

ABSTRACT

JEYARAJAH, ELIAS JOSEPH. Development and Validation of a ^1H NMR Method for Lipoprotein Quantification and Coronary Heart Disease Risk Assessment. (Under the direction of James Dorian Otvos, chair and Edmond F. Bowden, co-chair.)

There is abundant evidence that the subclasses within a given lipoprotein class differ in their associations with coronary heart disease. Since subclass distributions can vary widely from person to person, individuals with the same levels of LDL cholesterol and HDL cholesterol may be at different cardiovascular risk and respond differently to dietary and drug therapy. Unfortunately, existing laboratory methods of subclass measurement are too time-consuming and expensive to be used in routine clinical practice. Using a new approach to lipoprotein analysis that exploits the natural proton NMR spectroscopic differences exhibited by lipoprotein particles of different size, we have developed a new quantitative NMR technology for use in clinical laboratory medicine. The newly developed *NMR LipoProfile* assay rapidly and simultaneously quantifies the lipoprotein subclass particle concentrations of 10 lipoprotein species (3 VLDL, IDL, 3 LDL and 3 HDL) with good intraassay and interassay precision. Extensive validation studies were conducted that established robustness of the NMR lipoprotein particle assay. The average particle sizes of the major lipoprotein classes determined by NMR correlate very well with those estimated by gradient gel electrophoresis. Emerging clinical data from several coronary disease outcome studies indicate that NMR-derived lipoprotein particle parameters are superior predictors of cardiovascular disease risk compared to traditional cholesterol risk factors. The speed and efficiency of NMR lipoprotein subclass profiling make it a potentially valuable research tool and cost-effective means of assessing and managing heart disease risk in the general population.

**Development and Validation of a ^1H NMR Method for Lipoprotein Quantification and
Coronary Heart Disease Risk Assessment**

by

Elias Joseph Jeyarajah

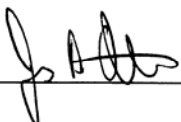
A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

CHEMISTRY

Raleigh

2004

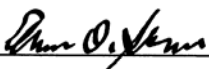
APPROVED BY:



Dr. James D. Otvos
Chair of Advisory Committee



Dr. Edmond F. Bowden
Co-chair of Advisory Committee



Dr. Edward O. Stejskal



Dr. Kenneth W. Hanck

DEDICATION

This dissertation is dedicated to the

People of Tamil Eelam and their struggle for Justice and Peace

BIOGRAPHY

Elias Joseph Jeyarajah was born in Naranthanai, Ceylon (now Sri Lanka) to Sebastiampillai and (late) Ponrose Elias on August 25, 1955. He grew up with sisters Nirmala and Vathsala, and, brothers Ponraj and Nithianandarajah. He studied at St. Anthony's College, Kayts and then St. Patrick's College, Jaffna. He earned his B.Sc. at University of Jaffna, in 1980 majoring in Chemistry. He received his Masters of Science in Biophysical Chemistry in 1985 at SUNY Stony Brook. He has worked at University of Wisconsin, Milwaukee (1987-1990), and North Carolina State University Biochemistry department (1990-2000) with Professor James D. Otvos researching lipoproteins using NMR spectroscopy. While at NC State he enrolled in the graduate program in the Analytical Division of the Department of Chemistry to earn his Ph.D. He is employed at LipoScience, Inc., (Raleigh, NC) as Director of NMR Applications. He is married to Shanthini and blessed with daughters Shoumini and Shiromini.

ACKNOWLEDGEMENTS

I would like to thank my research advisor, Dr. James D. Otvos, for his guidance and leadership during the course of this research, and during the long and fruitful association I have had with him. His unstinting support for my educational aspirations made this possible while earning a living and raising a family.

I thank Dr. Dennis Bennett of University of Wisconsin, Milwaukee for contributing to this research immensely through the development of the deconvolution software. I thank the Department of Chemistry at NC State and the faculty of the Analytical Chemistry division for molding me into the analytical chemist I am. My special thanks to my co-advisor Dr. Ed Bowden, for all his valuable support, help and advice.

I would like to thank Qun Zhou for her indispensable technical assistance in lipoprotein isolations. My thanks to Dr. Irina Shalaurova and Dr. David Morgan for their collaboration in advancing the NMR technology to clinical medicine.

I am grateful to my parents, my father Elias and my late mother PonRose, who made me who I am. Finally I thank my family, my daughters Shoumini and Shiromini, and my dear wife Shanthini who endeared many sacrifices for fulfilling this objective of mine. Shanthi, this work is truly the reflection of my love for you.

TABLE OF CONTENTS

ABBREVIATIONS	viii
LIST OF TABLES	ix
LIST OF FIGURES	x

Chapter 1: Introduction and Background 1

1.1 Lipids and Coronary Heart Disease	1
1.2 Lipoproteins: Definition, Structure, Function	3
1.2.1 Composition of Lipoproteins	3
1.2.2 Lipoprotein Structure	6
1.2.3 Nomenclature and Classification of Lipoproteins	6
1.2.4 Apolipoproteins and Lipoprotein Metabolism	8
1.3 Measurement of Lipoproteins	10
1.3.1 Measurement of lipids as surrogates for lipoproteins	10
1.3.2 Lipoprotein subclass measurement	11
1.3.3 Limitations of current methods for lipoprotein subclass analysis	12
1.4 NMR spectroscopy of blood plasma	13
1.4.1 Lipoprotein subclass measurement by NMR	13
1.4.2 Advantages of lipoprotein subclass analysis by NMR	18
1.4.3 Alternate NMR approaches	19
1.5 Overview of chapters to follow	19

Chapter 2: Experimental: Building Blocks of the *NMR LipoProfile* test 21

2.1 Isolation of lipoproteins	21
2.2 Characterization of lipoproteins	24
2.2.1 Chemical analysis	24
2.2.2 Electron microscopy	24
2.2.3 Gradient gel electrophoresis	27
2.3 NMR spectroscopy	27
2.4 Construction of fitting model	29

2.5	NMR lineshape deconvolution	32
2.6	Output of deconvolution: <i>NMR LipoProfile</i>	34
2.7	Correlation with chemical lipids	39

Chapter 3: Analytical Characterization and Validation of an Automated NMR Spectroscopic Method for Quantifying Lipoprotein Subclass Particles 41

Background	42
Methods	42
Results	43
Conclusion	43
Materials and Methods	47
Blood Samples	47
Lipoprotein Isolations	47
Chemical Analysis	48
Gradient Gel Electrophoresis	48
Electron Microscopy	49
NMR Spectroscopy	49
Deconvolution	50
Standard Addition Studies	51
Results	52
Lipoprotein Characterization	52
NMR Signal Area and Lipid Mass Concentrations	60
Standard Addition Studies: Specificity and Linearity of Response	61
Comparison of LDL and HDL Particle Size Distributions	
Determined by NMR and GGE	76
Correlation with Chemically Measured TG and HDL-C	79
LDL Particle Concentration and Apo-B	82
Precision Study	87
Normal Ranges and Intercorrelations	90
Discussion	93
References	99

Chapter 4: Measurement Issues Related to Lipoprotein Heterogeneity 102

Abstract	103
Lipids and Lipoproteins	104

Prevalence and Metabolic Origins of LDL Cholesterol Compositional Variability	106
Lipoprotein Quantification by NMR Spectroscopy	110
Clinical Implications of the Disconnect Between LDL Cholesterol and LDL Particles in Patients with Low HDL Cholesterol- Insights from the Framingham Offspring Study	113
Prevalence of the Disconnect Between LDL Cholesterol and LDL Particles	116
References	119
Chapter 5: Discussion	122
5.1 Advantages of NMR method for lipoprotein testing	122
5.2 Interferences	122
5.3 Stability of plasma for NMR analysis	124
5.4 Miscellaneous	125
Conclusion	126
References[†] ([†]for Chapters 1, 2, and 5)	127

ABBREVIATIONS

ApoB	apolipoprotein B
CAD	coronary artery disease
CE	cholesterol ester
CETP	cholesterol ester transfer protein
CHD	coronary heart disease
CV	coefficient of variation
GGE	gradient gel electrophoresis
HDL	high-density lipoprotein
IDL	intermediate density lipoprotein
LCAT	lecithin:cholesterol acyltransferase
LDL	low-density lipoprotein
LDL-P	LDL particle concentration
NCEP	national cholesterol education program
NMR	nuclear magnetic resonance
PCA	principal component analysis
PL	phospholipid
PLS	partial least squares regression
SVD	singular value decomposition
TC	total cholesterol
TG	triglyceride
TMA	trimethylacetate
VLDL	very-low-density lipoprotein

LIST OF TABLES

Chapter 1:

Table 1.1 Classification and Physical Properties of Lipoproteins 8

Table 1.2 Apolipoproteins and Functions 9

Chapter 3:

Table 1: Diameter Ranges for Lipoprotein Subclasses Measured by NMR 60

Table 2: Intraassay and Interassay Measurement Precision for
NMR LipoProfile-II 88

Table 3: Normal Ranges (10th – 90th percentile) for
NMR LipoProfile-II Parameters 91

Table 4: Inter-correlations Between Parameters in
NMR LipoProfile-II Report 92

Chapter 4:

Table 1: High –Risk Subjects in the Framingham Offspring Study
According to LDL Particle Concentration and LDL Cholesterol 118

Table 2: Low-Risk Subjects in the Framingham Offspring Study
According to LDL Particle Concentration and LDL Cholesterol 118

LIST OF FIGURES

Chapter 1:

Figure 1.1. Chemical structure of cholesterol and lipids	5
Figure 1.2. Schematic representation of a lipoprotein particle	6
Figure 1.3. Relationship of particle size and density of lipoprotein subclasses	7
Figure 1.4. Proton NMR spectrum of human blood serum	15
Figure 1.5. Lipoprotein subclasses quantified by NMR	17

Chapter 2:

Figure 2.1. Agarose column separation of major classes of lipoproteins	23
Figure 2.2. Electron micrograph of a homogenous VLDL subcomponent	26
Figure 2.3. Relationship of lipoprotein particle diameter and NMR chemical shift	30
Figure 2.4. Reference spectra comprising <i>NMR LipoProfile</i> fitting model	31
Figure 2.5. Plasma lineshape analysis results	35
Figure 2.6. Sample <i>NMR LipoProfile</i> assay report	37
Figure 2.7. Relations of LDL-C and HDL-C between NMR and beta quantification	40

Chapter 3:

Figure 1. Lipoprotein purification using agarose gel filtration	54
Figure 2. Electron microscopy for three purified lipoprotein components	56
Figure 3. Relationship between lipoprotein particle diameter and relative NMR chemical shift	58
Figure 4. NMR spectra of purified lipoprotein components in the <i>LipoProfile-II</i> Analysis model	59

LIST OF FIGURES continued

Figure 5. Correlation of NMR signal area to chemical lipids for LDL samples	61
Figure 6. GCE analysis of large and small LDL and HDL used for spiking studies	63
Figure 7. Standard addition plots- Spiking plasma with VLDL and LDL	67
Figure 8. Standard addition plots – Spiking plasma with HDL	71
Figure 9. NMR response to size perturbations induced by spiking	74
Figure 10. Comparison of NMR and GGE sizes for LDL and HDL	77
Figure 11. Comparison of NMR-derived lipids to chemical lipids for TG and HDL-C	80
Figure 12. Correlation of NMR LDL particle number with LDL apoB	84

Chapter 4:

Figure 1. Schematic representation of the metabolic origins of LDL particles containing less cholesterol than normal	107
Figure 2. Distribution of the measured ratios of cholesterol/triglyceride in the LDL fraction ($d=1.006-1.063$ kg/L) isolated by ultracentrifugation from 118 healthy subjects	108
Figure 3. Representation of the lipoprotein subclasses quantified by NMR and the information reported in the <i>NMR LipoProfile</i>	111
Figure 4. Relations in the Framingham Offspring cohort of HDL cholesterol levels to levels of HDL subclasses, LDL subclasses, LDL particles and cholesterol, and LDL particles and non-HDL cholesterol	114
Figure 5. Histograms for LDL cholesterol (Friedewald estimate) and NMR-measured LDL particle concentration from the Framingham Offspring Study	117

Chapter 1: Introduction and Background

1.1 Lipids and Coronary Heart Disease

Coronary heart disease (CHD) is the number one cause of death in the developed world.

Cholesterol, an essential constituent of cell membranes and a precursor of bile acids, vitamin D and other steroids, has long been implicated in the development of atherosclerosis - the narrowing of arteries caused by the deposition and build up of fatty plaque. Fatty acids (lipids) in the form of triglycerides and cholesterol esters from the diet or produced endogenously are packaged in particles called lipoproteins in the intestine and liver and transported in blood plasma to peripheral tissues. Total cholesterol at elevated levels in blood plasma has long been known to be associated with increased incidences of coronary heart disease, myocardial infarction and stroke (1-4). The cholesterol-carrying lipoproteins are classified into three broad categories based on density as very-low-density, low-density and high-density lipoproteins, or VLDL, LDL and HDL, respectively. In addition to total cholesterol, the LDL-cholesterol (LDL-C) is also known to have a positive association with CHD risk. The HDL-cholesterol (HDL-C), however, exhibits a negative association with CHD, meaning higher levels of HDL-C confer reduced risk for heart disease (5, 6). Due to their respective associations with CHD risk and their roles in cholesterol metabolism, LDL-C is commonly known as the “bad” cholesterol, and HDL-C has been dubbed the “good” cholesterol.

The Expert Panel of the National Cholesterol Education Program (NCEP) is responsible for the major public health initiatives for the identification of individuals at risk for CHD (7). The NCEP recommends screening for cholesterol and lipoprotein lipids for all adults above the age of 20 and once every 5 years. The original recommended guidelines

included initial classification into three categories of CHD risk based on the concentration of total cholesterol (TC): “desirable” – TC<200 mg/dL (5.17 mmol/L), “borderline high” – TC=200-239 mg/dL (5.17-6.18 mmol/L), and “high” – TC≥240 mg/dL (>6.21 mmol/L). Individuals in the “borderline high” and “high” categories require additional analyses for LDL-C and HDL-C to gauge the need for dietary or drug treatment. The initial NCEP guidelines to use total cholesterol instead of a complete lipid profile for CHD risk categorization was partly due to analytical cost considerations. An individual’s true concentration of cholesterol is subject to biological and analytical variations (8, 9) and as such requires repeated testing with carefully controlled analytical procedures for accurate risk assessment. People with “desirable” total cholesterol but with low levels of HDL-C will not be identified by this approach. Subsequent guidelines issued by the NCEP shifted the emphasis from total cholesterol to LDL-C. The most current communiqué from the NCEP’s Adult Treatment Panel, popularly known as ATP III guidelines, issued in May 2001 established clear guidelines for CHD risk reduction based on lowering of LDL-C levels (10). A multitude of factors are considered in assessing CHD risk. The presence of clinically manifest CHD such as history of heart attack, myocardial infarction, stroke etc. is considered a primary risk factor for future CHD. Then there are CHD risk equivalents such as the presence of diabetes mellitus or a greater than 20% risk of developing CHD based on use of the Framingham 10-year risk scoring system (11). Consideration is given then for the presence of major risk factors such as smoking, hypertension, low HDL-C, family history of premature CHD and age. Finally, an LDL treatment goal is arrived at based on all of these factors. The LDL-C reduction is achieved by nutritional and life style changes, drug therapy or both.

Though the emphasis on CHD prevention is based on LDL-C levels, in routine clinical practice LDL-C is not measured directly. The most widely used clinical procedure for measuring LDL-C concentrations is that of Friedewald (12) in which three separate determinations for total cholesterol, triglyceride, and HDL-C are first made. Then the LDL-C is estimated using the Friedewald formula, $LDL-C = TC - HDL-C - TG/5$. The Friedewald approximation of VLDL-C $\cong TG/5$ holds only for normal TG levels (i.e. $TG < 150 \text{ mg/dL}$), above which there are deviations that make the estimation unusable for $TG > 400 \text{ mg/dL}$. Furthermore, the accuracy of the LDL-C value, the key index for clinical decision making in the NCEP guidelines, depends on the combined reliability of the triglyceride, total cholesterol, and HDL-C measurements, the imprecision in all of which will add to the total error of the estimated LDL-C.

This dissertation deals with the development and validation of an alternate analytical procedure for measurement of lipoproteins in plasma or serum based on proton NMR spectroscopy. We have developed a protocol in which a linear least-squares fit of the plasma methyl lineshape provides an accurate assessment of the amplitudes of the signals from the component lipoproteins (chylomicrons, VLDL, IDL, LDL, and HDL). The derived amplitudes from the deconvolution of the proton NMR methyl spectra envelope correlate well with lipoprotein concentrations expressed in terms of lipoprotein lipid content. The rapid and simultaneous quantification of the lipoprotein mass concentrations and their average particle size dispersions by ^1H NMR spectroscopy offers significant advantages over existing methods and lends a powerful tool for the assessment and management of CHD risk.

1.2 Lipoproteins: Definition, Structure, Function

1.2.1 Composition of Lipoproteins

Human blood collected by veni-puncture in tubes containing anticoagulant (EDTA) can be spun at 2000g in a clinical centrifuge for 15 minutes to yield a clear liquid called plasma in the top half of the tube, separating it from the cells at the bottom. If the collection tube used has no anticoagulants and is left to clot before being spun, the resulting fluid is referred to as serum. Arterial plasma under normal conditions is maintained well buffered at $\text{pH } 7.40 \pm 0.05$. Plasma consists of many metabolites, lipoproteins and plasma proteins such as albumins, globulins and fibrinogens. Fibrinogens play an essential role in clotting, and serum separated after clot removal is essentially fibrinogen free. Plasma lipoproteins are spherical particles containing specific lipids and proteins. Dietary lipids contain fatty acids that have been esterified either with cholesterol, called cholesterol esters (a.k.a. cholesteryl esters), or with glycerol, called triglycerides (Figure 1.1). Fatty acids are long chain hydrocarbons that have a methyl (CH_3) group on one end and a carboxylic acid (COOH) moiety at the other end. The hydrophobic nature of lipids prohibits direct transport in plasma. The re-packaging of lipids *in vivo* into particles called lipoproteins facilitates their transport between organs and tissues. The dietary intake of fatty acids (lipids) in the form of triglycerides and cholesterol esters, and lipids synthesized in the body are packaged into lipoprotein particles in the intestine and liver and transported in blood plasma to peripheral tissues. As the name suggests, lipoproteins contain both lipids and proteins. The proteins contained in the lipoproteins are called apolipoproteins. Apolipoproteins play important roles in the metabolism of lipoproteins by binding to specific receptor sites, in addition to acting as cofactors for different enzymes.

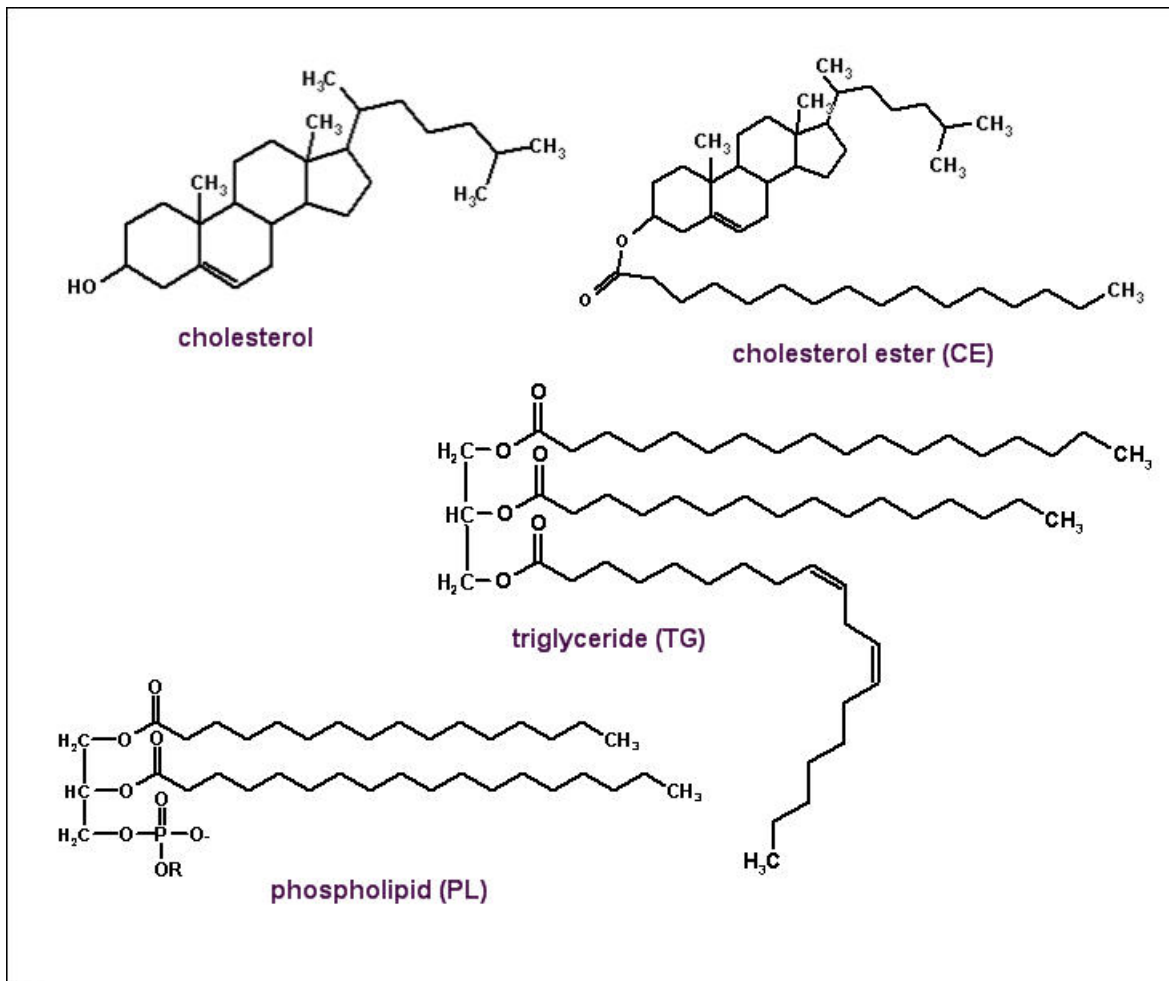


Figure 1.1. Chemical structure of cholesterol and lipids.

1.2.2 Lipoprotein structure

Lipoproteins are spherical particles that span a wide range of particle sizes. They have a hydrophobic core of cholesterol ester and triglycerides, surrounded by a hydrophilic shell of phospholipids with embedded apolipoproteins and some free (unesterified) cholesterol that provide structural stability. The phospholipid is in the form of a monolayer with an approximate thickness of 2 nm.

