilitating endocytosis of high-density lipoprotein. Nature medicine 5**:** 656-661.

Krieger M (2001). Scavenger receptor class B type I is a multiligand HDL receptor that influences diverse physiologic systems. The Journal of clinical investigation 108**:** 793-797.

Kunitake ST, Chen GC, Kung SF, Schilling JW, Hardman DA, & Kane JP (1990). Pre-beta high density lipoprotein. Unique disposition of apolipoprotein A-I increases susceptibility to proteolysis. Arteriosclerosis 10**:** 25-30.

Kunitake ST, & Kane JP (1982). Factors affecting the integrity of high density lipoproteins in the ultracentrifuge. Journal of lipid research 23**:** 936-940.

Kunitake ST, La Sala KJ, & Kane JP (1985). Apolipoprotein A-I-containing lipoproteins with pre-beta electrophoretic mobility. Journal of lipid research 26**:** 549-555.

Kunitake ST, La Sala, K. J., Mendel, C. M., Chen, G. C., and Kane, J. P. (1987). (Sept. 1987) National Institutes of Health Workshop on Lipoprotein Heterogeneity, . NIH Publication No 87-2646 87-2646**:** 419 – 427.

Kunitake ST, Mendel CM, & Hennessy LK (1992). Interconversion between apolipoprotein A-I-containing lipoproteins of pre-beta and alpha electrophoretic mobilities. Journal of lipid research 33**:** 1807-1816.

Kunitake ST, O'Connor, P., and Naya-Vigne, J. (1996). Methods in Enzymology (Bradley, G, and Segrest, Eds) Academic Press,SanDiego**:** 260-267.

Kwiterovich PO, Jr. (1998). The antiatherogenic role of high-density lipoprotein cholesterol. The American journal of cardiology 82**:** 13Q-21Q.

Lamon-Fava S, Herrington DM, Reboussin DM, Sherman M, Horvath KV, Cupples LA*, et al.* (2008). Plasma levels of HDL subpopulations and remnant lipoproteins predict the extent of angiographically-defined coronary artery disease in postmenopausal women. Arterioscler Thromb Vasc Biol 28**:** 575-579.

Lampman RM, Santinga JT, Bassett DR, Block WD, Mercer N, Hook DA*, et al.* (1980). Type IV hyperlipoproteinemia: effects of a caloric restricted type IV diet versus physical training plus isocaloric type IV diet. The American journal of clinical nutrition 33**:** 1233-1243.

Landschulz KT, Pathak RK, Rigotti A, Krieger M, & Hobbs HH (1996). Regulation of scavenger receptor, class B, type I, a high density lipoprotein receptor, in liver and steroidogenic tissues of the rat. The Journal of clinical investigation 98**:** 984-995.

Leaf DA KK (1988). Acute prolonged subanaerobic exercise on hypertriglyceridmics: Effect on plasma lipids, lipoproteins, and respiratory
exchange ratio slope. Ann Sports Med , 3**:** 242-246.

Leaf DA, Parker DL, & Schaad D (1997). Changes in VO2max, physical activity, and body fat with chronic exercise: effects on plasma lipids. Medicine and science in sports and exercise 29**:** 1152-1159.

Lee M, Metso J, Jauhiainen M, & Kovanen PT (2003). Degradation of phospholipid transfer protein (PLTP) and PLTP-generated pre-beta-high density lipoprotein by mast cell chymase impairs high affinity efflux of cholesterol from macrophage foam cells. The Journal of biological chemistry 278**:** 13539-13545.

Lee-Rueckert M, & Kovanen PT (2011). Extracellular modifications of HDL in vivo and the emerging concept of proteolytic inactivation of prebeta-HDL. Current opinion in lipidology 22**:** 394-402.

Lefevre M, Goudey-Lefevre JC, & Roheim PS (1987). Gradient gel electrophoresis-immunoblot analysis (GGEI): a sensitive method for apolipoprotein profile determinations. Journal of lipid research 28**:** 1495-1507.

Li JJ, & Fang CH (2004). Atherosclerotic is a more rational term for the pathological entity currently known as atherosclerosis. Medical hypotheses 63**:** 100-102.

Li Z, McNamara JR, Fruchart JC, Luc G, Bard JM, Ordovas JM*, et al.* (1996). Effects of gender and menopausal status on plasma lipoprotein subspecies and particle sizes. Journal of lipid research 37**:** 1886-1896.