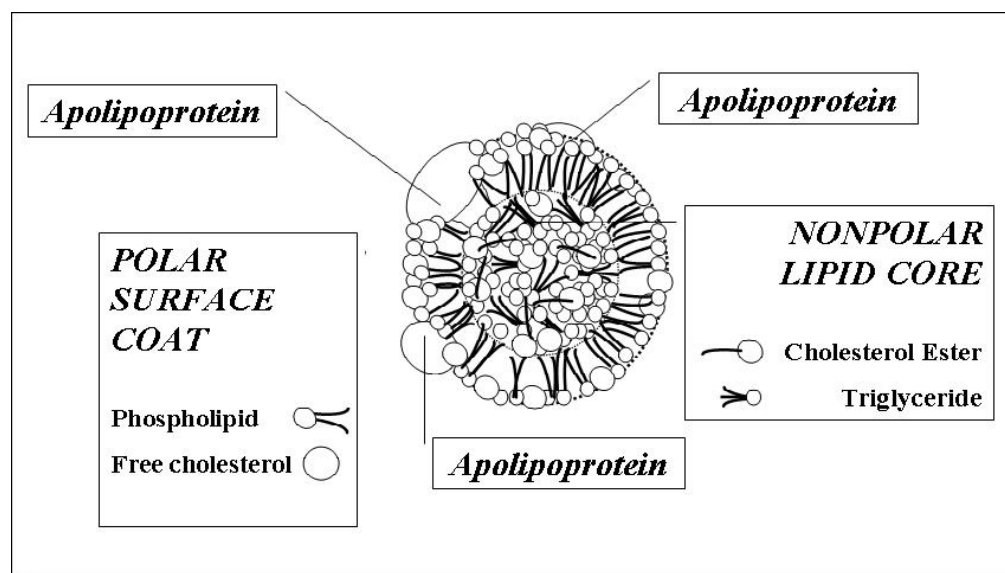


Figure 1.2. Schematic representation of a lipoprotein particle.

Lipoprotein particles have a spherical structure resembling a micelle. The non-polar core has cholesterol ester and triglyceride. The shell is made of phospholipids with the polar head group facing the surface along with one or more apolipoproteins and some unesterified cholesterol. Magnetically, the core is isotropic while the shell is ordered and anisotropic.

1.2.3 Nomenclature and Classification of Lipoproteins

Lipoproteins are classified in different ways based on their physical properties. They can be categorized based on density, size, electrophoretic mobility, flotation constant, and apolipoproteins. The most common classification is based on density into three major categories of very-low-density, low-density and high-density lipoproteins, or VLDL, LDL

and HDL, respectively. Two other related categories are chylomicrons that are less dense than VLDL, and intermediate-density-lipoproteins, or IDL, that falls between the density range of VLDL and LDL. The particle density increases from chylomicrons to HDL while the particle size decreases from chylomicrons to HDL. The relative protein content also increases going from chylomicrons to HDL. These properties are summarized on Figure 1.3 and Table 1.1.

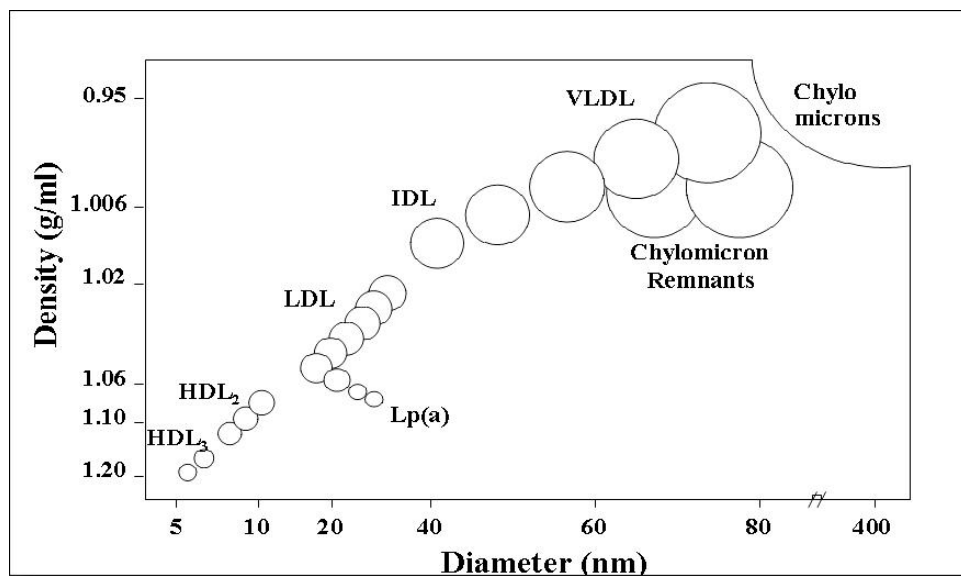


Figure 1.3. Relationship of particle size and density of lipoprotein subclasses. The particle size of lipoprotein subclasses have an inverse relationship to their hydrated densities. A common sub-classification of HDL consists of larger HDL₂ (d1.063-1.125kg/L), and smaller HDL₃ (d1.125-1.210kg/L). Lp(a) is a LDL-like particle with the apoB of LDL cross-linked by a disulfide bond to apo(a), a protein of variable mass that has homology to plasminogen.

Table 1.1 Classification and Physical Properties of Lipoproteins					
Lipoprotein	Density (kg/L)	Particle Diameter (nm)	Flotation Rate (Sf)	Electrophoretic Mobility	Amount of Protein (%)
Chylomicrons	< 0.95	80-1200	> 400	Stays at origin	2
VLDL	0.95-1.006	30-80	60-400	Pre-beta	8
IDL	1.006-1.019	23-35	20-60	Broad Beta	16
LDL	1.019-1.063	18-25	0-20	Beta	21
HDL	1.063-1.210	5-12	0-9	Alpha	55
VLDL= very-low density lipoprotein, IDL=intermediate-density lipoprotein, LDL= low-density lipoprotein, HDL= high-density lipoprotein; Sf= Svedberg units					

1.2.4 Apolipoproteins and Lipoprotein Metabolism

There is constant traffic of apolipoproteins between lipoprotein particles. They bind to specific receptors and act as cofactors to enzymes. The major enzymes involved in lipoprotein metabolism are lipoprotein lipase (LL), hepatic triglyceride lipase (HTGL), LDL receptor related protein (LRP), and lecithin:cholesterol acyltransferase (LCAT). Also there is exchange of TG and cholesterol ester between lipoprotein particles that are catalyzed by cholesteryl ester transfer protein (CETP). Table 1.2 details the known apolipoproteins, and the main functions (11).

Table 1.2 Apolipoproteins and Functions

Apolipoprotein	Main Functions
Apo AI	Accepts cholesterol. Structural for HDL. Ligand for HDL binding. LCAT cofactor.
Apo AII	Structural for HDL. Ligand for HDL binding. LCAT cofactor.
Apo AIV	Ligand for HDL binding. LCAT activator.
Apo(a)	Structural for Lp(a). Structural analogy with plasminogen.
Apo B-48	Structural for chylomicrons.
Apo B-100	Structural for VLDL, IDL, and LDL. LDL receptor ligand.
Apo CI	LCAT and LPL activator.
Apo CII	LCAT and LPL activator.
Apo CIII	LPL inhibitor. HTGL inhibitor. Modulator of TG-rich lipoproteins by LRP.
Apo E	Ligand for LDL receptors and LRP.

The following is a brief description of lipoprotein metabolism (14). Fats absorbed in the intestine are packaged into large triglyceride-rich particles known as chylomicrons. These undergo lipolysis (removal of TG) to form chylomicron remnants which are taken up by the liver via an apo E receptor. The liver can also secrete triglyceride-rich VLDL. Following lipolysis, these particles can be converted to LDL or be taken up by the liver via an apo E receptor. The LDL formed are catabolized mainly by the liver or by other tissues via LDL receptors that recognize both apo B-100 and apo E but not apo B-48. If LDL are modified, they also can be taken up by scavenger receptors on macrophages. HDL are synthesized by both the liver and the intestine. The HDL pick up lipid and protein constituents from chylomicrons and VLDL as these particles undergo lipolysis. HDL picks up free cholesterol from peripheral tissues and macrophages, a process known as reverse cholesterol transport, and are catabolized mainly in the liver.

1.3 Measurement of Lipoproteins

1.3.1 Measurement of lipids as surrogates for lipoproteins

Ever since lipid levels were linked to the prevalence of coronary artery disease (CAD), clinical tests were developed to measure lipids. Enzymatic assays have been available for over three decades to measure TC, TG, and HDL-C (15-19). The HDL assays involve a precipitation step to remove VLDL and LDL, or homogeneous assays that complex beta lipoproteins, followed by a cholesterol assay. As described before, LDL-C is estimated (12) as $TC - (HDL-C) - (TG/5)$, with concentrations expressed in mg/dL units. (For mmol/L units, $TG/2.2$ is used instead of $TG/5$).

What is often not appreciated is that measuring lipids is really a surrogate for measuring the lipoproteins that carry cholesterol and TG. The surrogate relationship of lipids (cholesterol and triglycerides) to lipoproteins was described in the 1967 landmark writings of Fredrickson, Levy, and Lees (20), who noted that "... all abnormalities in plasma lipid concentrations, or dyslipidemia, can be translated into dyslipoproteinemia" and shifting emphasis to lipoproteins "...offers distinct advantages in the recognition and management of such disorders." The reason that lipids, rather than lipoproteins, are the traditional focus of clinical attention was also discussed: "...there is no single test that infallibly separates all those who have dyslipoproteinemia from those who do not....the majority of laboratories still employ a combination of chemical measurements of plasma lipids for this purpose." It is known well that lipoprotein particles interacting with the arterial wall play key roles in the development of atherosclerosis (21). For reasons that are related primarily to the difficulty of measuring lipoprotein particles directly, triglycerides continue to serve as a surrogate measure of VLDL levels, and LDL and HDL cholesterol values as indicators of the

concentrations of LDL and HDL particles. The measurement of apolipoproteins, some of which have reasonably direct relationship to lipoprotein particle levels, have at best been used as extra information to add to the myriad of lipid information towards assessing CHD risk. Especially apolipoprotein B-100, which has a 1:1 relationship to LDL particle concentration, did not warrant much attention in the clinical science community fixated by its cholesterol focus (22).

1.3.2 Lipoprotein subclass measurement

While the relationships of the major lipoprotein classes (VLDL, LDL, and HDL) with CHD risk are well known, the measurement of subclasses within the major classes has brought further understanding to the study of atherosclerosis, diabetes, metabolic syndrome and other lipoprotein disorders. For example, within the LDL regime, the prevalence of smaller, denser LDL (pattern B phenotype) had been associated with as much as a 4-fold increase in CHD risk compared to a prevalence of larger, less dense LDL subclasses (pattern A phenotype) (23-26). Even in the case of the “cardioprotective” high density lipoproteins, only the large HDL subclass seems to give a beneficial effect while some smaller subclasses might be positively linked to CHD risk (27, 28, 61). This type of information has stimulated research interest in lipoprotein subclasses and the development of new, more efficient methods for their measurement.

Lipoprotein subclasses can be quantified by a variety of analytical techniques. The oldest and most common method is analytical ultracentrifugation (29). By appropriately adjusting the density of plasma, sequential flotation ultracentrifugation (30) can be used to isolate major lipoprotein classes as well as the subclasses within. The process involves

multiple steps and takes several days. Density gradient ultracentrifugation is another common method for the isolation of lipoprotein subclasses (31-33). Plasma is carefully layered with multiple density salt solutions and then spun in a swinging-bucket rotor. Depending on the purpose for isolation this can be done in several hours to a day. Gel filtration chromatography is another common way of isolation (34, 35). Agarose gels are used for this size exclusion process. Polyacrylamide gradient gel electrophoresis (GGE) is widely used for LDL and HDL subclass analysis, and has higher resolving power than the ultracentrifugal methods (36, 37). GGE is not, however, suited for preparative scale separations.

1.3.3 Limitations of current methods for lipoprotein subclass analysis

The traditional methods for subclass quantification reviewed above involve a two-step process whereby the subclasses are first physically separated from plasma and each other, and then a measurement is made to quantify the (partially) separated subclass. These are very labor-intensive processes that can take from half-a-day to several days to complete, the latter being more common. Even after the laborious separation, measuring only the cholesterol in the separated fractions does not achieve the objective of quantifying the lipoprotein particles themselves or the bulk lipid they contain. There are also documented concerns that the ultracentrifugation process itself modifies the physical properties of some lipoproteins (38). The powerful GGE techniques are fraught with reproducibility issues arising from the need to make uniform gradient gels and the need to run calibrators every time, all of which confine the technique to a few specialized laboratories. Due to the time and labor involved, these techniques remain cost-prohibitive for routine clinical adoption.

Several years ago, Otvos and coworkers proposed an entirely different approach to lipoprotein subclass analysis that exploits the natural proton nuclear magnetic resonance (NMR) spectroscopic differences existing between lipoprotein particles of different size (39, 40, 41). The proposed method completely eliminates the need for physical separation and measures simultaneously all of the lipoprotein subclass concentrations. There is also no need for any reagents. The method offers the potential for considerable cost and time savings over existing methods, and lends itself to automation and adoption for CHD risk evaluation.

1.4 NMR spectroscopy of blood plasma

NMR spectroscopy had been in use for several decades as a powerful tool for chemical and biomacromolecular structure elucidation. Proton (^1H) NMR spectroscopy where the nucleus being observed is the hydrogen atom (a spin $\frac{1}{2}$ nucleus) is the most commonly studied structural probe. With few exceptions it has been used as a qualitative, rather than quantitative, analytical tool. The first use of NMR to study lipoprotein samples was for the purpose of verifying the micellar structural model for serum lipoproteins (42). Proton NMR has been extensively used to identify the metabolites in plasma and other biological fluids, and also proposed to have utility in cancer diagnosis. An explosion in the field occurred starting in the mid eighties with the advent of good water suppression techniques (43-48). A comprehensive review by Ala-Korpela appeared in 1995 (49).

1.4.1 Lipoprotein subclass measurement by NMR

The proton NMR method of lipoprotein measurement takes advantage of the natural spectroscopic “fingerprints” of lipoprotein particles of varying sizes. Figure 1.4 shows a

proton NMR spectrum of a typical plasma/serum sample acquired at 400 MHz. The assignments for the origin of the multiple peaks from lipids and small molecule metabolites are given in the figure legend. The most prominent lipid peak comes from the repeating units of $-\text{CH}_2-$ (methylene) hydrogen atoms. However, the $-\text{CH}_3$ (methyl) signals appearing around 0.7-0.9 ppm are spectroscopically simpler in terms of their chemical environment, being at the terminal end of lipids. Efforts to measure lipoproteins by NMR thus focused on the methyl signal of plasma and its lipoprotein constituents. The signals in this region are emitted by methyl group protons of phospholipids, free cholesterol, cholesterol ester and triglyceride, which are spectroscopically indistinguishable from one another. The detected methyl signal is thus proportional to the bulk lipid carried in the lipoprotein particles.

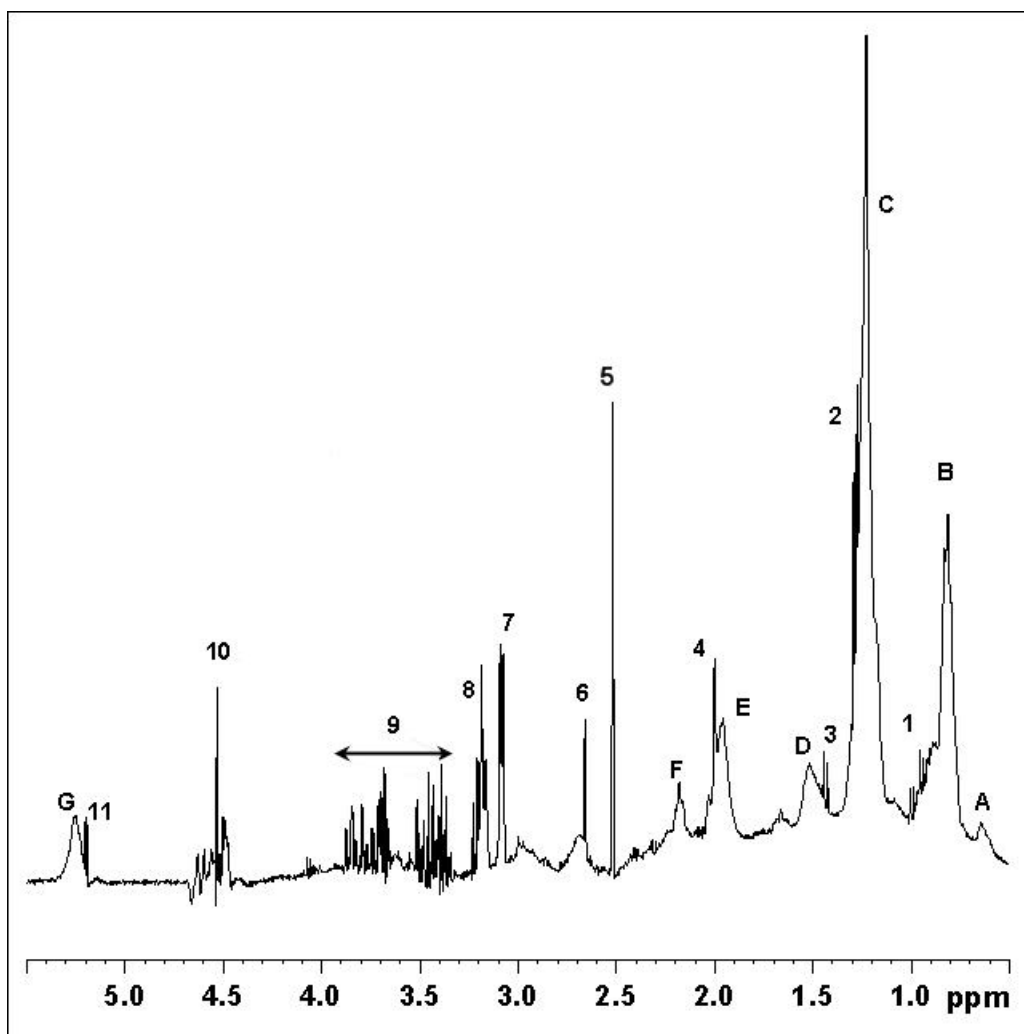


Figure 1.4. Proton NMR spectrum of human blood serum.

400 MHz ^1H NMR spectrum of a serum sample (diluted two fold with EDTA buffer) acquired at 47°C with 10 scans. Resonance assignments: **A**, $-\text{C}(18)\text{H}_3$ of cholesterol backbone; **B**, terminal methyl $-\text{CH}_3$ protons; **C**, methylene $-(\text{CH}_2)_n$ protons; **D**, methylene protons of C(3) carbon $-\text{CH}_2-\text{CH}_2-\text{COOC}-$; **E**, allylic methylene protons $-\text{CH}_2-\text{CH}=\text{CH}-$; **F**, methylene protons of the C(2) carbon $-\text{CH}_2-\text{COOC}-$; **G**, allylic carbon protons $-\text{CH}=\text{CH}-$; **1**, valine quartet; **2**, lactate doublet; **3**, alanine doublet; **4**, N_{acetyl} protons of N-acetylglucosamine of glycoproteins; **5**, ethylenic protons $-\text{N}-\text{CH}_2-\text{CH}_2-\text{N}-$ of CaEDTA; **6**, ethylenic protons $-\text{N}-\text{CH}_2-\text{CH}_2-\text{N}-$ of MgEDTA; **7**, acetate protons $-\text{CH}_2-\text{COO}^-$ of CaEDTA doublet; **8**, methyl $-\text{N}(\text{CH}_3)_3$ of choline head group of phospholipid; **9**, glucose multiplet; **10**, residual water H_2O ; **11**, α -glucose doublet.

By isolating the major classes of lipoprotein (VLDL, LDL and HDL), and the lipoprotein-free serum proteins, and adding the NMR spectra of these constituents together, we demonstrated that the shape and intensity of the intact plasma signal can be matched (48). By acquiring and utilizing a library of reference spectra of purified lipoprotein subclasses, it is feasible to work backwards from the composite plasma methyl signal using a specialized linear least squares fitting program (40). There are distinct chemical shift differences between the different classes of lipoproteins. The larger classes, and indeed subclasses, progressively shift downfield (to the left) from the smaller lipoprotein classes. While the core of the particle is isotropic at temperatures above the thermal order-disorder transition point ($>45^{\circ}\text{C}$), the phospholipid shell remains in an ordered state and this anisotropic magnetic susceptibility directly related to the radius of curvature of the particle has been established as the source of the chemical shift differences (50). Under current conditions, a total of 15 different lipoprotein subclasses consisting of 6 VLDL, 4 LDL including IDL, and 5 HDL are quantified by the NMR methodology with adequate precision and accuracy. The diameter ranges of the subclasses determined by NMR and characterized by electron microscopy and polyacrylamide gradient gel electrophoresis are given in Figure 1.5.

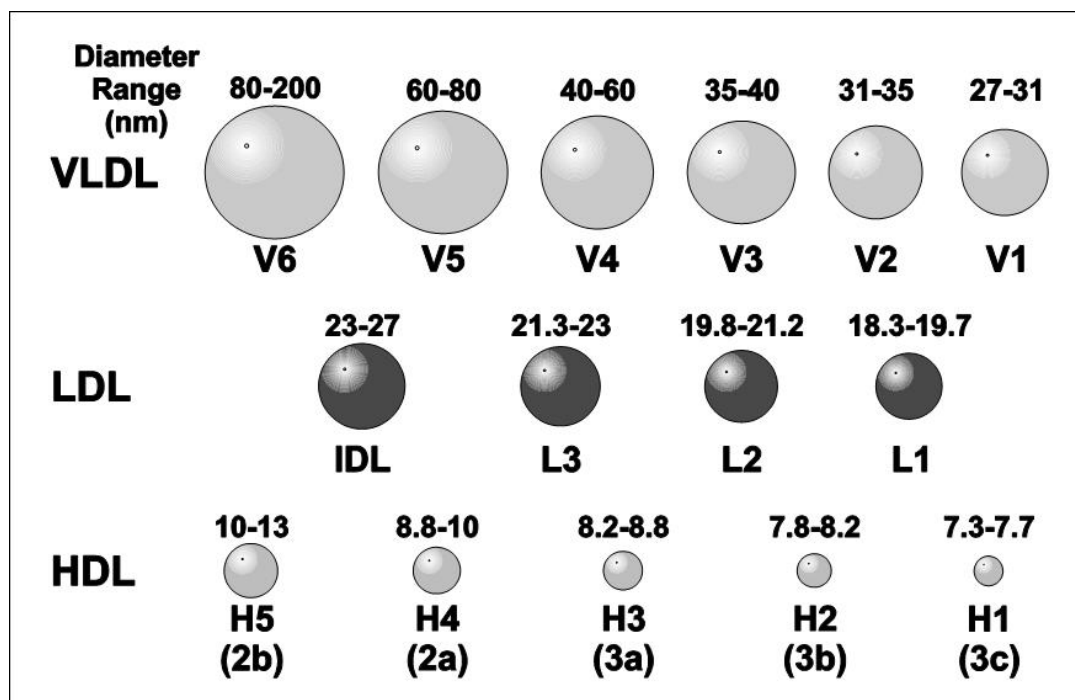


Figure 1.5. Lipoprotein subclasses quantified by NMR.

Subclass designations and diameter ranges for the fifteen subclasses quantifiable by NMR. The sizes of the original reference components were determined by GGE and electron microscopy. Another alternate classification is large VLDL (V6+V5), medium VLDL (V4+V3), small VLDL (V2+V1), IDL, large LDL (L3), medium-small LDL (L2), very small LDL (L1), large HDL (H5+H4), medium HDL (H3), and small HDL (H2+H1).

The deconvolution of the plasma spectra results in coefficients that tie the intensities of the plasma subclass signals to the reference subclass components used in the fitting model. These coefficients are then multiplied by conversion factors that produce the particle concentrations of the subclasses and the NMR-derived lipid concentrations. The most fundamental information gained by the NMR methodology is the particle concentrations of subclasses (mmol/L for VLDL and LDL, and $\mu\text{mol/L}$ for HDL), since the NMR intensity is directly proportional to the number of hydrogen nuclei of the methyl protons. The compositional changes resulting from CETP mediated TG – CE exchange processes have no bearing on the number of protons since both TG and CE have the same number of CH_3 groups contributing 9 protons per molecule. The NMR-derived lipid concentrations (in

mg/dL Chol or TG) on the other hand are obtained based on cholesterol and TG concentrations determined for the reference subclasses that assume normal composition of TC and TG (40-41, 51-52). Therefore, while generally good agreement will be seen for NMR-derived lipid values and chemical lipids, there will be significant differences when the plasma samples have lipoproteins with abnormal lipid compositions as in the case of TG-enriched, cholesterol-depleted particles. The third important piece of information resulting from the NMR deconvolution is the average particle sizes (in nm) for the VLDL, LDL, and HDL classes. These are computed as the weighted average of the sum of the diameter of each subclass multiplied by its relative mass percentage as estimated from the methyl NMR signal amplitude. There is very good agreement between NMR-determined and GGE-estimated particle sizes for LDL and HDL. The output incorporating all three classes of information (i.e. particle concentrations, lipid estimates, average particle size) is termed *NMR LipoProfile*.

1.4.2 Advantages of lipoprotein subclass analysis by NMR

The NMR method for subclass analysis is rapid and fully automatable. The major advantage of the method is in its avoidance of the need for physical separation of the lipoprotein classes and subclasses. NMR provides within minutes the data obtainable in days by traditional methods. On top of it, NMR methodology provides additional useful data like the LDL and HDL particle concentrations that are not directly accessible by any other method. The precision of the NMR method is also superior to the traditional methods. The efficiency with which lipoprotein subclass data can be generated opens new avenues in the assessment and management of CAD risk in the general population.

1.4.3 Alternate NMR approaches

In parallel to the development of the NMR method described above, a group in Finland led by Hiltunen and Ala-Korpela used a lineshape analysis program (FITPLA) where they tried to model the spectra of lipoprotein classes with multiple Lorentzian peaks (53-55).

Subsequently they introduced multivariate analysis approaches to quantify lipoprotein lipids (56-57). Chemometric techniques like Partial Least Squares regression (PLS) and Principal Component Analysis (PCA) were used to correlate NMR-derived values to plasma lipid levels. Later, Ala-Korpela's group employed artificial neural network (ANN) analysis to correlate NMR values to chemical lipids and apolipoproteins A1 and B. Another group in Norway led by Engan and Bathen extended the PLS and ANN approaches along with pattern recognition to separate CHD subjects from normal subjects, and to aid in cancer diagnosis (58-60). These novel chemometric approaches, however, have been tested with very small data sets, and require the need for a training data set and test (validation) set and the know-how not to over-train. More importantly, these are all attempts at quantifying lipids, and not quantifying lipoprotein subclasses at the particle level.

1.5 Overview of chapters to follow

Chapter 1 presented background information regarding CHD risk assessment and the existing need for measuring lipoprotein subclasses to aid diagnosis and management of coronary artery disease. Lipoprotein structure and function and the currently available methods for lipoprotein subclass analysis were reviewed. The NMR-based spectral deconvolution technique to measure lipoprotein classes and subclasses at a particle level, and the average particle sizes of VLDL, LDL and HDL classes was introduced.

Chapter 2 will deal with details of the methodology, especially focusing on the isolation of pure reference subclasses and the size characterizations of the same with GGE and electron microscopy. The NMR spectroscopy methodology will also be discussed in more detail. The clinical output of the results, the *NMR LipoProfile*, will be introduced.

Chapter 3 forms the bulk of the dissertation material and comes in the form of a paper to be submitted to Clinical Chemistry for publication. It provides comprehensive validation data for the NMR technique and contains precision and accuracy data establishing *NMR LipoProfile* as a clinical assay.

Chapter 4 consists of a paper published in the American Journal of Cardiology highlighting the importance of the newly available LDL particle concentration number. It demonstrates how traditional LDL cholesterol levels can underestimate the true risk of patients for certain metabolic conditions whereas the LDL particle number would have confirmed their excess LDL status. NMR data from the Framingham Offspring Study with 3400 participants is used for this analysis.

Chapter 5 entails a brief discussion, summary and conclusion. References for chapters 1, 2 and 5 are given at the end, starting on page 127.

Chapter 2: Experimental: Building blocks of the *NMR LipoProfile* test

2.1 Isolation of lipoproteins

Lipoprotein preparations were obtained by sequential ultracentrifugation as previously described (39, 62, 63). The density ranges for the major subclasses isolated were Chylomicrons ($<0.94\text{kg/L}$), VLDL ($0.94\text{-}1.006\text{ kg/L}$), IDL ($1.006\text{-}1.109\text{ kg/L}$), LDL ($1.019\text{-}1.063\text{ kg/L}$) and HDL ($1.063\text{-}1.210\text{ kg/L}$). Plasma proteins with $d>1.225\text{kg/L}$ were also isolated. Where appropriate, the preparations with densities higher than that of plasma were dialyzed against plasma diluent buffer containing 120mM KCl, 5mM EDTA, 1mM CaCl_2 , 50mM Na_2HPO_4 and 0.2g/L NaN_3 buffered at pH 7.4.

For the purposes of generating highly purified lipoprotein subfractions with as homogeneous a range of particle diameters as possible, a combination of ultracentrifugation and agarose gel filtration chromatography was used based on modifications to the general procedures of Rudel (34) and Sata (35). Meter-long columns (1.5cm id) packed with a variety of agarose beads (Bio-Rad, Hercules, CA or Agarose Bead Technologies, Tampa, FL) were set up in a cold room maintained at 4°C . Separate columns of Bio-Gel type A-150 (1 %), A-50 (2%), A-15 (4%) and A-1.5 (8 %) agarose gel beads were used for the isolation and/or purification of Chylomicrons, VLDL, LDL and HDL subclasses, respectively. The lipoprotein isolations typically started with 20-25 mL of plasma from several normo- and dyslipidemic subjects, one individual's plasma at a time except for IDL, for which plasma from several hypertriglyceridemic subjects was pooled. For VLDL, the top fraction from a density 1.006kg/L spin ($180,000\text{ xg}$, 18h) was used on the A-50 agarose column. For LDL and HDL the plasma, or plasma without the VLDL, was adjusted to a density of 1.225kg/L

by the dissolution of solid NaBr, ultracentrifuged at 4⁰C and 50,000 rpm for 48 hours, (Beckman Coulter, Optima TLX) and the top fractions collected using a Beckman tube slicer. For IDL, the fraction with density range 1.006 – 1.019kg/L was first isolated from pooled plasma using sequential ultracentrifugation. The floated lipoproteins were concentrated to 1-2mL using Centricon-10 microconcentrators (Amicon Inc, Danvers, MA) and loaded onto the A-15 column and eluted with plasma buffer. Fractions of 3-4 mL each were collected and the OD measured at 280nm to record the elution of the lipoprotein particles.

The A-15 columns yielded baseline separations of VLDL, LDL and HDL. The tubes containing LDL and HDL were pooled separately, concentrated and loaded back on the A-15 or A-1.5 columns, respectively, for further purification of the LDL and HDL. The quality of the separations improved further when the first half and second half of the LDL and HDL peaks were re-chromatographed separately on their respective columns. Each column run required one to three days to complete. Figure 2.1 shows the A-15 agarose column profile of the d<1.225kg/L fraction from a hypertriglyceridemic subject. Note that the chylomicron remnants were eluted in the excluded volume of the column. Further column profiles are included in Figures 1 and 2 of Chapter 3.

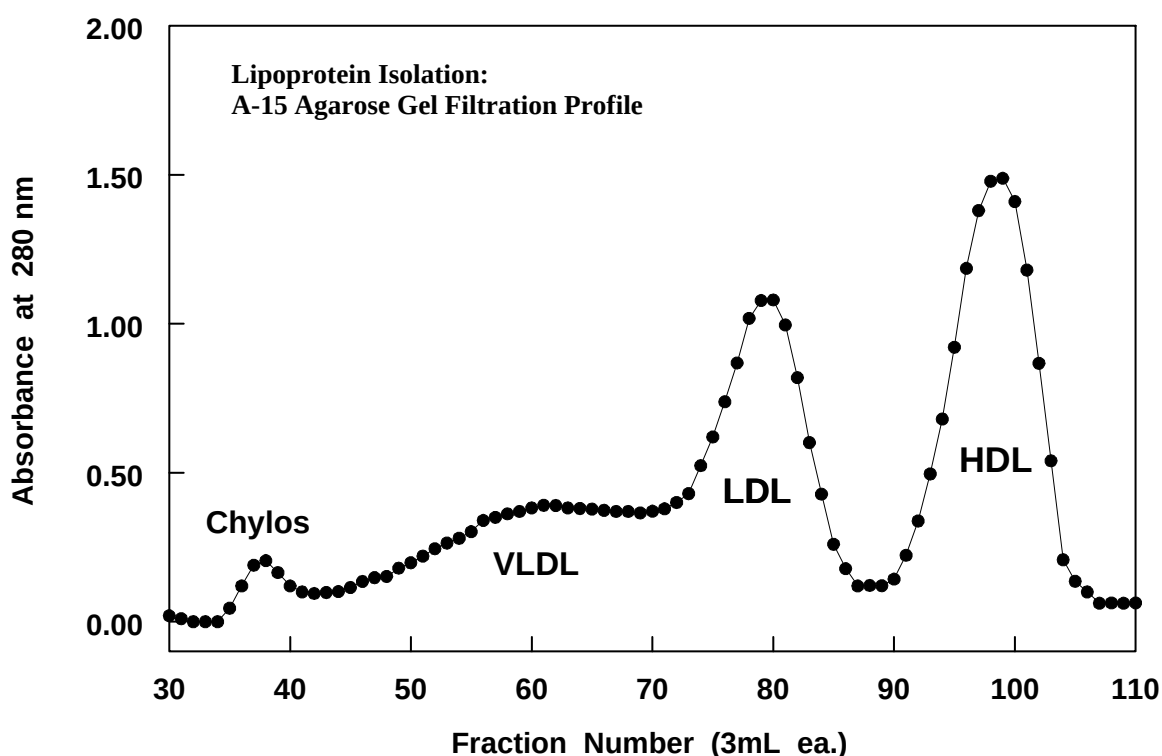


Figure 2.1. Agarose column separation of major classes of lipoproteins. A-15 agarose gel filtration profile of density $< 1.225\text{kg/L}$ portion from a hypertriglyceridemic subject. The absorbance at 280 nm was plotted against fraction number (3 mL ea.). Peaks from chylomicrons, VLDL, LDL, and HDL are clearly visible, with the chylomicrons appearing in the void volume of the column.

The A-15 column (4% Agarose beads) gave the best overall separation of all three classes of lipoproteins. However, in light of the overlap in the VLDL/LDL regime, procedures were modified to first isolate VLDL by ultracentrifugation and then achieve further purification by chromatographing on an A-50 column. The fraction with VLDL removed from it was centrifuged at $d=1.225\text{kg/L}$ before being loaded on the A-15 column for purification of LDL and HDL. The fractions eluting on either side of the main peaks were considered to have greater subclass homogeneity. The fractions were concentrated to 0.5-1mL and kept refrigerated until characterized by NMR, chemical lipid analysis and gradient gel electrophoresis or electron microscopy.

2.2 Characterization of lipoproteins

2.2.1 Chemical analysis

Chemical lipid analyses for TC, TG and HDL-C were performed enzymatically on a Bayer RA-1000 analyzer at the Lipid Analytic Laboratory of the Wake Forest University School of Medicine (Winston Salem, NC). All TG analyses were run with glycerol blanking. The analysis for TC and TG was performed on the isolated lipoprotein subcomponents after special calibration for measuring concentrations lower than normally seen in plasma. Apolipoprotein B (apoB) measurements were performed on a Beckman Synchron CX-7 (Beckman Coulter Inc., Fullerton, CA) analyzer using a commercial turbidimetric immunoassay (Wako Chemicals, Osaka, Japan) (64). Compositional analysis to measure CE, TG, unesterified cholesterol and protein were carried out at Wake Forest University School of Medicine (Winston Salem, NC) with the assistance of Dr. Martha Wilson.

2.2.2 Electron microscopy

Lipoprotein particle size measurements for purified VLDL and LDL subclasses were carried out at the Center for Electron Microscopy at North Carolina State University using a JEOL 100S (Peabody, MA) transmission electron microscope with the assistance of Ms. Valerie Knowlton. Samples were prepared following negative staining procedures described by Forte (65). Briefly, samples of VLDL/LDL in saline buffer were diluted (~1mg/mL, final) and 1 drop of sample was placed on a 200-mesh Formvar-carbon-coated grid. After a minute, extra sample was blotted off with filter paper, and one drop of 2% sodium phosphotungstic acid (PTA) was placed on the grid, blotted dry after a minute, and air-dried for 10 minutes.

Magnifications of 25,000 – 60,000 x and a further photographic enlargement of 2.5 – 3.0 x were used. Diameters on the photographs were measured using a HiPad Digitizer (Houston Instruments, Austin, TX). Mean diameters were derived by measuring the diameters of at least 200 lipoprotein particles on two or more grids. The VLDL particles are very susceptible to aggregation and needed to be analyzed in a timely manner, preferably within 1 week of isolation. Aggregates with the appearance of threaded beads were seen on aged samples of VLDL subclass components. Figure 2.2 illustrates a typical electron micrograph. This was obtained for a small VLDL sample with an average diameter of 33 ± 3 nm. More examples can be found in Chapter 3, Figures 2A, 2B, 2C.

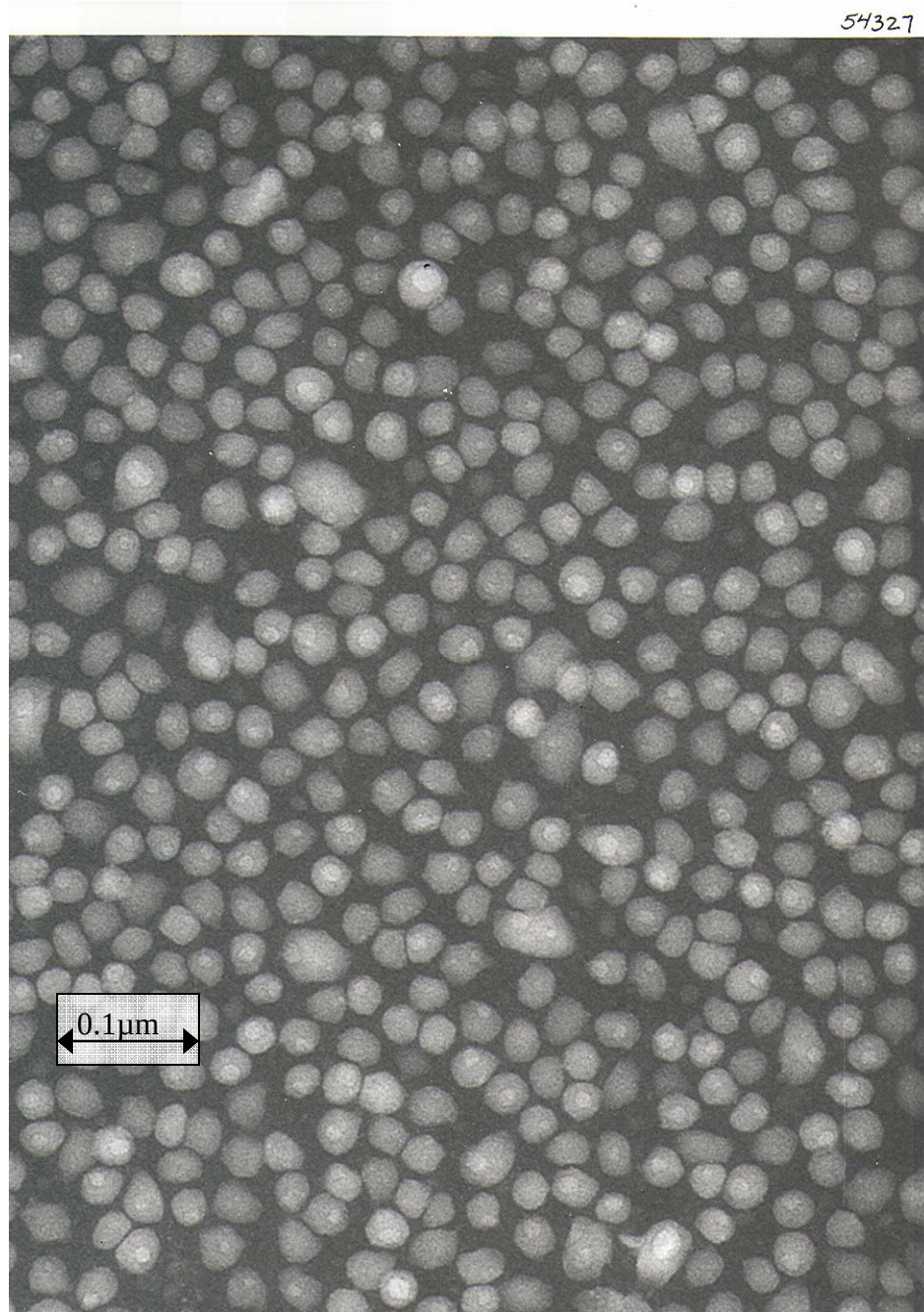


Figure 2.2. Electron micrograph of a homogeneous VLDL subcomponent.

Transmission electron micrograph (JEOL 100S) of a VLDL lipoprotein subclass component separated and purified by combination of ultracentrifugation and agarose column chromatography. Sample was diluted with saline to approx. 1mg/mL and applied on 200-mesh Formvar-carbon-coated grids and stained with 2% sodium phosphotungstic acid (PTA). Total magnification $\times 135,000$. Diameters on the photographs were measured with a HiPad Digitizer counting 200 particles to generate the frequency distribution and mean diameters. The particle diameter (mean $\pm$ SD) for the lipoprotein component was 33 ± 3 nm.

2.2.3 Gradient gel electrophoresis

Nondenaturing gradient gel electrophoresis was used to characterize isolated HDL subfractions in the laboratory of Dr. Larry Rudel at Wake Forest University School of Medicine (34, 36). Further GGE analysis of plasma samples and LDL and HDL preparations for the standard addition studies were performed at Dr. David Rainwater's laboratory at Southwest Foundation for Biomedical Research (San Antonio, TX) (37, 66, 67). Generally, in the Rainwater laboratory, twelve microliters of isolated LDL or HDL fraction was applied to a 3-31% gradient gel. After electrophoresis, the gel was stained for lipid with Sudan black B.

Calibrators for LDL diameters were: latex microspheres (38 nm), two LDL bands (27.5 & 26.6 nm, calibrated by Berkeley HeartLab, Inc., and thyroglobulin (17 nm); for HDL: thyroglobulin (17 nm), ferritin (12.2 nm), LDH (8.16 nm), and albumin (7.2 nm). A photograph of a typical GGE scan and its densitometer trace is shown in Chapter 3, Figure 5.

2.3 NMR spectroscopy

Proton NMR spectra of all plasma samples and isolated lipoprotein subclasses were acquired on 400 MHz Avance spectrometers (Bruker Bio-Spin, Billerica, MA) at the CLIA approved facilities of LipoScience Inc., (Raleigh, NC). The NMR system was supplied with a special

INCA (Integrated NMR Chemical Analyzer) enclosure consisting of an actively shielded magnet, an automatic Gilson-215 (Madison, WI) sample handler and a flow probe with a 120uL active volume. All NMR measurements for lipoprotein analyses were performed at 47⁰C; the flow path was heated to minimize the time needed for temperature equilibration of the sample inside the probe. Automatic sample handling methods and procedures developed in-house were used in tandem with Bruker's ICON NMR software module. Sample preparations were executed automatically by a Tecan Genesis RSP-100 (Tecan US, RTP, NC) aliquotting station. The bar-coded sample racks were kept refrigerated and loaded on the NMR analyzer after daily startup and QC procedures were completed. When the racks are cooled, up to 256 samples can be analyzed unattended.

As part of the startup procedure a NMR standard sample of TMA (trimethylacetate, sodium salt) was first injected. The TMA standard mimics the ionic strength of plasma and contains 15mM TMA, 5mM EDTA, 3mM CaCl₂, 120mM KCl and 10%v/v D₂O, pH 9.0. The TMA was used for "shimming" to achieve acceptable magnetic field homogeneity and to calibrate the detection sensitivity of the spectrometer. Spectra were acquired following shimming the magnetic field on the field-frequency lock signal provided by the D₂O in the TMA sample to achieve a Lorentzian lineshape with a linewidth at half-height of < 1.2 Hz. The methyl peak of the TMA standard was integrated and the peak area used to correct for day-to-day variations in spectrometer sensitivity and to normalize multiple spectrometers and flow probes. The frequency shift of the water with respect to the CaEDTA peak at 2.519 ppm was used as an internal thermometer to maintain the measurement temperature at 47.0⁰C.

On runs involving patient samples, aliquots of two levels of serum control material were analyzed in the automation mode under identical conditions to that of the analysis of the

test samples. The serum control materials were purchased from Soloman Park Research Laboratories (Kirkland, WA) where the two pools were prepared encompassing high and low ranges of lipid levels. The *NMR LipoProfile* results obtained for the serum controls were the input for Westgard's multirule quality control procedures (69, 70).

NMR acquisition conditions were similar to previous descriptions (39, 40). A single pulse scout scan was used to determine the water frequency. This was followed by a standard one-pulse sequence preceded by a 1.5-s pre-acquisition delay and 1.5-s selective presaturation pulse at the water frequency. A spatially selective composite 90° observation pulse ($90_x - 90_y - 90_{-x} - 90_{-y}$) was used to minimize water suppression artifacts (68). The acquisition time was 1.0-s with a pulse repetition rate of 4-s per scan. Other settings for data acquisition were: spectral width, 4496 Hz; time domain data size, 9024; composite pulse length 5-8 μ s; and constant receiver gain. For routine *NMR LipoProfile* analysis of plasma and serum samples NMR data were acquired unlocked in 5 blocks of two scans each. For isolated lipoprotein components with relatively low lipid content, samples were run locked acquiring 16-128 scans in a single block. The NMR analysis time for the plasma samples was 40-s and a further 1.5 to 2 minutes was required for sample loading and cleaning the flow path between samples. The time-domain data were zero-filled to 16K, multiplied by appropriate Gaussian and exponential apodization functions to enhance resolution, and Fourier-transformed with identical scaling. The resulting real and imaginary files form the input for the deconvolution program to generate the *NMR LipoProfile*.

2.4 Construction of fitting model

After a sufficient number of lipoprotein subcomponents of the different subclasses of the major lipoprotein classes of VLDL, LDL, and HDL had been characterized, the plasma

lineshape fitting model was then constructed. Selecting enough subcomponents (30-40) to provide representation of all lipoprotein species likely to be encountered in a patient's plasma sample is essential. The subcomponents were then grouped into subclasses as described in Chapter 1 and 3 forming 6 VLDL, IDL, 3 LDL and 5 HDL subclasses. Figure 2.3 illustrates the chemical shift relationship of the lipoproteins to the particle size for representative subclasses included in the model.