Liadaki KN, Liu T, Xu S, Ishida BY, Duchateaux PN, Krieger JP*, et al.* (2000). Binding of high density lipoprotein (HDL) and discoidal reconstituted HDL to the HDL receptor scavenger

receptor class B type I. Effect of lipid association and APOA-I mutations on receptor binding. The Journal of biological chemistry 275**:** 21262-21271.

Lopez A, Vial R, Balart L, & Arroyave G (1974). Effect of exercise and physical fitness on serum lipids and lipoproteins. Atherosclerosis 20**:** 1-9.

Lowry OH, Rosebrough NJ, Farr AL, & Randall RJ (1951). Protein measurement with the Folin phenol reagent. The Journal of biological chemistry 193**:** 265-275.

Luhrmann PM, Herbert BM, Gaster C, & Neuhauser-Berthold M (1999). Validation of a self-administered 3-day estimated dietary record for use in the elderly. European journal of nutrition 38**:** 235-240.

Manninen V, Tenkanen L, Koskinen P, Huttunen JK, Manttari M, Heinonen OP*, et al.* (1992). Joint effects of serum triglyceride and LDL cholesterol and HDL cholesterol concentrations on coronary heart disease risk in the Helsinki Heart Study. Implications for treatment. Circulation 85**:** 37-45.

Manttari M, Huttunen JK, Koskinen P, Manninen V, Tenkanen L, Heinonen OP*, et al.* (1990). Lipoproteins and coronary heart disease in the Helsinki Heart Study. European heart journal 11 Suppl H**:** 26-31.

McVicar JP, Kunitake ST, Hamilton RL, & Kane JP (1984). Characteristics of human lipoproteins isolated by selected-affinity immunosorption of apolipoprotein A-I. Proceedings of the National Academy of Sciences of the United States of America 81**:** 1356-1360.

Mehta NJ, & Khan IA (2002). Cardiology's 10 greatest discoveries of the 20th century. Texas Heart Institute journal / from the Texas Heart Institute of St Luke's Episcopal Hospital, Texas Children's Hospital 29**:** 164-171.

Miida T, Nakamura Y, Inano K, Matsuto T, Yamaguchi T, Tsuda T*, et al.* (1996). Pre beta 1-high-density lipoprotein increases in coronary artery disease. Clinical chemistry 42**:** 1992-1995.

Miller GJ, & Miller NE (1975). Plasma-high-density-lipoprotein concentration and development of ischaemic heart-disease. Lancet 1**:** 16-19.

Mineo C, Deguchi H, Griffin JH, & Shaul PW (2006). Endothelial and antithrombotic actions of HDL. Circulation research 98**:** 1352-1364.

Miyazaki O, Kobayashi J, Fukamachi I, Miida T, Bujo H, & Saito Y (2000). A new sandwich enzyme immunoassay for measurement of plasma pre-beta1-HDL levels. Journal of lipid research 41**:** 2083-2088.

Morton RE, & Greene DJ (1997). Suppression of lipid transfer inhibitor protein activity by oleate. A novel mechanism of cholesteryl ester transfer protein regulation by plasma free fatty acids. Arterioscler Thromb Vasc Biol 17**:** 3041-3048.

Nakamura Y, Kotite L, Gan Y, Spencer TA, Fielding CJ, & Fielding PE (2004). Molecular mechanism of reverse cholesterol transport: reaction of pre-beta-migrating high-density lipoprotein with plasma lecithin/cholesterol acyltransferase. Biochemistry 43**:** 14811-14820.

Nanjee MN, & Brinton EA (2000). Very small apolipoprotein A-I-containing particles from human plasma: isolation and quantification by high-performance size-exclusion chromatography. Clinical chemistry 46**:** 207-223.

Navab M, Berliner JA, Watson AD, Hama SY, Territo MC, Lusis AJ*, et al.* (1996). The Yin and Yang of oxidation in the development of the fatty streak. A review based on the 1994 George Lyman Duff Memorial Lecture. Arterioscler Thromb Vasc Biol 16**:** 831-842.

Navab M, Gharavi N, & Watson AD (2008). Inflammation and metabolic disorders. Current opinion in clinical nutrition and metabolic care 11**:** 459-464.

Nye ER, Carlson K, Kirstein P, & Rossner S (1981). Changes in high density lipoprotein subfractions and other lipoproteins by exercise. Clinica chimica acta; international journal of clinical chemistry 113**:** 51-57.