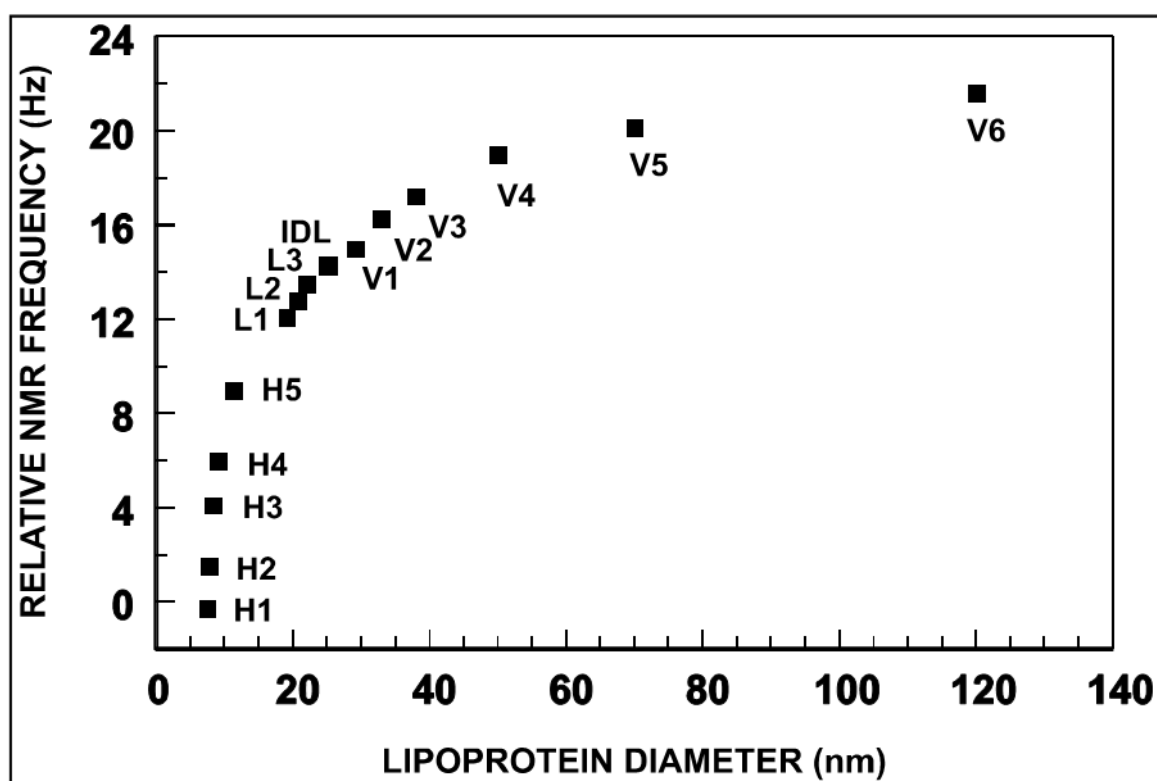


Figure 2.3. Relationship of lipoprotein particle diameter and NMR chemical shift. Plot of relative NMR shift (with respect to the smallest HDL) of fully characterized lipoprotein subclasses against lipoprotein particle diameter.

The fitting model representative of the 10 major subclasses of lipoproteins is given in Figure 2.4. The NMR spectra of the methyl region are plotted adjusted to the same vertical scale. The particle-size related chemical shift differences and lineshape differences between

the different classes of lipoproteins, including subtle differences within each class are clearly discernable.

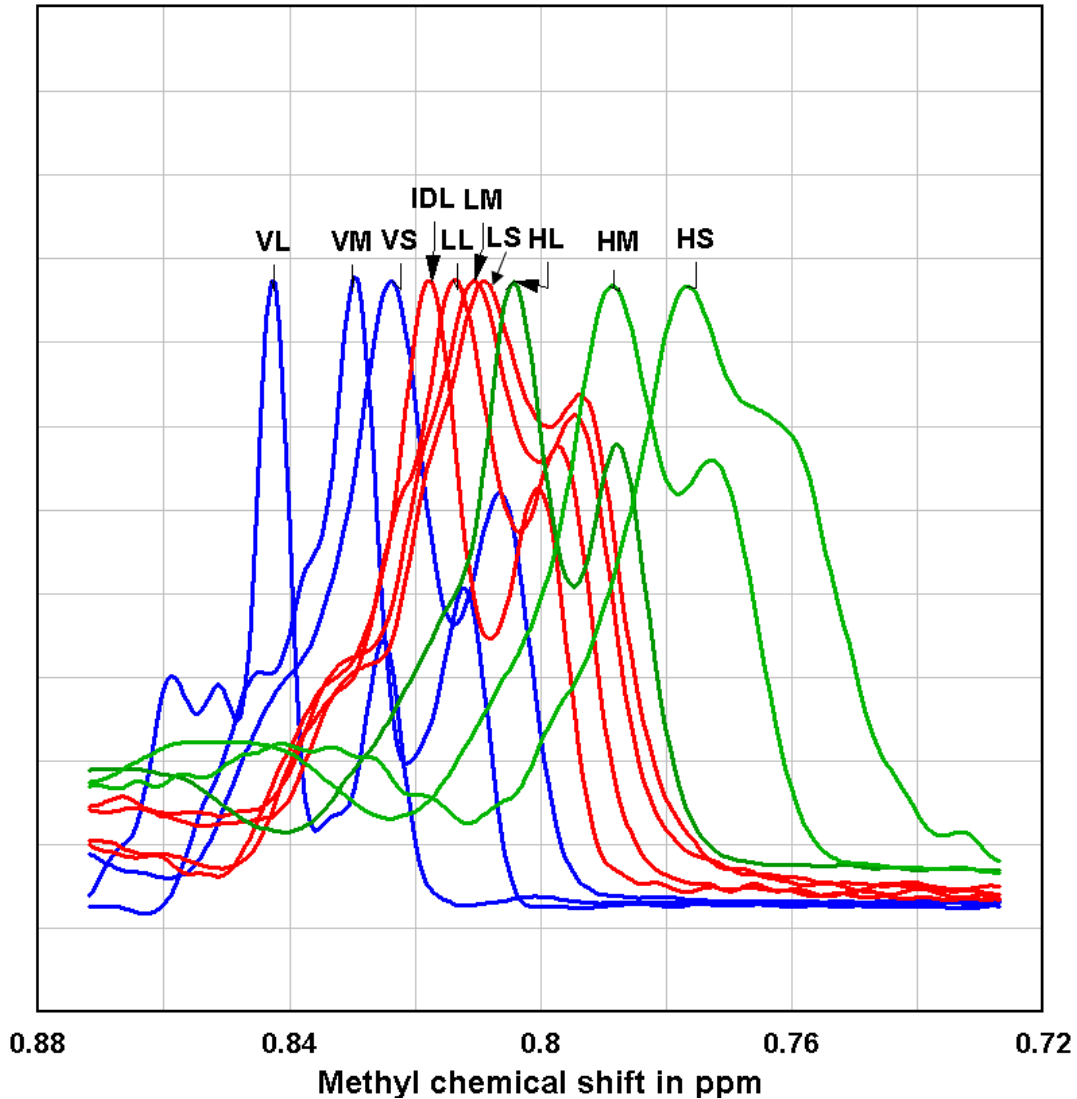


Figure 2.4. Reference spectra comprising *NMR LipoProfile* fitting model. Methyl region ^1H NMR spectra of representative lipoprotein subclasses making up *NMR LipoProfile* lineshape analysis model. VL=large VLDL, VM=medium VLDL, VS=small VLDL, IDL=intermediate density lipoproteins, LL=large LDL, LM=medium LDL, LS=small LDL, HL=large HDL, HM=medium HDL, and HS=small HDL.

2.5 NMR lineshape deconvolution

The lipid methyl region, from 0.914 to 0.718 ppm, was the spectral region employed for deconvolution of the experimental NMR lineshape. The experimental spectrum was modeled as a linear combination of over 30 lipoprotein subcomponent spectra obtained as described above. Contribution of each subcomponent NMR lineshape in the basis set to the lineshape of the experimental spectrum was determined by singular value decomposition (71, 72, 73), constrained so that concentrations could not be less than zero.

The digitized data covering the deconvolution region from each of the reference lipoprotein spectra was stored in separate arrays of computer memory. Both the real and imaginary part of the experimental plasma (analyte) spectrum was then read into the array. The analyte NMR lineshape was deconvoluted using multivariate analysis with non-negative constraints. The lineshape consisted of a vector of m discrete NMR intensities, each representing an incremental change in the NMR chemical shift that was directly related to the size of lipoprotein particles. The experimental lineshape was approximated as a linear combination of reference component lineshapes, with each component a vector spanning the same region as the experimental spectrum:

$$P_i^R \approx \sum_{j=1}^{n-2} c_j V_{ji}^R + c_k V_{ki}^R + c_p P_i^I$$

R and I represent the real and imaginary parts of the NMR spectrum. $\mathbf{P}$ is the experimental analyte vector, with m data points where $m = 1, 2, \dots, 300$. The $\mathbf{V}_j^R$ are vectors corresponding to the real parts of a carefully phased set of $n-2$ lipoprotein component spectra spanning particle size range from 200 nm to 7.3 nm, covering large VLDL/Chylomicrons to small

HDL. The vector $\mathbf{P}^I$ is the imaginary part of the analyte spectral vector and $\mathbf{V}_k$ is the spectral vector of the non-lipoprotein ‘plasma protein’ component. These vectors constitute a basis set for least squares analysis, combined into a design matrix for multicomponent regression:

$$\mathbf{V} = \begin{bmatrix} V_{11} & V_{12} & L & V_{1m} \\ V_{21} & V_{22} & L & V_{2m} \\ M & M & O & M \\ V_{n1} & V_{n2} & L & V_{nm} \\ V_{k1} & V_{k2} & L & V_{km} \\ P_1^I & P_2^I & L & P_m^I \end{bmatrix}$$

The c_j , c_k , and c_p are the relative contributions to the calculated spectrum for each component vector of the design matrix and constitute a solution vector $\mathbf{c}$, such that $\mathbf{V}^T \mathbf{c} \approx \mathbf{P}^R$, where $\mathbf{P}^R$ is the vector corresponding to the real part of the analyte spectrum. The best fit (in the least squares sense) is when the Euclidean norm of the residual vector, $\mathbf{r} = \mathbf{P}^R - \mathbf{V}^T \mathbf{c}$, is minimized. The condition for this is that $\mathbf{V}\mathbf{V}^T \mathbf{c} = \mathbf{V}\mathbf{P}^R$ and $\mathbf{c} = (\mathbf{V}\mathbf{V}^T)^{-1} \mathbf{V}\mathbf{P}^R$; in principle the desired vector $\mathbf{c}$ can be obtained using standard matrix algebra.

However, many of the component spectra have similar lineshapes, and the matrix $\mathbf{V}^T$ is often close enough to singularity that the presence of noise in the analyte spectrum creates instability in the conventional least squares solution. This instability is overcome by decomposing the matrix $\mathbf{V}^T$ using singular value decomposition (73): $\mathbf{V}^T = \mathbf{Q}\mathbf{S}\mathbf{P}^T$, where $\mathbf{Q}$ is a $m \times m$ orthogonal matrix and $\mathbf{P}$ a $n \times n$ matrix of singular vectors. $\mathbf{S}$ is a $m \times n$ matrix consisting of a diagonal matrix of singular values in the upper $n \times n$ portion of the matrix, with the remainder of the matrix containing zeros. $\mathbf{Q}$ and $\mathbf{P}$ effectively change the basis $\mathbf{V}^T$, with the magnitude of singular values corresponding to the vectors in this new basis

determining their relative contribution to the solution. When these values are very small, so that the contribution of their corresponding vectors to the solution has the same magnitude as the noise, they are eliminated by setting the singular values to zero, thus removing statistically meaningless contributions to the fit.

Once the coefficients c_j relating analyte data to the amplitude fraction of the reference components were solved, conversion factors relating reference spectra signal amplitude to subclass concentrations expressed in particle concentration units or in lipid mass concentration units (cholesterol or TG) were then applied. Particle concentrations (nmol/L for VLDL and LDL; $\mu\text{mol/L}$ for HDL) were calculated for each subclass standard by measuring the total concentration of core lipid (cholesterol ester plus TG) and dividing the volume occupied by these lipids by the core volume per particle calculated from the particle diameter of the isolated lipoprotein subclass reference. Lipid mass concentration estimates (VLDL in mg/dL TG and LDL and HDL in mg/dL cholesterol) were obtained by applying conversion factors that contained the lipid concentrations measured on each reference subclass. Weighted average VLDL, LDL and HDL particle sizes (nm diameter) were computed as the sum of the diameter of each subclass multiplied by its relative mass percentage as estimated from the intensity of its methyl NMR signal.

2.6 Output of deconvolution: *NMR LipoProfile*

Once the model is established the actual deconvolution takes only a fraction of a second. The visual output in Figure 2.5 shows the experimental spectrum (red) fitted with the components (lower curves), with the calculated spectrum (black) closely matching the plasma spectrum.

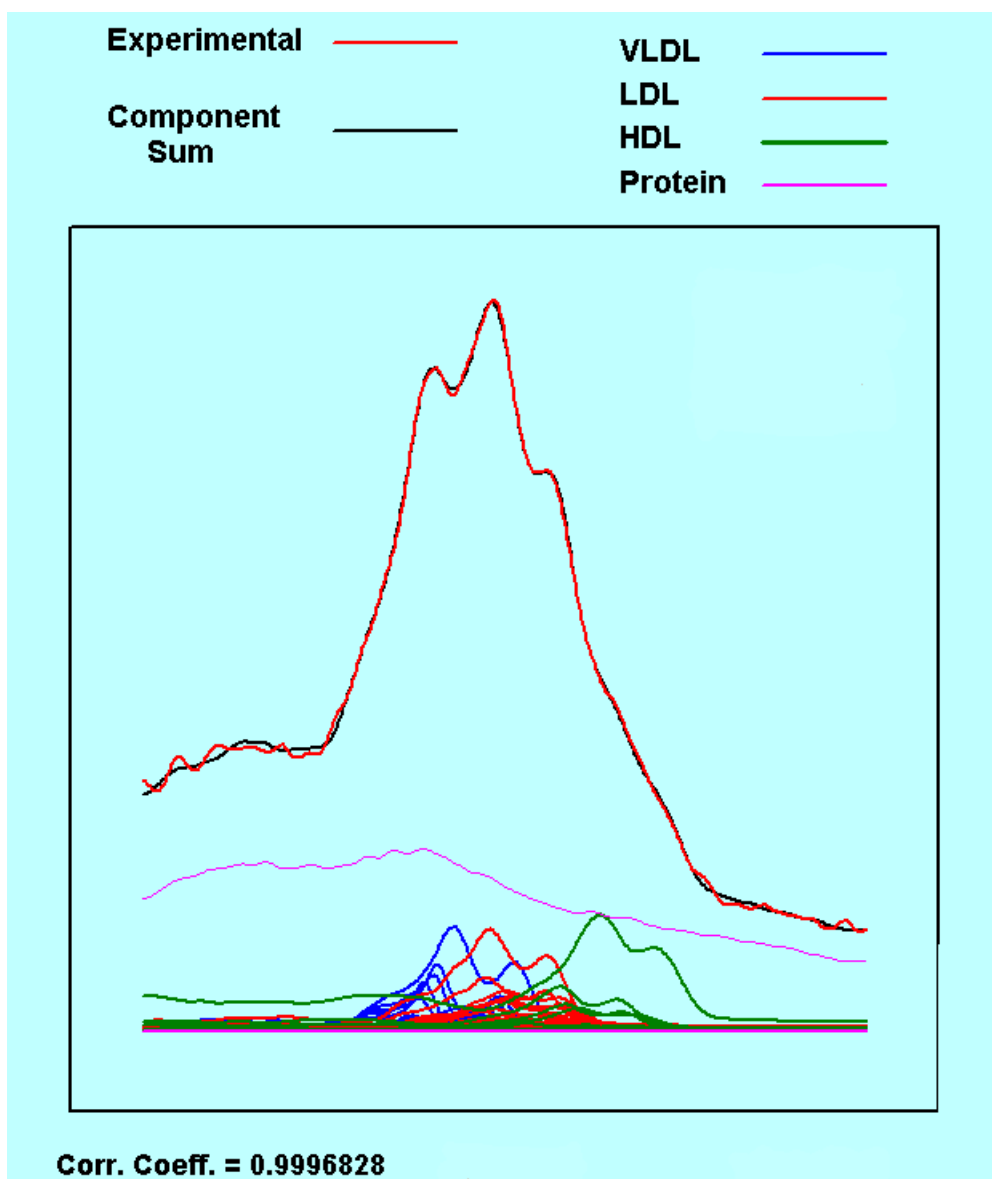


Figure 2.5. Plasma lineshape analysis results.

Visual output of a plasma lineshape analysis. The calculated (black) line closely match the experimental spectrum (red) with $r=0.99968$. The lower curves show the constituent subclasses used in the fit, VLDL (blue), LDL (red), HDL (green). The large featureless curve (purple) represents the plasma protein peak included in the fit as a component.

The results of deconvolution are then automatically converted as described earlier to provide particle concentrations, estimated lipid values, and average VLDL, LDL, and HDL particle sizes. A selection of these results is encapsulated in the clinical *NMR LipoProfile* report for

the purpose of assessing and managing the CHD risk of patients. Figure 2.6 A, B contains a representative *NMR LipoProfile* report.

A

Produced under patent licenses
to U.S. Patent Nos. 4,933,844
and 5,343,389

© LipoScience, Inc. 2002

NMR LipoProfile®

LipoScience, Inc.
3009 New Bern Avenue
Raleigh, NC 27610
877-547-6837
www.liposcience.com

Page 1 of 2

Patient Name			Sex	Age	Clinician
Patient ID	Birth Date	Accession Number		Client Name and Address	
				Client Name	
				Address 1	
				Address 2	
				City, State	
				ZIP	Client #/Route #
				Phone:	Fax:
Date Collected	Date Received	Report Date and Time		Requisition Number	Comment

LIPOPROTEIN PANEL		Coronary Heart Disease (CHD) Risk Categories				
	nmol/L	Optimal* under 1100	Near or above optimal 1100 - 1399	Borderline-high 1400 - 1799	High 1800 - 2100	Very high over 2100
LDL Particle Number	1654					
<i>* Goal for patients with CHD or CHD risk equivalents</i>						
	nm	Pattern A (large LDL) 22.0 - 20.6 Lower-Risk		Pattern B (small LDL) 20.5 - 19.0 Higher-Risk		
LDL Size	19.1					
	mg/dL	Negative Risk Factor greater than 30		Intermediate 30 - 11	Positive Risk Factor less than 11	
Large HDL (cholesterol)	10					
	mg/dL	Lower-Risk less than 7		Intermediate 7 - 27	Higher-Risk greater than 27	
Large VLDL (triglyceride)	35					

RISK ASSESSMENT PANEL		Elevated numbers of LDL particles and the lipoprotein traits of the metabolic syndrome affect CHD risk interactively. Risk is highest when both are present.		
Elevated LDL Particle Number	>1400 nmol/L	☒		
Lipoprotein Traits of the Metabolic Syndrome*	≥2 traits	☒		
		Small LDL Pattern B (≤20.5 nm)	Reduced Large HDL (<11 mg/dL)	Elevated Large VLDL (>27 mg/dL)
		☒	☒	☒

* Other identifiers of the metabolic syndrome include abdominal obesity, elevated blood pressure, and fasting glucose ≥110 mg/dL.

B

Produced under patent licenses
to U.S. Patent Nos. 4,933,844
and 5,343,389
© LipoScience, Inc. 2002

NMR LipoProfile®

LipoScience, Inc.
3009 New Bern Avenue
Raleigh, NC 27610
877-547-6837
www.liposcience.com

Page 2 of 2

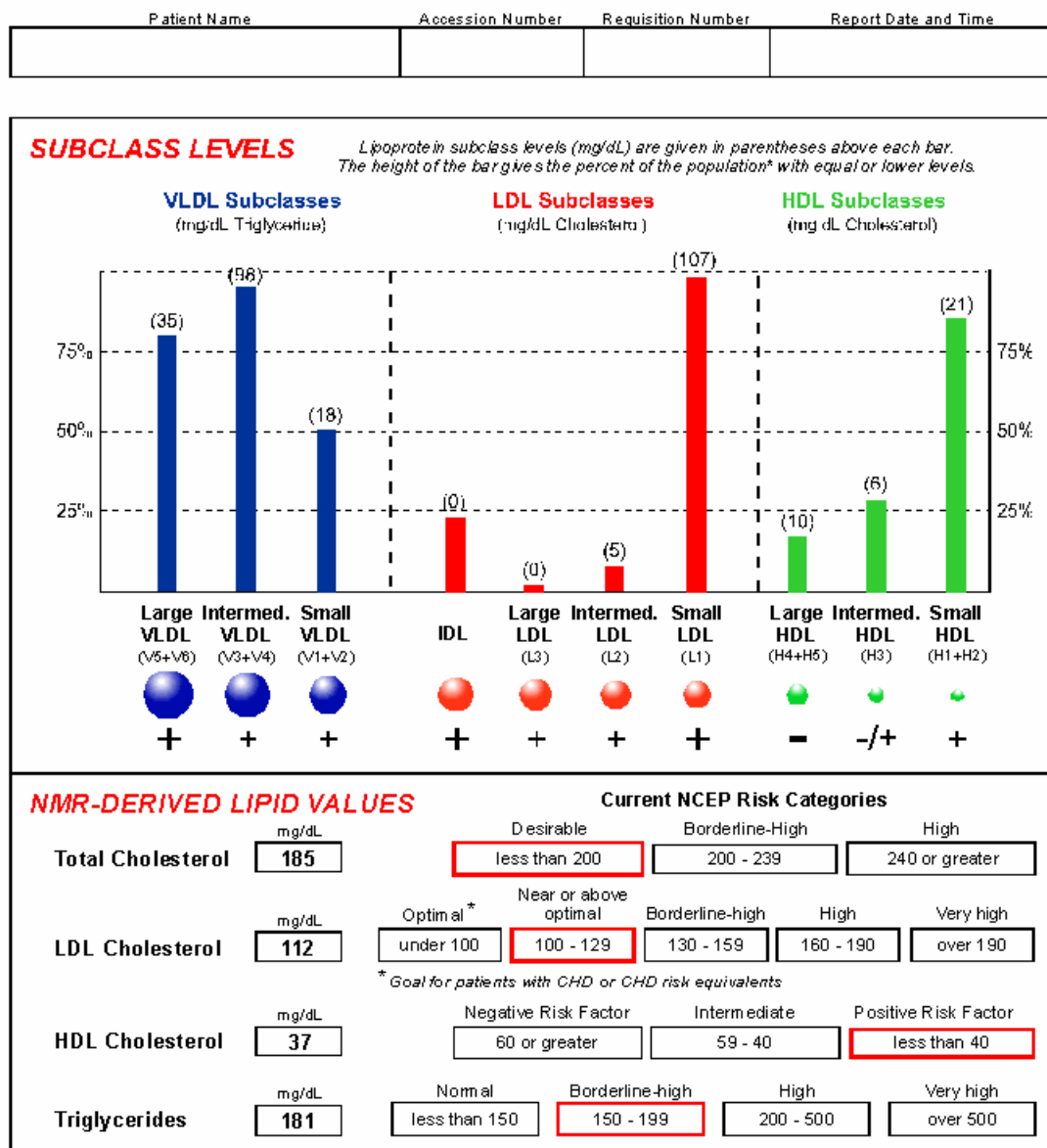


Figure 2.6. Sample of NMR LipoProfile assay report
NMR LipoProfile report consists of (A) a Lipoprotein Panel and Risk Assessment Panel, and, (B) bar graphs with Subclass Levels and a panel with NMR-Derived Lipid Values.

2.7 Correlation with chemical lipids

At the early stages of the development of the *NMR LipoProfile* test, it was considered important to determine correlations between NMR-derived and chemically-measured lipid values. Figure 2.7 shows correlation plots for HDL-C and LDL-C between the two methods for 118 fasting samples obtained from volunteers at NC State. To obtain gold standard LDL-C values, the plasma samples were fractionated by ultracentrifugation to remove VLDL (beta quant procedure) and the chemical LDL-C was calculated as the difference between the TC and HDL-C in the bottom fractions. The correlation coefficients for HDL-C and LDL-C were 0.96 and 0.94, respectively. For triglyceride, the correlation was 0.98. Studies providing further validation of the NMR lipoprotein assay are presented in Chapter 3.

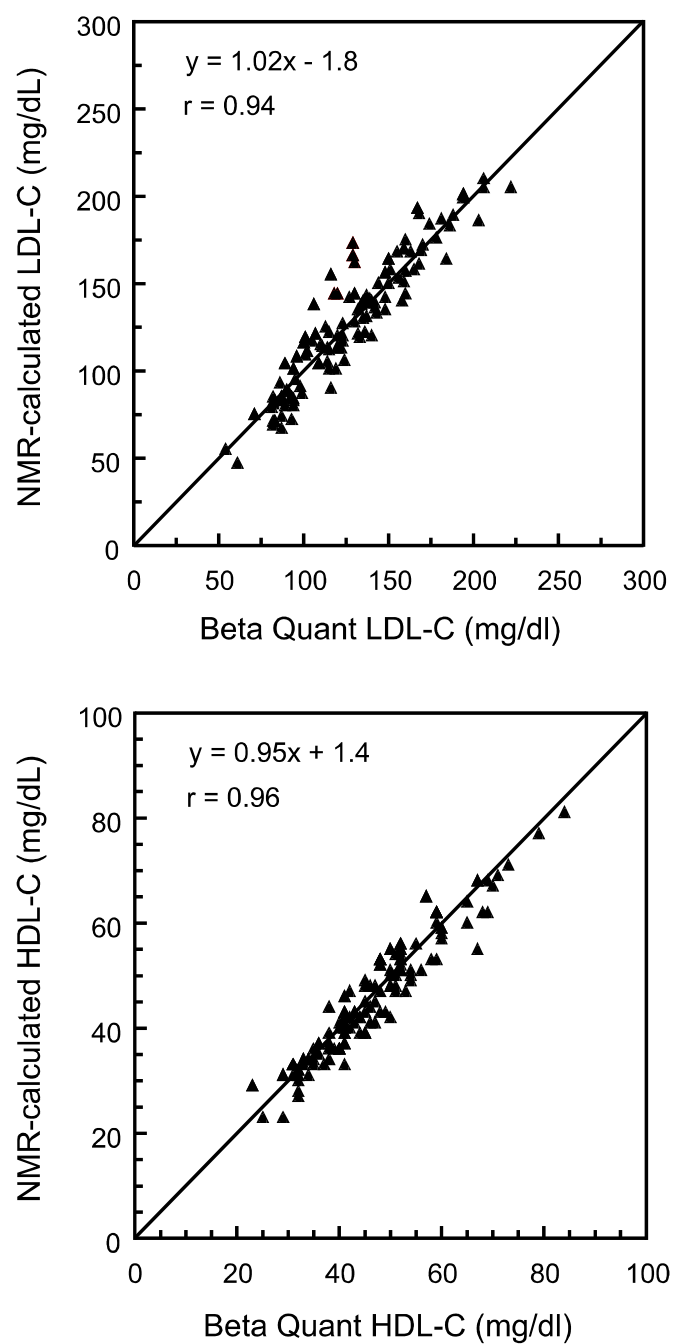


Figure 2.7 Relations of LDL-C and HDL-C between NMR and beta quantification. The graphs show the line of identity and measured correlation coefficient and regression equation. (To convert mg/dL to mmol/L, multiply by 0.0259).

Chapter 3

Analytical Characterization and Validation of an Automated NMR Spectroscopic Method for Quantifying Lipoprotein Subclass Particles

Elias J. Jeyarajah¹, Dennis W. Bennett³, Irina Shalaurova², Lawrence L. Rudel⁴, David L. Rainwater⁵, and James D. Otvos^{2*}

¹ Department of Chemistry, North Carolina State University, Raleigh, NC.

² LipoScience Inc., Raleigh, NC.

³ Department of Chemistry, University of Wisconsin, Milwaukee.

⁴ Department of Biochemistry, Wakeforest University School of Medicine, Winston Salem, NC.

⁵ Department of Genetics, Southwest Foundation for Biomedical Research, San Antonio, TX.

* Address for correspondence to this author at: LipoScience Inc., 2500 Sumner Blvd, Raleigh, NC 27616, USA.

(manuscript to be submitted to Clinical Chemistry)

Analytical Characterization and Validation of an Automated NMR Spectroscopic Method for Quantifying Lipoprotein Subclass Particles

Elias J. Jeyarajah¹, Dennis W. Bennett³, Irina Shalaurova², Lawrence L. Rudel⁴, David L. Rainwater⁵, and James D. Otvos^{2*}

¹ Department of Chemistry, North Carolina State University, Raleigh, NC.

² LipoScience Inc., Raleigh, NC.

³ Department of Chemistry, University of Wisconsin, Milwaukee.

⁴ Department of Biochemistry, Wakeforest University School of Medicine, Winston Salem, NC.

⁵ Department of Genetics, Southwest Foundation for Biomedical Research, San Antonio, TX.

* Address for correspondence to this author at: LipoScience Inc., 2500 Sumner Blvd, Raleigh, NC 27616, USA.

Background: The measurement of blood lipid levels has been central to the clinical prediction of coronary heart disease (CHD) for many years. Traditionally, plasma lipid and lipoprotein lipid concentrations have been measured chemically, but several recent studies have established that lipoprotein subclass particle concentrations measured by proton nuclear magnetic resonance (NMR) spectroscopy are a superior predictor of risk for CHD (1-6). The NMR lipoprotein assay directly measures the particle concentrations of lipoprotein subclasses of different size and, from this information, the average particle sizes of the major lipoprotein classes. A description of the NMR lipoprotein particle assay and the results of studies that provide analytic validation of the method are reported here.

Methods: Lipoprotein subclasses were isolated from human plasma using a combination of ultracentrifugation and agarose column chromatography. The purified subfractions were characterized by NMR, lipid analysis, gradient gel electrophoresis (GGE), and electron microscopy. The proton NMR spectra of plasma samples were deconvoluted as linear

combinations of the purified lipoprotein subclass spectra using non-negative least squares analysis.

Results: NMR-determined particle sizes correlated well with those estimated by GGE. Total LDL particle concentration measured by NMR was shown to be highly correlated with LDL apolipoprotein B levels measured by immunoassay. Standard addition tests established the linearity range and robustness of the assay. Intraassay and interassay precision data demonstrated that NMR-derived lipoprotein particle concentrations and sizes are reproducible. Normal ranges and intercorrelations for all of the NMR-determined lipoprotein parameters were determined.

Conclusion: The *NMR LipoProfile* assay provides for a robust, accurate and precise tool for the measurement of lipoprotein parameters important in the assessment of CHD risk. Particle concentrations of 10 lipoprotein subclasses can be rapidly generated with good analytical precision and accuracy.

Lipoproteins play a key role in the development of coronary heart disease (CHD). The assessment and management of CHD risk has been invariably tied to the measurement of cholesterol carried by lipoprotein particles, primarily due to the ease with which cholesterol can be measured using standard chemical assays. However, the total cholesterol in blood plasma has turned out to be an unreliable predictor of an individual's risk for CHD, and the need for measuring the very low density, low density and high density lipoprotein (VLDL, LDL and HDL) classes has emerged with the understanding that these major lipoprotein classes have differing associations with heart disease risk. For example, it has been established that while LDL and VLDL are positively associated with CHD, the risk

association is reversed for HDL, with higher levels of HDL affording protection against heart disease. Furthermore, the lipoprotein particles of different size (subclasses) that make up each major lipoprotein class appear to have different CHD risks associated with them. These observations have led to the motivation to develop an analytical tool to quantify VLDL, LDL and HDL subclasses in a more automated and efficient manner than provided by existing separation-based methods, which are relatively laborious and time-consuming.

Traditionally, lipoprotein subclass concentrations have been measured by separating the lipoprotein fractions either by density, typically using density gradient ultracentrifugation, or by size, using gradient-gel electrophoresis. Both methods are labor intensive and therefore relatively costly, and produce data of limited precision. Even measurement of the concentrations of the main lipoprotein classes by traditional methods is somewhat problematic analytically. Despite its clinical importance, the LDL cholesterol (LDL-C) concentration is difficult to measure chemically, and the most common method employed in most clinical laboratories is to estimate the LDL-C concentration using the Friedewald approximation (7), in which LDL-C is calculated from a measurement of total cholesterol (TC), HDL cholesterol (HDL-C) and total triglycerides (TG) in a fasting blood sample. The need for a fasting specimen, the requirement for TG to be under 400mg/dL, the heterogeneity of TG content of VLDL, and the cumulative error from the measurement of three different parameters all diminish the reliability of LDL-C derived from the Friedewald relationship. Despite these considerable analytical disadvantages, the National Cholesterol Education Program (NCEP) guidelines have made treatment goals for LDL-C measured in this way the centerpiece of national recommendations to reduce CHD risk in the U.S. population. Unfortunately, the uncertainty in LDL-C measurements has complicated these

guidelines to the extent that physicians are encouraged to consider non-HDL cholesterol levels (TC - HDL-C) as an alternate target for therapy (8).

In the early nineties we introduced a nuclear magnetic resonance (NMR) spectroscopic method as an alternative analytical tool for the measurement of lipoproteins in blood plasma and serum (9-11). This method enabled the rapid quantification of several lipoprotein classes and subclasses and determination of the average VLDL, LDL, and HDL particle sizes, all without requiring any physical separation of the different lipoproteins. The NMR method is based on the observation that the protons (hydrogen nuclei) in triglycerides, ~~and~~ cholesterol esters, and phospholipids experience differences in chemical shift which depend on the diameter of the lipoprotein particles in which they reside. These differences are attributable to variations in anisotropic magnetic susceptibility resulting from the orientational order of the phospholipid shells of different size that surround the neutral lipid core (12).

Deconvolution of the lipid methyl signal envelope of the plasma spectrum as a linear combination of the amplitudes of the methyl signals from a library of stored standard subclass spectra results in the determination of the quantitative contribution made by each subclass to the composite plasma spectrum. In order to perform this analysis, a representative library of VLDL, LDL, and HDL subclass standards needed to first be isolated and characterized chemically and physically, as well as by NMR analysis to provide the needed reference spectra for the computational deconvolution.

The lipoprotein subclass information from the NMR assay was made available for clinical use in the United States in 1999 in the form of the ***NMR LipoProfile®*** test report (11,14). Briefly, the report contains the following four sections: Lipoprotein Panel, Risk

Assessment Panel, Subclass Levels and NMR-Derived Lipid Values. The NMR-derived lipid values for TC, TG, LDL-C and HDL-C are computed from the total lipid values for a given subclass, based on the assumption that the normal triglyceride:cholesterol ratio of a particular subclass is invariant in the population, and are expressed in familiar mg/dL lipid units. The panel also highlights the risk status of the patient according to the NCEP guidelines. The Lipoprotein Panel section contains the emerging risk factors LDL particle number, large HDL and large VLDL subclass levels, and average LDL particle size with pattern A/B classification. The section of the report titled Subclass Levels gives the concentrations of large, medium and small VLDL, LDL and HDL along with IDL in a bar chart format, while also depicting the concentrations as percentiles of population data based on results from the Framingham Offspring Study. The Risk Assessment Panel section contains check boxes that identify the patient as having higher risk, based on the four parameters in the Lipoprotein Panel and the presence of lipoprotein traits associated with the metabolic syndrome or atherogenic dyslipidemia. In the last five years, physicians have ordered and obtained over one million *NMR LipoProfile* tests.

The NMR assay has been improved significantly from the time of its initial introduction. Instead of using four major lipoprotein subclass spectra that were digitally shifted to cover the span of size ranges from large VLDL to small HDL, more than 30 discrete purified subcomponents have now been included as a basis set. The exclusive use of real lipoprotein components isolated from normal and dyslipidemic subjects covering the lineshape and size heterogeneity of the general population has markedly improved the quality of the lineshape analysis and the accuracy of the NMR assay.

In this paper we report results which establish normal ranges for all lipoprotein subclass particle concentrations, since, as previously stated, they are the parameters which exhibit the highest predictive value for the diagnosis of CHD. In addition, we present data which shows a strong correlation between LDL particle concentration and LDL apoB concentration. Good agreement for LDL and HDL particle sizes between NMR and GGE is also established. The merits of using NMR-derived total triglycerides and HDL-C in place of chemically measured lipids is presented. Finally, the results of standard addition experiments and precision studies are described which demonstrate the analytical robustness of the NMR assay.

Materials and Methods

BLOOD SAMPLES

Blood was collected in commercially available evacuated tubes containing EDTA from healthy volunteers after 10 to 14-h fasting. For harvesting chylomicrons, blood was drawn 1 to 3-h after a fat load (15). Informed consents were obtained following protocols approved by the committee for protection of human subjects of the NC State University. Plasma was separated by centrifugation (2000xg, 15 min) and kept refrigerated at 4°C.

LIPOPROTEIN ISOLATIONS

Lipoprotein preparations were obtained by sequential ultracentrifugation as previously described (9,16). For the purposes of generating highly purified lipoprotein subfractions with very narrow size distributions, a combination of ultracentrifugation and agarose gel filtration chromatography were used following procedures modified from Rudel (17) and Sata (18).

Briefly, the d(1.006-1.225) kg/L plasma fraction loaded on A-15 agarose columns yielded baseline separations of LDL and HDL. The LDL- and HDL-containing tubes were pooled separately, concentrated and loaded back on A-15 or A-1.5 columns, respectively, for further purification of the LDL and HDL. In some cases the first half and second half of the LDL and HDL peaks were rechromatographed on their respective columns to achieve greater purity of the individual subfractions. The fractions eluting on either side of the main peaks were considered to have the greatest subclass homogeneity (Figure 1). Once isolated, the individual fractions were concentrated by ultrafiltration to 0.5-1mL and refrigerated prior to characterization by NMR, chemical lipid analysis, and gradient gel electrophoresis or electron microscopy.

CHEMICAL ANALYSIS

Chemical lipid analysis for TC, TG and HDL-C was performed enzymatically on a Bayer RA-1000 analyzer at the Lipid Analytic Laboratory at the Wake Forest University School of Medicine (Winston Salem, NC). All TG analyses were performed with glycerol blanking. Apolipoprotein B (apoB) measurements were conducted on a Beckman Synchron CX-7 (Beckman Coulter Inc., Fullerton, CA) analyzer using a commercially available turbidimetric immunoassay (Wako Chemicals, Osaka, Japan) (19).

GRADIENT GEL ELECTROPHORESIS

Nondenaturing gradient gel electrophoresis (GGE) was used to characterize isolated HDL subfractions at the laboratory of Dr. Larry Rudel at Wake Forest University School of Medicine (17, 20). Further GGE analyses of plasma samples and LDL and HDL preparations

for the standard additions study were performed in Dr. David Rainwater's laboratory at the Southwest Foundation for Biomedical Research (San Antonio, TX) (21, 22, 23).

ELECTRON MICROSCOPY

Lipoprotein particle size measurements of purified VLDL and LDL subclasses were determined at the Center for Electron Microscopy at North Carolina State University using a JEOL 100S (Peabody, MA) transmission electron microscope. Samples were prepared following negative staining procedures described by Forte (24). Mean diameters were derived by counting at least 200 lipoprotein particles measured on two or more grids.

NMR SPECTROSCOPY

Proton NMR spectra of all plasma samples and isolated lipoprotein subclasses were acquired on 400 MHz Avance spectrometers (Bruker Bio-Spin, Billerica, MA) at the CLIA approved facilities of LipoScience Inc., (Raleigh, NC). The NMR system was supplied with a special INCA (integrated NMR chemical analyzer) enclosure consisting of an actively shielded magnet, an automatic Gilson-215 (Madison, WI) sample handler and a flow probe with a 120uL active volume. All NMR measurements for lipoprotein analyses were performed at 47⁰C; the flow path was heated to minimize the time needed for temperature equilibration of the sample inside the probe. Automatic sample handling methods and procedures developed in-house function in tandem with Bruker's ICON NMR software module. Sample preparations were executed automatically by a Tecan Genesis RSP-100 (Tecan US, RTP, NC) aliquotting station.

NMR acquisition conditions were similar to previous descriptions (9, 10, 25). For routine *NMR LipoProfile* analysis of plasma and serum samples NMR data were acquired unlocked in 5 blocks of two scans each. For isolated lipoprotein components with relatively low lipid content, samples were run locked acquiring 16-128 scans in a single block. The methyl peak of a trimethyl acetate standard (TMA) was integrated and the area used to correct for day-to-day variations in spectrometer sensitivity and to normalize the results of multiple spectrometers and flow probes. Aliquots of two levels of serum control material were analyzed in the automation mode under identical conditions to that of the analysis of the test samples. The serum control materials were purchased from Soloman Park Research Laboratories (Kirkland, WA) where the two pools were prepared encompassing high and low ranges of lipid levels. The *NMR LipoProfile* results obtained for the serum controls were the input for Westgard's multirule quality control procedures (26, 27).

DECONVOLUTION

The lipid methyl region, from 0.914 to 0.718 ppm, was the spectral region employed for deconvolution of the experimental NMR lineshape. The experimental spectrum was modeled as a linear combination of over 30 lipoprotein subcomponent spectra obtained as described above. Contribution of each subcomponent NMR lineshape in the basis set to the lineshape of the experimental spectrum was determined by singular value decomposition (28, 29, 30), constrained so that concentrations could not be less than zero.

Previously, 16 lipoprotein subcomponents consisting of chylomicrons, 6 VLDL (V1-V6), IDL, 3 LDL (L1-L3), and 5 HDL (H1-H5) were used as reference spectra. In the current embodiment (*NMR LipoProfile-II*) the number of subcomponents has been expanded to more

than 30 to give a better representation of the continuum of particle subspecies present in plasma. By appropriate grouping and summation of the levels of the expanded subcomponent set, concentrations for 10 lipoprotein subclasses (large, medium, and small VLDL, LDL, and HDL, plus IDL) are obtained. The diameter ranges for the new grouping of subclasses are given in Table 1.

To relate the measured subclass signal amplitudes to subclass particle concentrations (numbers of particles per unit volume, expressed as moles of particles per liter), a set of conversion factors was derived using the calculated particle concentration of each lipoprotein subclass standard whose methyl NMR signal is part of the spectral deconvolution library. As described earlier, when each of these subclass standard samples was isolated, independent determinations were made of particle size distribution and lipid composition. The particle concentration of each subclass sample was calculated by taking the measured concentration of core lipid (cholesterol ester plus triglyceride) and dividing the volume occupied by these lipids (based on their partial specific volumes) by the volume of the subclass particle core, as estimated from the measured mean subclass particle diameter. These calculations make use of existing knowledge about the commonality of lipoprotein structure linking particle diameter to lipid content (13, 33), and hence to the expected amplitude of the NMR signal emitted by the lipid methyl groups of each subclass particle.

STANDARD ADDITION STUDIES

Plasma samples were spiked with varying amounts of purified subclass preparations of large and small VLDL, LDL and HDL. The sizes of the large and small LDL and HDL were independently characterized by GGE. The elution profile from a size exclusion agarose A-50

column was used to select a predominantly larger or smaller size dispersion of VLDL subclasses. NMR analysis was done in the usual manner.

Results

LIPOPROTEIN CHARACTERIZATION

The lipoprotein components that form the basis set for the *NMR LipoProfile* analysis were painstakingly isolated and characterized by physical and chemical methods including NMR spectroscopy, electron microscopy, nondenaturing polyacrylamide gradient gel electrophoresis and chemical lipid analysis. Figure 1 shows the gel filtration profile of a typical isolation of LDL and HDL, from a plasma sample from which VLDL ($d < 1.006$ kg/L) and plasma proteins ($d > 1.225$ kg/L) had first been removed by 2 preparative ultracentrifugation separations. The LDL and HDL were well separated on the A-15 agarose gel column (Figure 1A). The fractions containing LDL were pooled, concentrated and re-chromatographed on the A-15 column, further purifying the LDL. The fractions eluting on either side of the main peaks were individually retained for further characterization, since they contain a relatively narrow size distribution of subclass particles. (Figure 1B).

The mean particle diameters of the purified subcomponents of VLDL and LDL subclasses were measured by electron microscopy. Representative electron micrographs of isolated samples containing predominantly medium-size VLDL, small VLDL and large LDL subclasses are shown in Figure 2A. Mean diameters were derived by measuring the diameters of at least 200 lipoprotein particles. The measured frequency distribution of the particle diameters for these three subcomponents are illustrated in Figure 2B. The mean particle

diameters were 40.2 ± 6.6 nm, 30.6 ± 3.5 nm and 22.6 ± 2.5 nm for the medium VLDL, small VLDL and large LDL, respectively.

NMR analyses were done on the purified subfractions as described earlier. The relationship between the lipoprotein particle diameters as determined by electron microscopy or gradient gel electrophoresis and proton NMR chemical shifts (line position) is depicted in Figure 3. The relative shifts (compared to smallest HDL) of lipoprotein subfractions of VLDL (V6-V1), IDL, LDL (L3-L1), and HDL (H5-H1) subclasses increase as a function of particle diameter. Signals from larger particles appear downfield (higher frequency) from those of smaller particles. The relationship also illustrates that the resolving power of NMR is greater for the VLDL and HDL subclasses than for the LDL subclasses.

NMR spectra of the fully characterized lipoprotein subfractions were then incorporated into the spectroscopic analysis model for determination of lipoprotein subclass concentrations from spectra of whole plasma. The diameter ranges for lipoprotein subclasses measured by *NMR LipoProfile* analysis are given in Table 1. The ten subclasses of lipoproteins that can be quantified with acceptable precision by *NMR LipoProfile-II* analysis are: large, medium and small VLDL, IDL, large, medium-small and very-small LDL, and large, medium and small HDL. The NMR spectra of the methyl region of representative lipoprotein subclasses that form the basis of the *NMR LipoProfile-II* analysis model are depicted in Figure 4. The small but distinct differences in both the lineshape and chemical shift going from the larger to the smaller subclasses are easily discernable.