O'Connor PM, Naya-Vigne JM, Duchateau PN, Ishida BY, Mazur M, Schoenhaus SA*, et al.* (1997). Measurement of prebeta-1 HDL in human plasma by an ultrafiltration-isotope dilution technique. Analytical biochemistry 251**:** 234-240.

O'Connor PM, Zysow BR, Schoenhaus SA, Ishida BY, Kunitake ST, Naya-Vigne JM*, et al.* (1998). Prebeta-1 HDL in plasma of normolipidemic individuals: influences of plasma lipoproteins, age, and gender. Journal of lipid research 39**:** 670-678.

O'Kane MJ, Wisdom GB, McEneny J, McFerran NV, & Trimble ER (1992). Pre-beta high-density lipoprotein determined by immunoblotting with chemiluminescent detection. Clinical chemistry 38**:** 2273-2277.

Oram JF, & Lawn RM (2001). ABCA1. The gatekeeper for eliminating excess tissue cholesterol. Journal of lipid research 42**:** 1173-1179.

Pencina MJ, D'Agostino RB, Sr., D'Agostino RB, Jr., & Vasan RS (2008). Evaluating the added predictive ability of a new marker: from area under the ROC curve to reclassification and beyond. Statistics in medicine 27**:** 157-172; discussion 207-112.

Phillips MC, Gillotte KL, Haynes MP, Johnson WJ, Lund-Katz S, & Rothblat GH (1998). Mechanisms of high density lipoprotein-mediated efflux of cholesterol from cell plasma membranes. Atherosclerosis 137 Suppl**:** S13-17.

Posner BM, Martin-Munley SS, Smigelski C, Cupples LA, Cobb JL, Schaefer E*, et al.* (1992). Comparison of techniques for estimating nutrient intake: the Framingham Study. Epidemiology 3**:** 171-177.

Pullinger CR, Hennessy LK, Chatterton JE, Liu W, Love JA, Mendel CM*, et al.* (1995). Familial ligand-defective apolipoprotein B. Identification of a new mutation that decreases LDL receptor binding affinity. The Journal of clinical investigation 95**:** 1225-1234.

Rader DJ, & Ikewaki K (1996). Unravelling high density lipoprotein-apolipoprotein metabolism in human mutants and animal models. Current opinion in lipidology 7**:** 117-123.

Rico-Sanz J, Moosavi M, Thomas EL, McCarthy J, Coutts GA, Saeed N*, et al.* (2000). In vivo evaluation of the effects of continuous exercise on skeletal muscle triglycerides in trained humans. Lipids 35**:** 1313-1318.

Robins SJ, Collins D, Wittes JT, Papademetriou V, Deedwania PC, Schaefer EJ*, et al.* (2001). Relation of gemfibrozil treatment and lipid levels with major coronary events: VA-HIT: a randomized controlled trial. JAMA : the journal of the American Medical Association 285**:** 1585-1591.

Rosenson RS (2010). Functional assessment of HDL: Moving beyond static measures for risk assessment. Cardiovascular drugs and therapy / sponsored by the International Society of Cardiovascular Pharmacotherapy 24**:** 71-75.

Sady SP, Thompson PD, Cullinane EM, Kantor MA, Domagala E, & Herbert PN (1986). Prolonged exercise augments plasma triglyceride clearance. JAMA : the journal of the American Medical Association 256**:** 2552-2555.

Sakai T, Kamanna VS, & Kashyap ML (2001). Niacin, but not gemfibrozil, selectively increases LP-AI, a cardioprotective subfraction of HDL, in patients with low HDL cholesterol. Arterioscler Thromb Vasc Biol 21**:** 1783-1789.

Santamarina-Fojo S, Haudenschild C, & Amar M (1998). The role of hepatic lipase in lipoprotein metabolism and atherosclerosis. Current opinion in lipidology 9**:** 211-219.

Schmitz G, & Langmann T (2001). Structure, function and regulation of the ABC1 gene product. Current opinion in lipidology 12**:** 129-140.

Seip RL, Moulin P, Cocke T, Tall A, Kohrt WM, Mankowitz K*, et al.* (1993). Exercise training decreases plasma cholesteryl ester transfer protein. Arteriosclerosis and thrombosis : a journal of vascular biology / American Heart Association 13**:** 1359-1367.

Semprini A, Maddalena-Feruzzi A, & Giunta S (1992). Immunofixation electrophoresis (IFE) of serum apolipoproteins: a new tool for probing lipoprotein disorders and the atherosclerosis risk. Archives of gerontology and geriatrics 15 Suppl 1**:** 325-332.