FIGURE 1

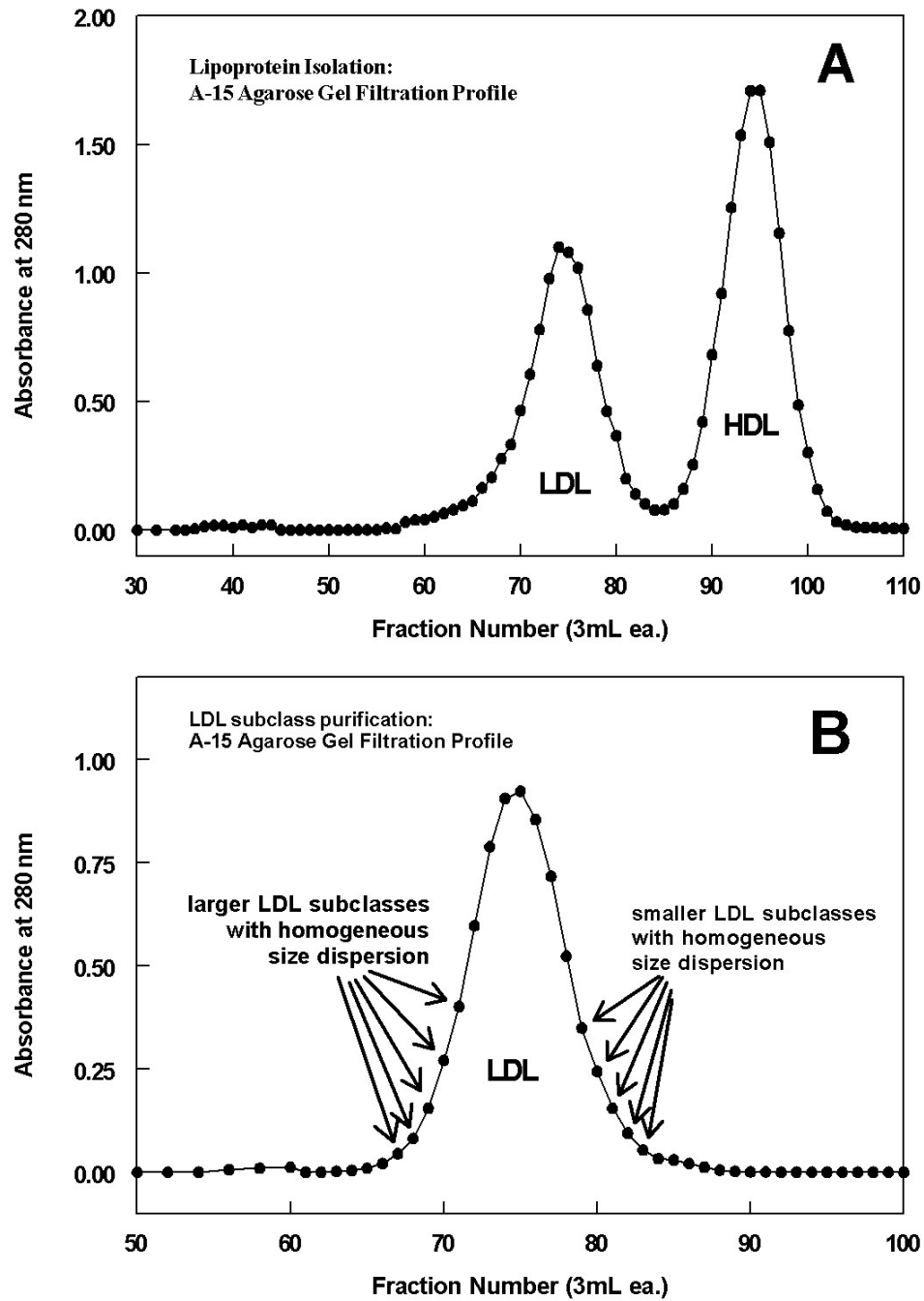
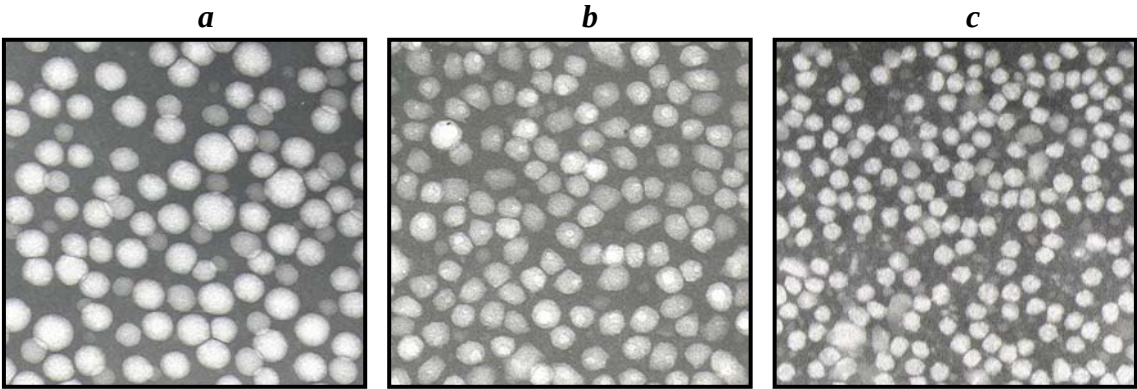


Figure 1. Lipoprotein purification using agarose gel filtration.

(A) Agarose gel filtration profile on a A-15 column of the density 1.006-1.225kg/L fraction of a plasma (30mL) consisting of LDL and HDL. The VLDL and plasma proteins were removed beforehand by ultracentrifugation. The absorbance at 280 nm was plotted for successive 3 mL fractions eluting from the column. **(B)** Purification of LDL subclass components on A-15 column. Fractions 60-81 from **A** were pooled, concentrated and re-loaded on the A-15 column. Subclasses with homogeneous size dispersions appearing on the wings of the major peak were individually concentrated and characterized by NMR. The HDL fractions were further purified on a A-1.5 column in a similar way (profile not shown).

FIGURE 2

A



B

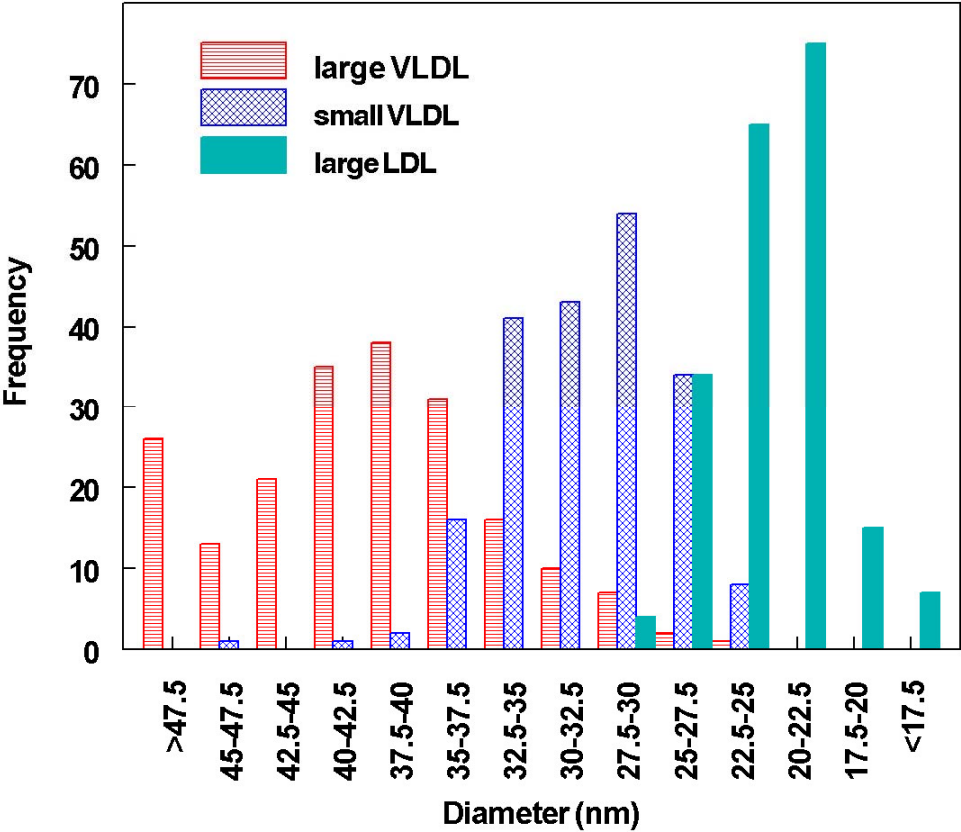


Figure 2. Electron microscopy for three purified lipoprotein components.

(A) Transmission electron micrographs (JEOL 100S) of three lipoprotein components separated and purified by combination of ultracentrifugation and agarose column chromatography: **a** medium VLDL, **b** small VLDL, **c** large LDL. Samples were diluted with saline to approx. 1mg/mL and applied on 200-mesh Formvar-carbon-coated grids and stained with 2% sodium phosphotungstic acid (PTA). Total magnification x135,000.

(B) Frequency distribution as a function of particle diameter for the medium VLDL, small VLDL and large LDL particles pictured in figure 2 A. Diameters on the photographs were measured using a HiPad Digitizer counting 200 particles to generate the frequency distribution and mean diameters. The particle diameters (mean $\pm$ SD) for the three lipoprotein components were 40.2 ± 6.6 nm for the medium VLDL, 30.6 ± 3.5 nm for the small VLDL, and 22.6 ± 2.5 nm for the large LDL.

FIGURE 3

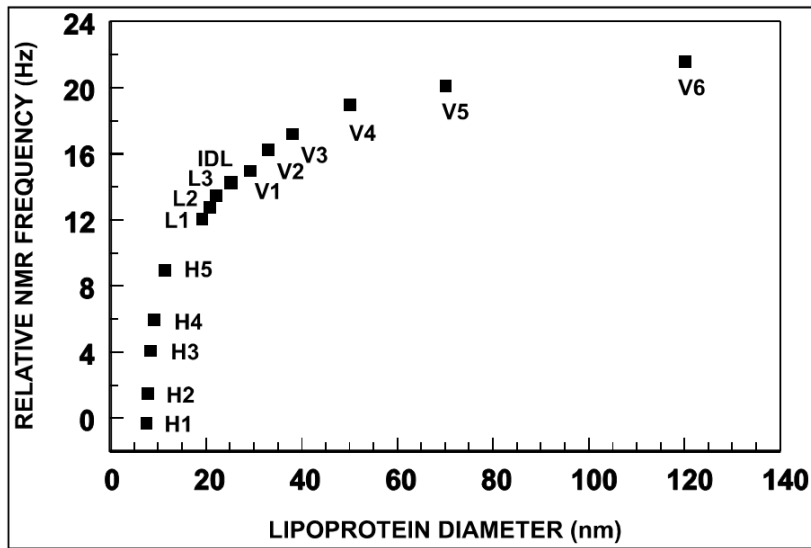


Figure 3. Relationship between lipoprotein particle diameter and relative NMR chemical shift.

Relative shift of proton NMR signals of purified lipoprotein subfractions are plotted against the lipoprotein particle diameters determined by electron microscopy or gradient gel electrophoresis. Signals from larger particles appear downfield (higher frequency) from those of smaller particles (H, L, and V designate subfractions of HDL, LDL, and VLDL, respectively).

FIGURE 4

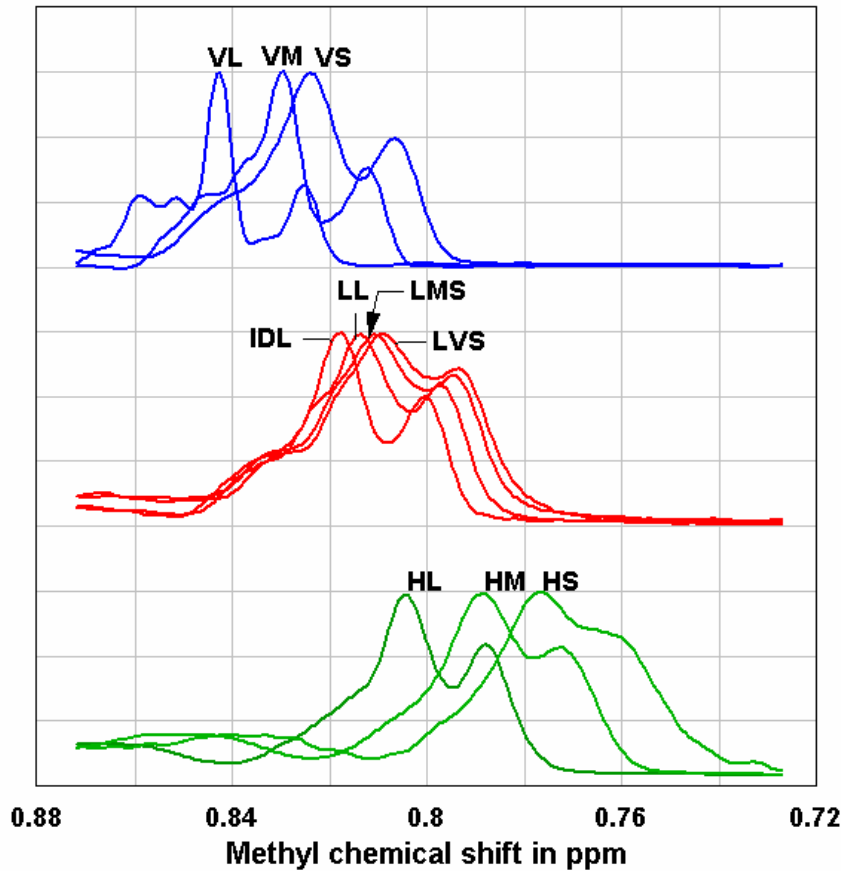


Figure 4. NMR spectra of purified lipoprotein components in the LipoProfile-II analysis model.

^1H NMR (400MHz) spectra were recorded for purified lipoprotein components at 47°C. The methyl resonance signal is plotted here for 10 lipoprotein subcomponents representative of the basis set in use. The VLDL subclasses (top, in blue), LDL subclasses (middle, in red) and HDL subclasses (bottom, in green) are plotted with vertical offsets between the 3 major subclasses for easy viewing. VL=large VLDL, VM=medium VLDL, VS=small VLDL, IDL=intermediate density lipoproteins, LL=large LDL, LMS=medium-small LDL, LVS=very-small LDL, HL=large HDL, HM=medium HDL and HS=small HDL.

**Table 1: Diameter Ranges for Lipoprotein Subclasses
Measured by NMR**

<u>NMR Lipoprotein Parameter</u>	<u>Diameter Range (nm)</u>
VLDL	
Large VLDL/Chylos	>60
Medium VLDL	35-60
Small VLDL	27-35
LDL	
IDL	23-27
Large LDL	21.2-23
Small LDL	18-21.2
Medium-Small LDL	19.8-21.2
Very-Small LDL	18-19.8
HDL	
Large HDL	8.8-13
Medium HDL	8.2-8.8
Small HDL	7.3-8.2

NMR SIGNAL AREA AND LIPID MASS CONCENTRATIONS

The proton NMR measurement of plasma culminates in a direct quantification technique for the total lipid mass concentrations. The CH₃ (methyl) region of the NMR spectra of lipoproteins consists of signals emanating from the terminal CH₃ protons of triglycerides and cholesteryl esters (CE) of the core and the phospholipids of the shell. Figure 5 demonstrates the relationship of NMR peak area and chemically determined lipid mass concentrations. A total of 14 LDL samples were isolated from subjects with a wide range of TC/TG ratios (2.7 – 8.7) for which the cholesterol and TG concentrations were measured chemically. The methyl NMR signal area correlates well with cholesterol concentrations, $r=0.967$ (Figure 5A). The correlation further improved significantly to 0.995 when the total lipids (TC+TG)

measured in the isolated LDL fractions were plotted against the NMR signal area (Figure 5B), underlining the capacity of NMR to quantify the total number of CH₃ protons encapsulated in the lipoprotein particle regardless of its lipid composition.

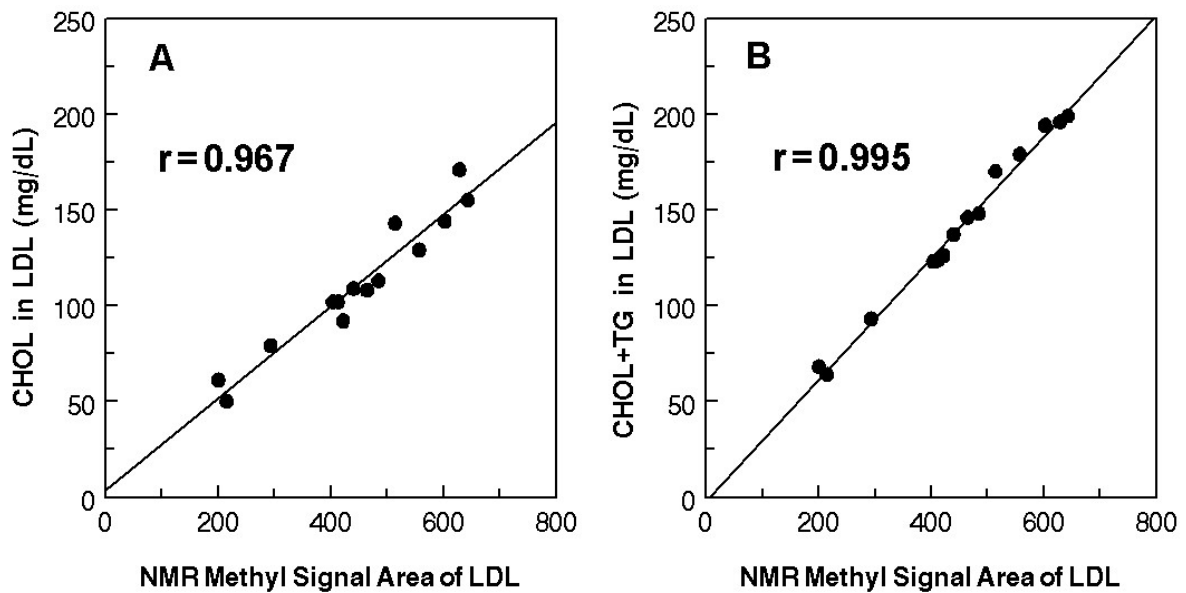


Figure 5. Correlation of NMR signal area to chemical lipids for LDL samples. LDL samples (n=14) isolated by sequential ultracentrifugation, d(1.006-1.063)kg/L, were subjected to NMR and chemical lipid analysis. **(A)** NMR methyl signal areas show linear relationship to the cholesterol carried by LDL, $r = 0.967$. **(B)** NMR methyl signal areas correlate even stronger with the total lipid mass (CHOL + TG), $r = 0.995$.

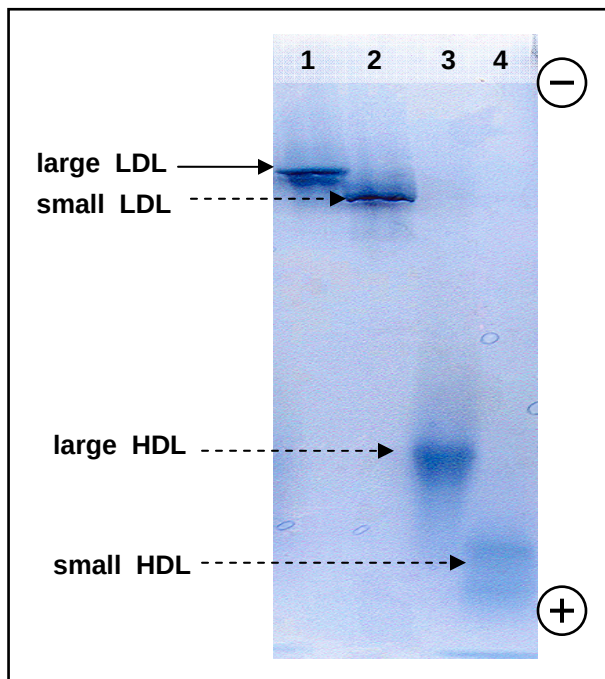
STANDARD ADDITION STUDIES: SPECIFICITY AND LINEARITY OF RESPONSE

As a way of assessing the accuracy of the *NMR LipoProfile-II* analysis results in quantifying lipoprotein levels and size distributions, standard addition studies were conducted where plasma samples were spiked with known amounts of purified lipoprotein subclasses. Purified lipoprotein preparations that consisted of predominantly large or small subclasses of VLDL, LDL or HDL were quantitatively added to plasma, and *NMR LipoProfile-II* analysis was

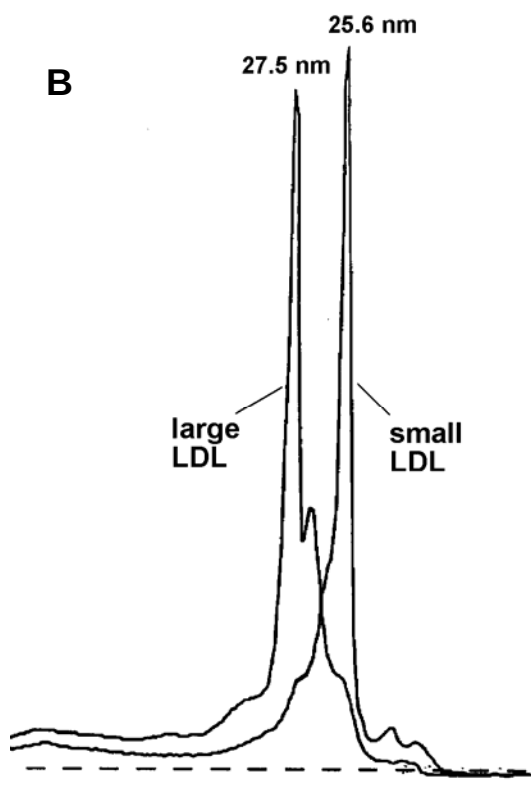
carried out. The large and small LDL and HDL size preparations were characterized by gradient gel electrophoresis, as depicted in Figure 6.

FIGURE 6

A



B



C

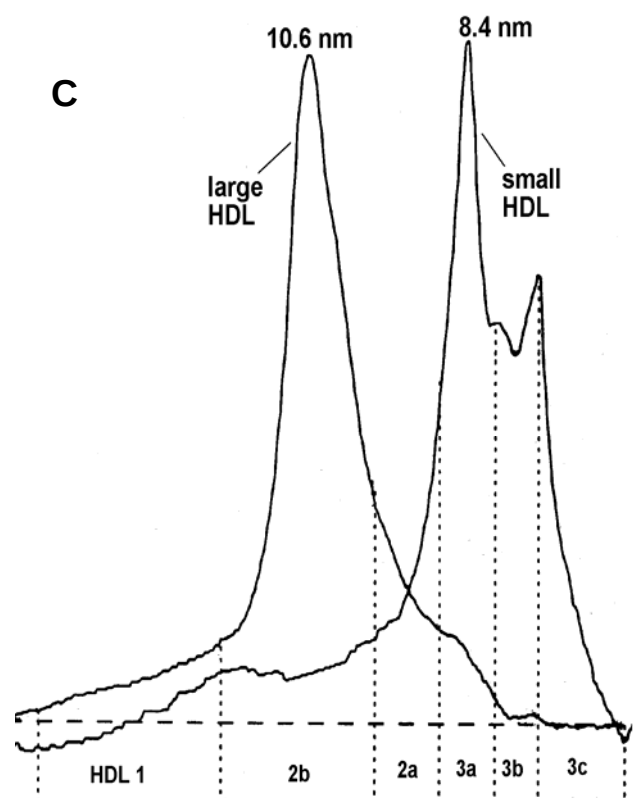


Figure 6. GGE analysis of large and small LDL and HDL used for spiking studies. **(A)** Non-denaturing polyacrylamide gradient gel electrophoresis (GGE) scan. Twelve microliters of isolated large LDL, small LDL, large HDL and small HDL (lanes 1,2,3,4 respectively) were applied on to a 3-31% gradient gel. After electrophoresis the gel was stained for lipid with Sudan black B. **(B)** Laser densitometer scans of the large and small LDL bands in lanes 1 and 2 showing a predominantly large (27.5 nm) and predominantly small (25.6 nm) LDL fractions. **(C)** Densitometry scans of HDL bands in lanes 3 and 4 show large (10.6 nm) and small (8.4 nm) HDL fractions.

The large and small lipoprotein subclasses were isolated from plasma of two donors known to have either large or small subclasses, respectively. A combination of ultracentrifugation and agarose column separations were used as described in the methods. The gel and densitometer scans in figure 6 clearly show the size separation between the two LDL fractions (27.5 nm vs 25.6 nm) and similarly between the two HDL preparations (10.6 nm vs 8.4 nm). There is, however, some overlap between the particles present in the large and small lipoprotein subclass stock solutions, in particular for the LDL sample (fig. 6B). The NMR-determined average particle sizes for the stock subclass samples were 21.4 nm and 19.5 nm for the large and small LDL samples, and 10.7 nm and 8.2 nm for the large and small HDL samples, respectively. The LDL particle sizes determined using electron microscopy are consistently measured to be 5 to 6 nm smaller than when they are calibrated with GGE analysis (31, 32, 33). The agreement between average particle sizes for isolated LDL and HDL preparations determined by NMR and GGE (Rainwater's lab) is excellent. The two VLDL preparations used in the spiking study were characterized as consisting predominantly of large and small particles by their separation by agarose gel column chromatography, as well as by NMR. The NMR-determined particle size for the large VLDL was 70.0 nm, and 40.8 nm for the small VLDL. The small VLDL, while devoid of any large VLDL, contained a mix of medium and small VLDL particles, and as such the combined medium/small particle concentrations will be used as input for analysis of samples spiked with this VLDL stock.

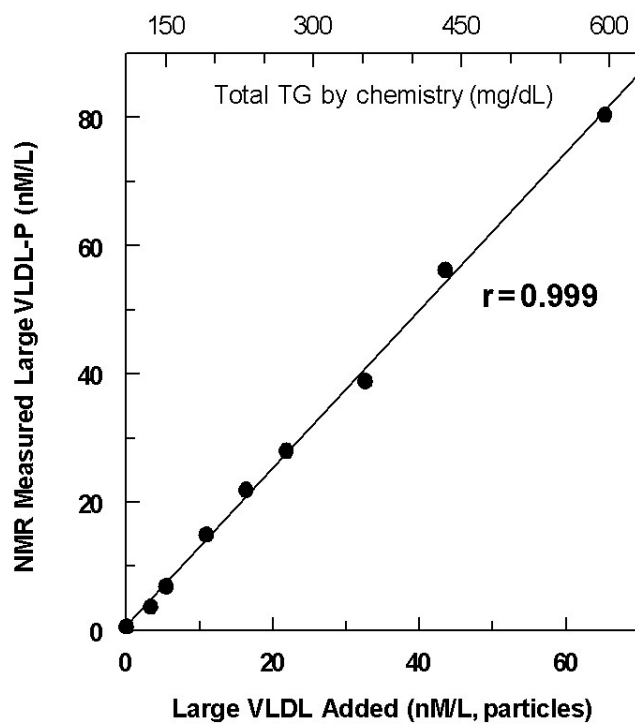
Fasting plasma was aliquoted and an increasing amount of large or small lipoprotein stock solutions were added along with plasma diluent buffer maintaining the total volume and original concentration of the experimental plasma. The matrix varied from pure plasma

to plasma mixed with varying amounts of a particular lipoprotein subclass to pure subclass. NMR analysis was done in the usual manner and chemical lipid analysis for TC, TG, HDL-C and direct LDL-C was also performed. The particle concentrations determined by NMR analysis of the individual lipoprotein stock solutions were used to estimate the amount of lipoprotein particles added into the matrix.

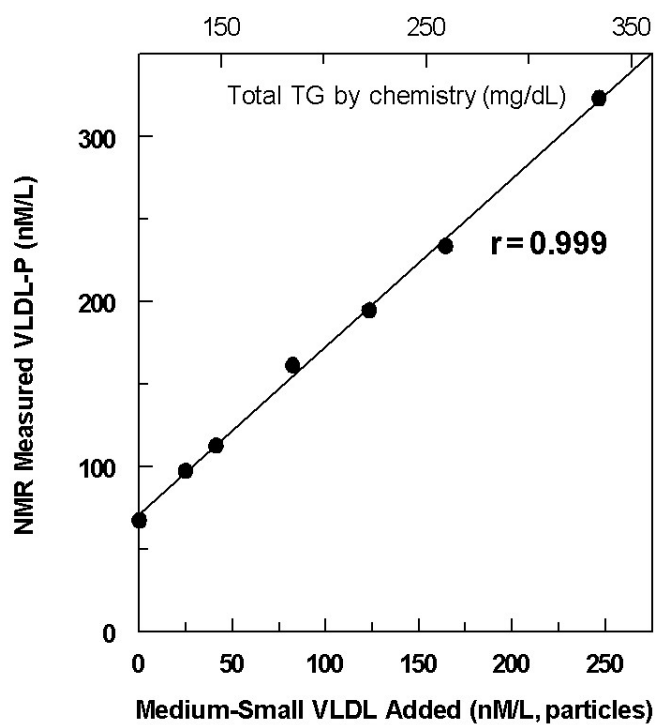
Figures 7A-D and 8A-B show the results of the standard addition experiments. The lipoprotein subclass being added is plotted on the bottom x axes in particle concentration units (nanomoles/L for VLDL and LDL, and micromoles/L for HDL). The NMR-measured total particle concentrations (VLDL-P, LDL-P and HDL-P) of the type of subclass added is plotted on the y axes. The plots exhibit excellent linearity for the *NMR Lipoprofile* assay over a wide range of lipoprotein concentrations. The correlations between the added lipoprotein subclass and the detected lipoprotein concentrations ranged from 0.994 to 0.999 (fig. 7A-D, fig. 8A, 8B). To give perspective for the linearity with respect to physiologically relevant ranges, the chemically-measured concentrations in mg/dL of the lipids are presented on the top x axes. For the standard additions of VLDL subclasses the total TG levels (fig. 7A, 7B), for LDL additions the LDL-C levels (fig. 7C, 7D), and for the HDL additions the HDL-C levels (fig. 8A, 8B) are given on the top x axes. For example, in fig. 7A, the addition of large VLDL particles (up to 65 nmol/L) established that the linearity is maintained at least up to 600 mg/dL of total triglyceride concentrations. Similarly, in figure 7C, the enrichment in small LDL particles up to 4000 nmol/L encompassing LDL cholesterol concentrations of at least 400 mg/dL did not affect the accuracy of the NMR assay.

FIGURE 7

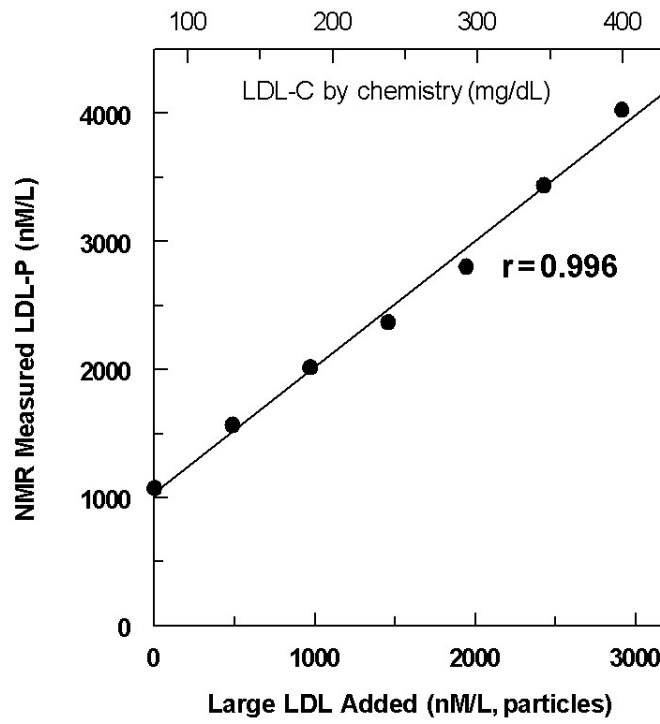
A



B



C



D

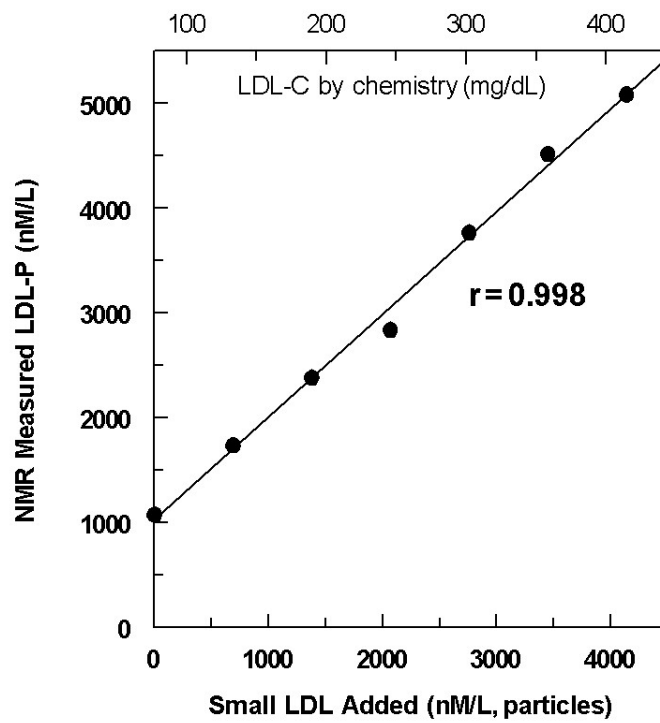


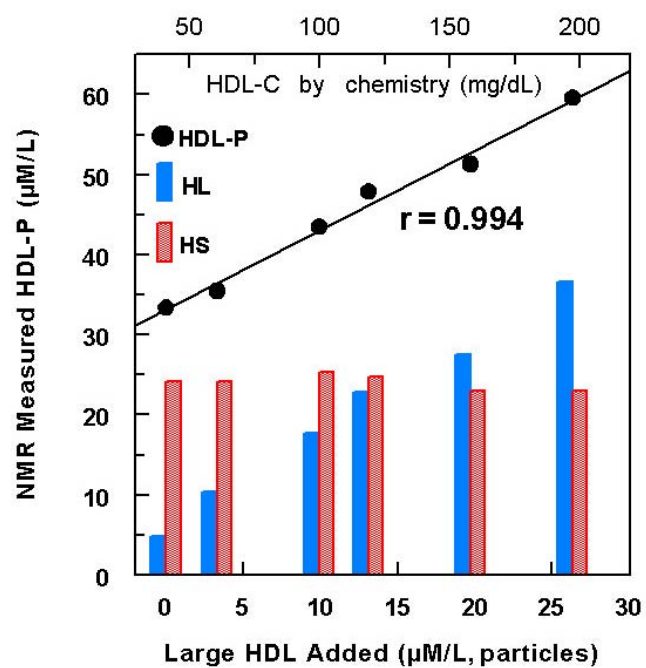
Figure 7. Standard addition plots – Spiking plasma with VLDL and LDL.

Plots of NMR determined subclass particle concentrations versus known concentrations of large and small size lipoprotein stock added to plasma: Addition of (A) large VLDL, (B) medium-small VLDL, (C) large LDL and (D) small LDL, all added particle concentrations on bottom x axes. The chemically determined TG (A, B) and LDL-C (C, D) are represented on the top x axes. The linear correlations of NMR measured lipoprotein concentrations to the concentrations of added lipoproteins are very strong with $r > 0.99$.

The bar graphs shown in the lower halves of figures 8A and 8B show the changes to the distribution of large and small HDL particle concentrations as increasing amounts of large HDL (fig. 8A) and small HDL (fig. 8B) are added to plasma. The first data point shows that the pure plasma before spiking has a relatively low concentration (4.8 $\mu\text{mol/L}$) of large HDL (HL, solid bar) and a significant amount (24.2 $\mu\text{mol/L}$) of small HDL (HS, cross-hatched bar). With the addition of large HDL, the NMR-detected large HDL concentrations steadily increase while the concentration of the small HDL is not altered significantly (fig. 8A). Similarly when the plasma is spiked with small HDL (fig. 8B) the NMR-measured small HDL increases linearly while the NMR-detected large HDL concentrations remain relatively constant. Again the top axis with chemical HDL-C concentrations shows that the NMR method's subclass specificity is maintained up to HDL-C concentrations of 200mg/dL, a good measure above the normal upper range of physiological levels.

FIGURE 8

A



B

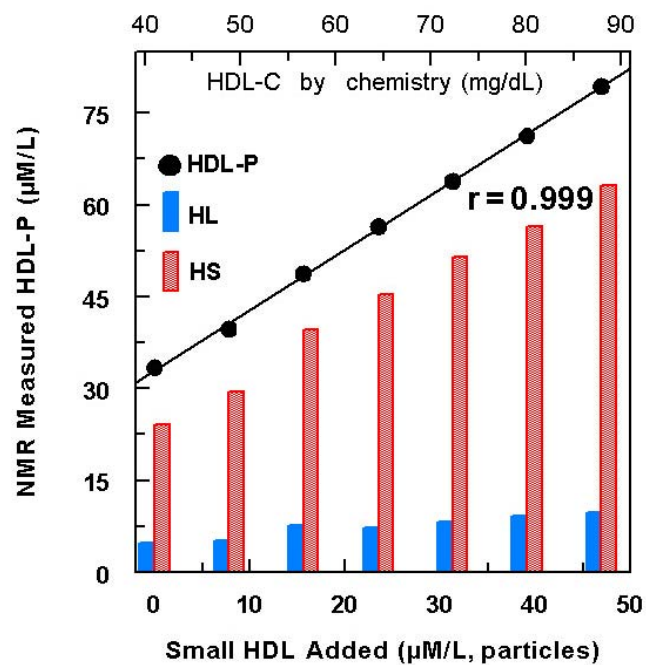


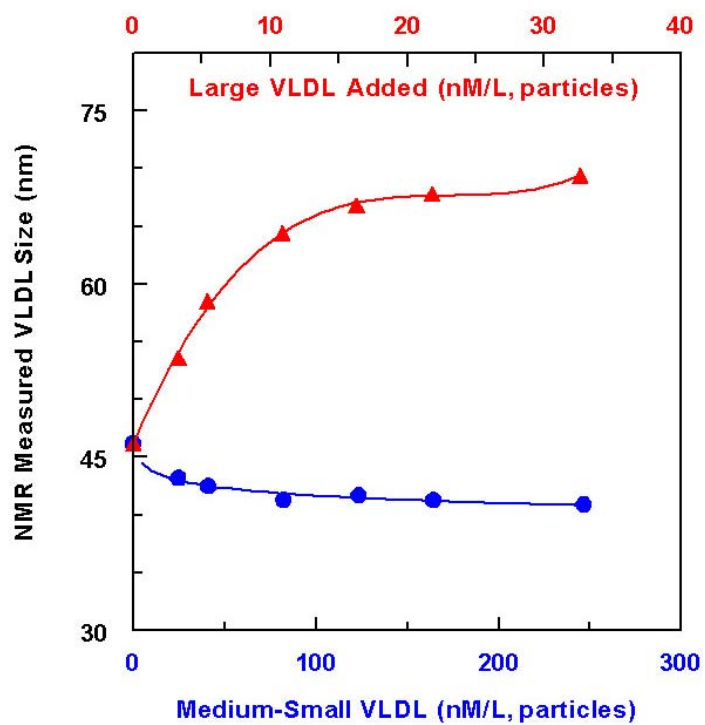
Figure 8. Standard addition plots – Spiking plasma with HDL.

Plots of NMR determined HDL subclass particle concentrations (HDL-P, ●) versus known concentrations of large (A) and small (B) HDL lipoproteins. The chemically determined HDL-C is represented on the top x axes. The correlations between NMR measured HDL-P concentrations and added HDL particles are very strong, $r > 0.99$. The bar graphs represent the distribution of large (HL, ■) and small (HS, ■) HDL particle concentrations. Concentrations of HL and HS change linearly when large or small HDL is added, or remain constant when the added fraction does not contain HL or HS attesting to the specificity of the NMR LipoProfile test.

The qualitative accuracy of NMR-determined average particle sizes for VLDL, LDL and HDL subclasses is demonstrated in figure 9A-B. In figure 9A, NMR-measured VLDL size is plotted against the quantity of large VLDL added (upper curve and top x axis) and against the amount of medium-small VLDL added to the plasma (lower curve, bottom x axis). The progressive addition of large VLDL increases the average VLDL particle size from 46.2 nm to 69.4 nm, approaching the size (70.0 nm) of the large VLDL stock (fig. 9A, upper). Similarly, when medium-small VLDL is added, the average VLDL particle size of the plasma mixture decreases from 46.2 nm to 40.9 nm, approaching the size of 40.8 nm determined for the small VLDL stock (fig. 9A, lower). Figure 9B illustrates the effect of LDL and HDL particle size as large LDL and large HDL were added to plasma. As expected the NMR-determined LDL particle size increases from 20.2 nm to 21.5 nm (fig. 9B upper curve) upon addition of large LDL. A similar trend is observed when large HDL is added (fig. 9B, lower curve) where the average particle size for HDL increase from 8.7 nm to 10.5 nm.

FIGURE 9

A



B

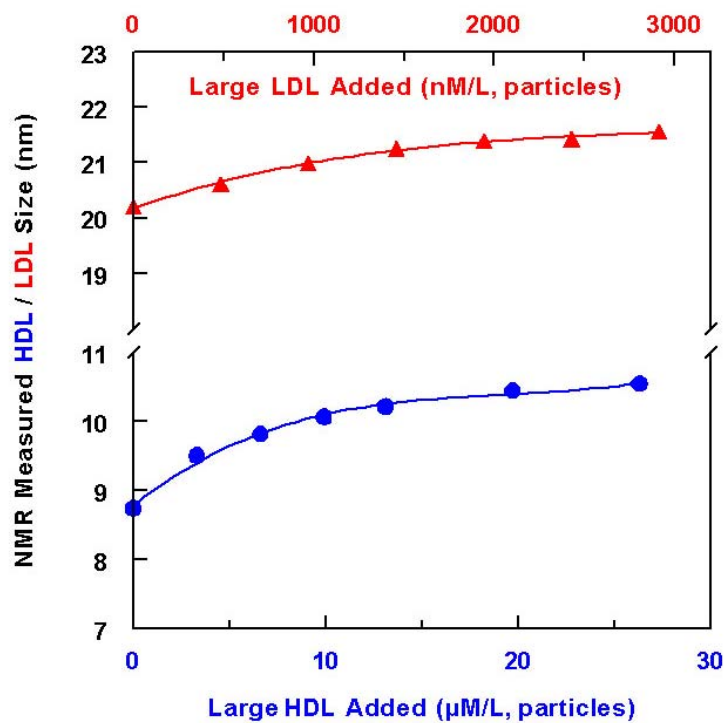


Figure 9. NMR response to size perturbations induced by spiking.

(A) Effect of adding large (▲ top curve, top x axis) or small (● lower curve, bottom x axis) VLDL to plasma results in increase or decrease, respectively, of NMR determined particle size of VLDL as anticipated. (B) Spiking with large LDL (▲ top curve, top x axis) or large HDL (● lower curve, bottom x axis) results in progressive increase of NMR determined average particle sizes for LDL and HDL subclasses, conforming to the expected trend.

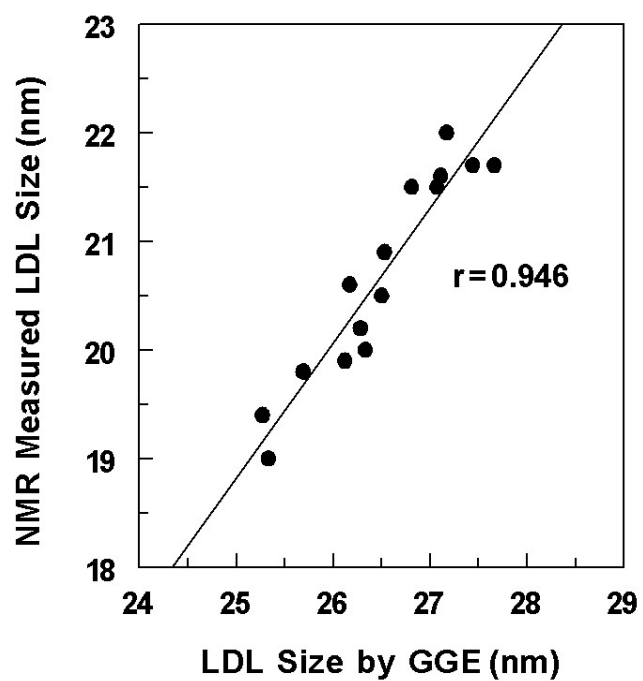
COMPARISON OF LDL AND HDL PARTICLE SIZE DISTRIBUTIONS DETERMINED BY NMR AND GGE

The *NMR LipoProfile* analysis of plasma generates average particle sizes for the lipoprotein subclasses of VLDL, LDL and HDL. The particle sizes are reported in units of nm diameter. The LDL size in particular as measured originally by GGE is considered to aid in the clinical diagnosis of CHD risk. We conducted a comparison study of LDL and HDL size determined by NMR and GGE. Fifteen fasting samples consisting of 8 serum and 7 plasma specimens were analyzed by NMR in the usual manner and another aliquot was sent to Dr. Rainwater's lab (Southwest Foundation for Biomedical Research, San Antonio, TX) for GGE analysis as described in the methods section (21). The GGE analysis was performed in duplicate and the values were averaged. The comparison between NMR- and GGE-determined sizes for LDL and HDL is given in Figures 10A and 10B.

The LDL size correlation between the two methods is very good with $r = 0.946$ (fig. 10A). The NMR-derived LDL sizes, however, are uniformly lower than the GGE numbers. The NMR particle sizes were originally calibrated against particle diameters measured by electron microscopy. The fairly constant differences of 5-6 nm between electron microscopy and GGE for LDL samples has been noted before (31-33). The mean values for the 15 samples are 20.69 nm for NMR and 26.50 nm for GGE. The NMR-determined HDL sizes correlate extremely well with GGE sizes, with $r = 0.953$ (fig. 10B). The mean values for HDL sizes for the two methods are nearly identical at 8.99 nm by NMR and 9.03 nm by GGE.