Sethi AA, Sampson M, Warnick R, Muniz N, Vaisman B, Nordestgaard BG*, et al.* (2010). High pre-beta1 HDL concentrations and low lecithin: cholesterol acyltransferase activities are strong positive risk markers for ischemic heart disease and independent of HDL-cholesterol. Clinical chemistry 56**:** 1128-1137.

Sviridov D, Miyazaki O, Theodore K, Hoang A, Fukamachi I, & Nestel P (2002). Delineation of the role of pre-beta 1-HDL in cholesterol efflux using isolated pre-beta 1-HDL. Arterioscler Thromb Vasc Biol 22**:** 1482-1488.

Tall AR, Yvan-Charvet L, Terasaka N, Pagler T, & Wang N (2008). HDL, ABC transporters, and cholesterol efflux: implications for the treatment of atherosclerosis. Cell metabolism 7**:** 365-375.

Taskinen MR, & Nikkila EA (1980). Effect of acute vigorous exercise on lipoprotein lipase activity of adipose tissue and skeletal muscle in physically active men. Artery 6**:** 471-483.

Thompson PD (1990). What do muscles have to do with lipoproteins? Circulation 81**:** 1428-1430.

Thompson PD, Crouse SF, Goodpaster B, Kelley D, Moyna N, & Pescatello L (2001). The acute versus the chronic response to exercise. Medicine and science in sports and exercise 33**:** S438-445; discussion S452-433.

Thompson PD, Cullinane E, Henderson LO, & Herbert PN (1980). Acute effects of prolonged exercise on serum lipids. Metabolism 29**:** 662-665.

Thompson PD, Cullinane EM, Sady SP, Flynn MM, Bernier DN, Kantor MA*, et al.* (1988). Modest changes in high-density lipoprotein concentration and metabolism with prolonged exercise training. Circulation 78**:** 25-34.

Thompson PD, Yurgalevitch SM, Flynn MM, Zmuda JM, Spannaus-Martin D, Saritelli A*, et al.* (1997). Effect of prolonged exercise training without weight loss on high-density lipoprotein metabolism in overweight men. Metabolism 46**:** 217-223.

TSAI CMHaC-L (1989). Regression and time series model selection in small samples
Biometrika 76**:** 297-307.

Tsopanakis C, Kotsarellis D, & Tsopanakis A (1988). Plasma lecithin: cholesterol acyltransferase activity in elite athletes from selected sports. European journal of applied physiology and occupational physiology 58**:** 262-265.

Valacchi G, Sticozzi C, Lim Y, & Pecorelli A (2011). Scavenger receptor class B type I: a multifunctional receptor. Annals of the New York Academy of Sciences 1229**:** E1-7.

von Eckardstein A, Nofer JR, & Assmann G (2001). High density lipoproteins and arteriosclerosis. Role of cholesterol efflux and reverse cholesterol transport. Arterioscler Thromb Vasc Biol 21**:** 13-27.

Wang N, Lan D, Chen W, Matsuura F, & Tall AR (2004). ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins. Proceedings of the National Academy of Sciences of the United States of America 101**:** 9774-9779.

Warnick GR, Benderson J, & Albers JJ (1982). Dextran sulfate-Mg2+ precipitation procedure for quantitation of high-density-lipoprotein cholesterol. Clinical chemistry 28**:** 1379-1388.

Watson AD, Berliner JA, Hama SY, La Du BN, Faull KF, Fogelman AM*, et al.* (1995). Protective effect of high density lipoprotein associated paraoxonase. Inhibition of the biological activity of minimally oxidized low density lipoprotein. The Journal of clinical investigation 96**:** 2882-2891.

Willnow TE, Nykjaer A, & Herz J (1999). Lipoprotein receptors: new roles for ancient proteins. Nature cell biology 1**:** E157-162.

Wood PD, Stefanick ML, Dreon DM, Frey-Hewitt B, Garay SC, Williams PT*, et al.* (1988). Changes in plasma lipids and lipoproteins in overweight men during weight loss through dieting as compared with exercise. The New England journal of medicine 319**:** 1173-1179.

Zhong S, Goldberg IJ, Bruce C, Rubin E, Breslow JL, & Tall A (1994). Human ApoA-II inhibits the hydrolysis of HDL triglyceride and the decrease of HDL size induced by hypertriglyceridemia and cholesteryl ester transfer protein in transgenic mice. The Journal of clinical investigation 94**:** 2457-2467.