FIGURE 10

A



B

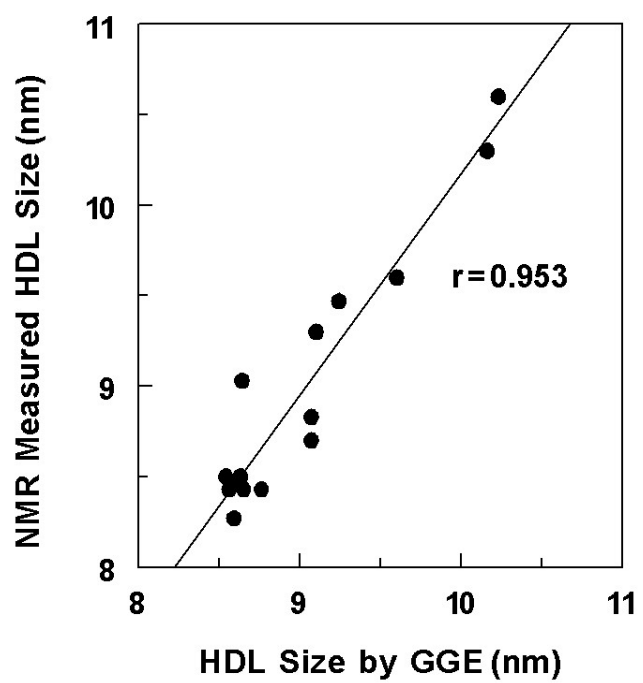


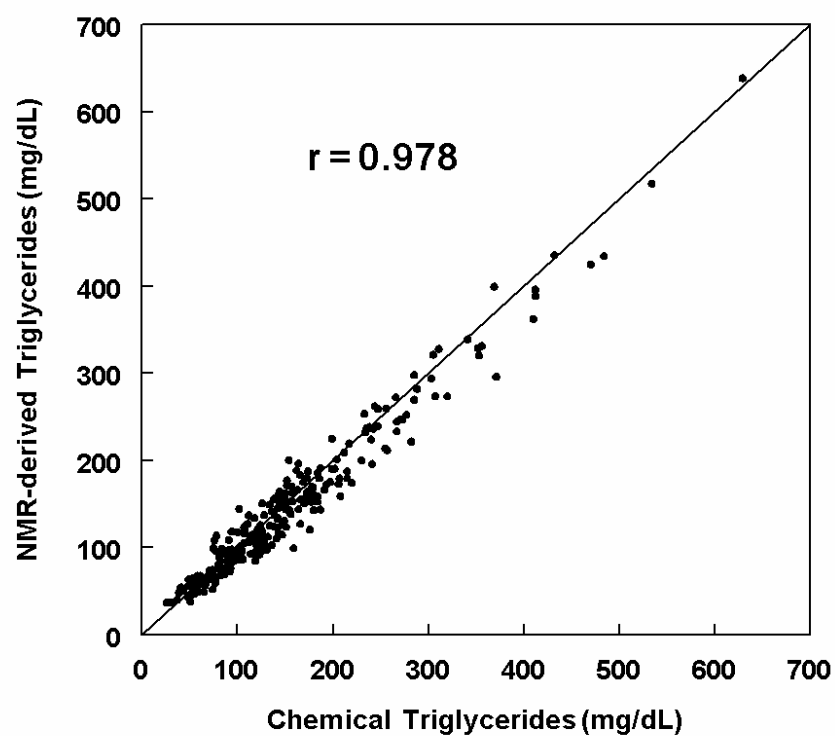
Figure 10. Comparison of NMR and GGE sizes for LDL and HDL. Average particle sizes determined by NMR and median particle diameter measured by GGE correlate very well. **(A)** $r = 0.946$ for LDL, and **(B)** $r = 0.953$ for HDL. $N=15$.

CORRELATION WITH CHEMICALLY MEASURED TG AND HDL-C

We have reported previously that the NMR-derived total triglycerides and HDL cholesterol values correlate well with chemically measured TG and HDL-C for samples from normal healthy volunteers (11, 14). In the current study it was extended to patient population consisting of a mix of primary prevention patients without coronary disease and secondary prevention patients with disease. A total of 255 patient samples received at LipoScience were analyzed on the same day across 11 different NMR analyzers and also subjected to chemical analysis for TG and HDL-C (homogeneous assay). Two samples with TG>1200 mg/dL were excluded. The correlation plots for NMR-derived and chemically determined triglycerides and HDL-C is given in Figure 11. The correlation for total triglycerides between the two methods is excellent with $r=0.978$ (fig. 11A). Similarly good correlation is obtained for HDL-C with $r=0.959$ (fig. 11B).

FIGURE 11

A



B

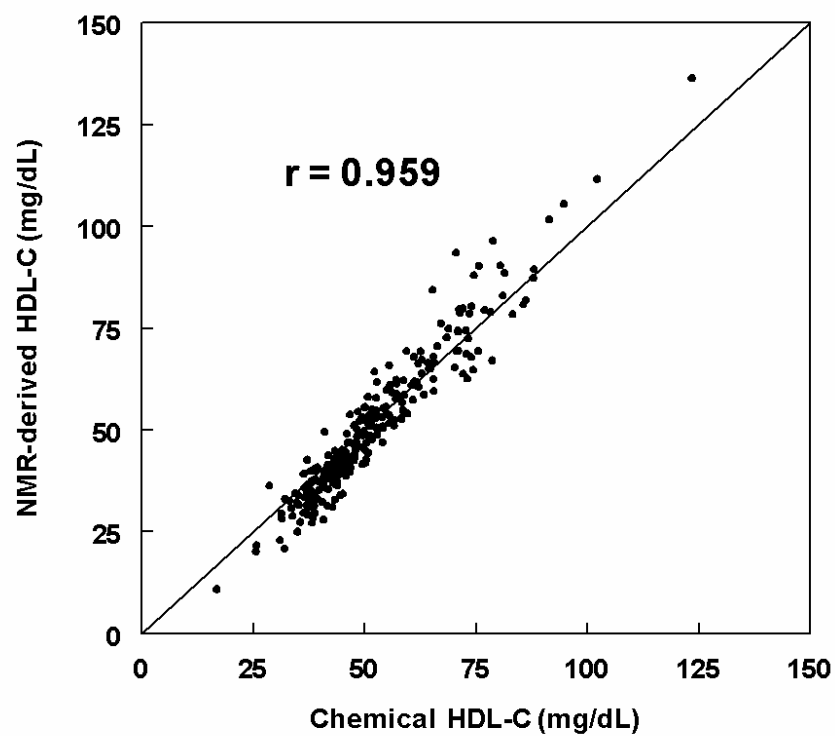


Figure 11. Comparison of NMR-derived lipids to chemical lipids for TG and HDL-C. Chemically determined total triglycerides and HDL-C (homogeneous assay) correlate very well with NMR-derived TG and HDL-C. Line drawn is line of identity. N=253. **(A)** $r = 0.978$ for TG; regression line $y=0.94x+2.7$, and **(B)** $r = 0.959$ for HDL-C; regression line $y=1.16x-9.3$.

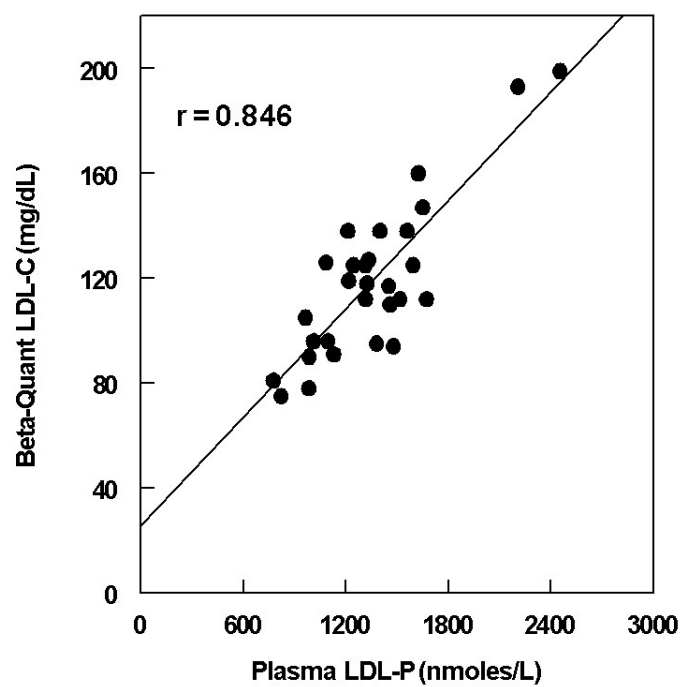
LDL PARTICLE CONCENTRATION AND APO-B

The LDL particle concentration expressed in nanomole per liter units is perhaps the most clinically useful of all the information provided by the *NMR LipoProfile* test, based on prospective CHD outcome studies. Since each LDL particle is known to have one apoB molecule, the LDL particle concentration might be expected to correlate well with LDL apoB level. However, in common clinical practice it is the plasma apoB level that is usually measured, to avoid the ultracentrifugation step to remove VLDL. Though there is also one apoB molecule on every VLDL particle, plasma apoB is considered to give a reasonably good approximation of LDL particle numbers since LDL apoB always comprises at least 90% of total plasma apoB (19, 34, 35). A total of 29 fasting plasma samples were analyzed by NMR to obtain LDL particle concentrations. Another aliquot of the plasma samples was subjected to ultracentrifugal separation to remove VLDL. Total cholesterol, HDL-C and apoB were measured in the bottom fractions. LDL-C was calculated as the difference between the TC and HDL-C values in the bottom fraction. The plasma LDL particle concentration measured by NMR is compared to LDL apoB and LDL-C in Figure 12.

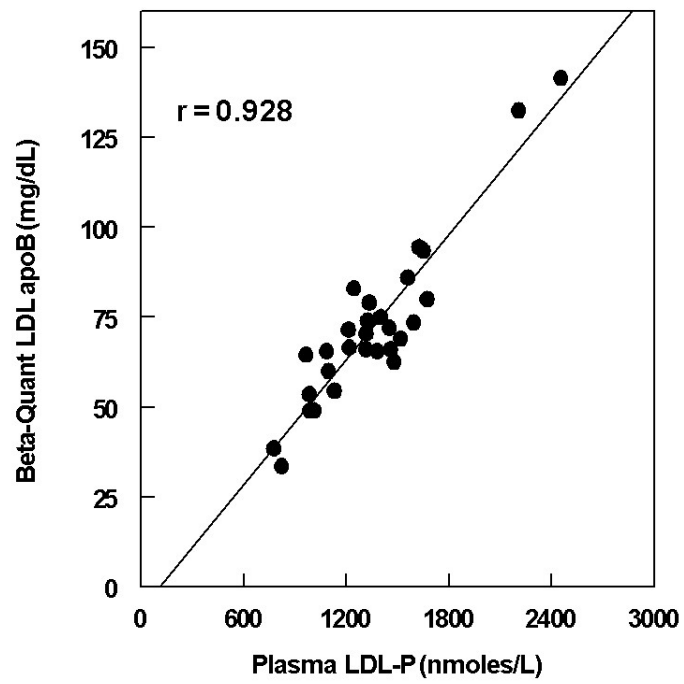
The LDL particle number (LDL-P) correlates well with LDL-C, with $r=0.846$ (fig. 12A). There is, as expected, a better correlation between LDL-P and LDL apoB, $r=0.928$ (fig. 12B). In order to evaluate whether small LDL samples correlate differently than the large LDL samples the data was separated based on NMR-determined LDL size (size >20.5 nm, pattern A, $n=18$; size <20.5 nm, pattern B, $n=11$). As seen in figure 10C, the correlations between LDL-P and LDL apoB were enhanced when the pattern A and pattern B samples were looked at separately, with correlation coefficients of 0.958 and 0.945 respectively. The fact that the regression line for the samples with predominantly small LDL is shifted

somewhat to the right compared to that for large LDL suggests that the apoB assay, relative to the NMR assay, under-quantifies small LDL particles.

FIGURE 12
A



B



C

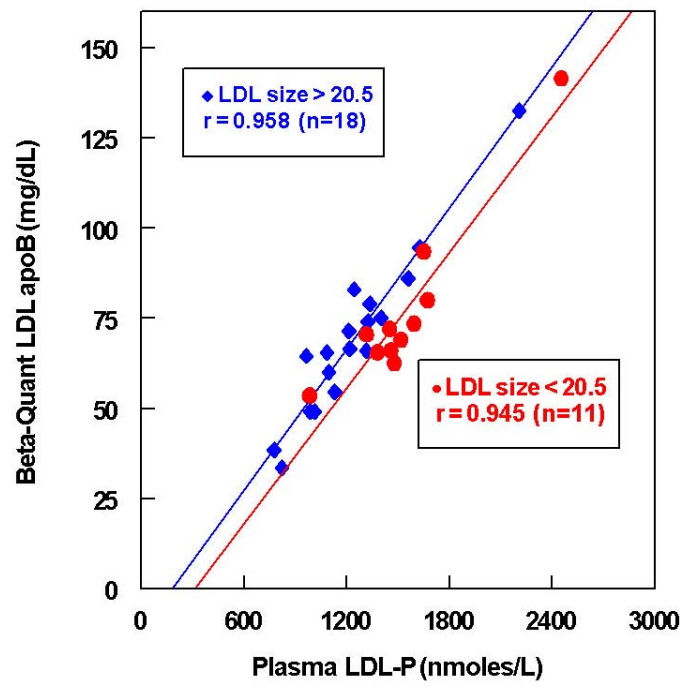


Figure 12. Correlation of NMR LDL Particle number with LDL apoB. Relationships of NMR derived LDL particle number (LDL-P) with beta-quant LDL-C (**A**) and beta-quant LDL apoB (**B, C**). $n=29$. LDL-P correlates better with LDL apoB than with LDL-C ($r=0.928$ vs 0.846). (**C**) The correlations are even stronger when LDL size >20.5 nm (pattern A) samples ($n=18$, $r = 0.958$) were compared separately from LDL size <20.5 nm (pattern B) samples ($n=11$, $r = 0.945$).

PRECISION STUDY

Precision studies were conducted to estimate the intraassay (within-run) and interassay precision (or, total imprecision) of the NMR-measured lipoprotein parameters. Two plasma pools were prepared, one with nominally high triglyceride and low HDL (pool A), and the other with low TG and high HDL (pool B). The pools were aliquoted and frozen. The intraassay precision was determined by thawing and analyzing 20 replicates of each of the two pools on one instrument following standard protocols. The interassay precision was evaluated by analyzing a frozen aliquot of each of the two pools for 20 consecutive days across six different NMR analyzers. The results of both intra and interassay precision are given in Table 2 separately for the two pools.

The total imprecision is only slightly worse than the intraassay imprecision. The CVs for the particle concentrations of VLDL, LDL, and HDL classes are 4% or less and are around 2% in most cases. The CVs for the individual subclasses (large, medium, and small) making appreciable contributions to these totals are generally under 6%. The calculated lipids of total TG, VLDL-TG and HDL-C are in the 1 to 2 % range. The LDL and HDL particle sizes have CVs of about 0.5%.

Table 2: Intraassay and Interassay Measurement Precision for *NMR LipoProfile-II***POOL A**

NMR Lipoprotein		Intra-assay Precision ¹			Interassay Precision ²		
Parameter	(Units)	Mean	SD	%CV	Mean	SD	%CV
VLDL (nmol/L)							
VLDL Particles (total)		94.2	1.3	1.4	96.5	3.0	3.1
Large VLDL/Chylos		10.1	0.2	2.4	10.0	0.5	5.1
Medium VLDL		47.5	1.5	3.2	48.6	2.0	4.1
Small VLDL		36.6	2.0	5.4	37.9	2.7	7.1
LDL (nmol/L)							
LDL Particles (total)		1876	44.3	2.4	1913	39.4	2.1
IDL		94	9.7	10.3	89	11.6	13.1
Large LDL		509	32.4	6.4	522	33.1	6.3
Small LDL (total)		1273	70.8	5.6	1301	60.8	4.7
Medium Small LDL		233	12.7	5.4	238	10.9	4.6
Very Small LDL		1039	59.4	5.7	1063	50.8	4.8
HDL (μmol/L)							
HDL Particles (total)		33.2	0.4	1.2	33.6	0.5	1.5
Large HDL		7.7	0.4	5.6	7.6	0.4	5.9
Medium HDL		2.5	1.0	**	2.8	0.9	**
Small HDL		23.0	0.9	4.1	23.1	0.8	3.7
Mean Particle Sizes (nm)							
VLDL size		63.9	0.5	0.8	63.1	1.1	1.8
LDL size		20.53	0.10	0.5	20.54	0.09	0.4
HDL size		8.57	0.04	0.5	8.56	0.05	0.6
Calculated Lipids (mg/dL)							
Total Triglycerides		229	1.3	0.6	229	2.4	1.1
VLDL Triglycerides		180	0.7	0.4	180	2.7	1.5
HDL Cholesterol		46	0.5	1.1	46	0.8	1.8

¹ Intraassay measurement precision was based on analysis of 20 replicates of each of two plasma pools (A & B).

² Interassay precision (measure of total imprecision) was derived from the analysis of frozen aliquots of each of two plasma pools for twenty days across 6 instruments.

** RSD (%cv) not reported since mean values for these parameters for the samples analyzed are very small and similar to SD.

Table 2 continued

POOL B

NMR Lipoprotein Parameter	(Units)	Intra-assay Precision ¹			Interassay Precision ²		
		Mean	SD	%CV	Mean	SD	%CV
VLDL (nmol/L)							
VLDL Particles (total)		48.8	3.1	6.3	49.5	2.8	5.7
Large VLDL/Chylos		1.9	0.3	**	1.9	0.2	**
Medium VLDL		14.8	1.6	10.7	15.6	1.8	11.7
Small VLDL		32.1	4.1	12.8	32.1	3.5	11.0
LDL (nmol/L)							
LDL Particles (total)		1090	44.0	4.0	1109	47.5	4.3
IDL		7	6.8	**	6	6.4	**
Large LDL		561	24.0	4.3	571	30.5	5.3
Small LDL (total)		523	62.4	11.9	532	70.2	13.2
Medium Small LDL		115	13.9	12.1	119	18.4	15.5
Very Small LDL		408	51.2	12.5	413	54.2	13.1
HDL (μmol/L)							
HDL Particles (total)		36.8	0.3	0.9	37.2	0.5	1.5
Large HDL		11.0	0.4	3.7	11.1	0.4	4.0
Medium HDL		1.5	0.7	**	1.6	0.7	**
Small HDL		24.3	0.6	2.7	24.6	0.7	3.0
Mean Particle Sizes (nm)							
VLDL size		51.6	1.4	2.7	50.8	1.2	2.3
LDL size		21.55	0.12	0.5	21.54	0.12	0.6
HDL size		9.06	0.05	0.6	9.05	0.05	0.6
Calculated Lipids (mg/dL)							
Total Triglycerides		87	1.5	1.7	88	1.8	2.1
VLDL Triglycerides		52	1.3	2.5	53	1.7	3.2
HDL Cholesterol		59	0.7	1.2	59	0.9	1.5

¹ Intraassay measurement precision was based on analysis of 20 replicates of each of two plasma pools (A & B).

² Interassay precision (measure of total imprecision) was derived from the analysis of frozen aliquots of each of two plasma pools for twenty days across 6 instruments.

** RSD (%cv) not reported since mean values for these parameters for the samples analyzed are very small and similar to SD.

NORMAL RANGES AND INTERCORRELATIONS

Normal ranges for the parameters contained in the *NMR LipoProfile-II* report are given in Table 3. The data were compiled from over 7300 randomly selected fasting patient plasma samples analyzed at LipoScience in November/December, 2003. The patients come mainly from the Southeastern U.S. and are a mix of primary prevention patients without coronary disease and secondary prevention patients with disease. Ages range from 20-94 years, with a median age of 58 years. Data are provided separately for men (n=4054) and women (n=3317) along with the combined (Overall, n=7371) group. The mean and median values are reported along with the normal ranges covering the 10th to 90th percentile.

Many lipoprotein subclasses are metabolically interrelated and as such their levels are not independent of each other. To assist in evaluation and interpretation of NMR-derived lipoprotein subclass particle data, intercorrelations among the 20 reportable parameters from *NMR LipoProfile-II* are provided in Table 4. The Spearman correlations are based on the data from the same set of 7371 samples used to generate normal ranges.

Table 3: Normal Ranges (10th – 90th percentile) for *NMR LipoProfile-II* Parameters

Lipoprotein Parameter	Men (n=4054)			Women (n=3317)			Overall (n=7371)		
	Mean $\pm$ SD	Median	Range	Mean $\pm$ SD	Median	Range	Mean $\pm$ SD	Median	Range
VLDL (nmol/L)									
VLDL Particles (total)	84.8 $\pm$ 67.0	71.5	17.2 – 162.9	68.1 $\pm$ 62.5	54.6	8.3 – 141.0	77.3 $\pm$ 65.5	64.0	12.3 – 154.0
Large VLDL/Chylomicrons	3.4 $\pm$ 8.9	0.8	0.1 – 8.5	2.5 $\pm$ 5.8	0.6	0.1 – 6.4	3.0 $\pm$ 7.7	0.7	0.1 – 7.6
Medium VLDL	51.9 $\pm$ 55.2	36.8	4.3 – 114.9	47.3 $\pm$ 4.9	26.3	2.2 – 87.4	46.2 $\pm$ 52.2	31.2	3.0 – 103.8
Small VLDL	29.5 $\pm$ 26.3	24.4	1.4 – 61.6	26.5 $\pm$ 25.7	20.4	0 – 58.9	28.1 $\pm$ 26.0	22.8	0.5 – 60.3
LDL (nmol/L)									
LDL Particles (total)	1535 $\pm$ 490	1468	972 – 2195	1489 $\pm$ 487	1419	949 – 2118	1514 $\pm$ 489	1445	961 – 2161
IDL	28 $\pm$ 43	7	0 – 86	26 $\pm$ 45	0	0 – 86	27 $\pm$ 44	5	0 – 86
Large LDL	339 $\pm$ 241	297	70 – 657	524 $\pm$ 289	496	172 – 912	422 $\pm$ 279	381	99 – 792
Small LDL (total)	1169 $\pm$ 542	1122	516 – 1886	938 $\pm$ 564	870	242 – 1698	1065 $\pm$ 564	1021	370 – 1818
Medium Small LDL	256 $\pm$ 116	246	119 – 402	212 $\pm$ 123	200	63 – 371	236 $\pm$ 121	228	88 – 390
Very Small LDL	913 $\pm$ 433	874	393 – 1483	727 $\pm$ 447	675	172 – 1329	829 $\pm$ 449	793	280 – 1425
HDL (μmol/L)									
HDL Particles (total)	28.1 $\pm$ 6.7	27.8	19.9 – 36.5	33.4 $\pm$ 7.7	33.0	24.2 – 43.5	30.5 $\pm$ 7.6	30.1	21.2 – 40.3
Large HDL	5.3 $\pm$ 3.5	4.6	1.6 – 10.1	9.1 $\pm$ 4.9	8.3	3.5 – 16.1	7.0 $\pm$ 4.6	6.0	2.2 – 13.6
Medium HDL	2.3 $\pm$ 3.4	0.9	0 – 6.8	3.1 $\pm$ 4.0	1.5	0 – 8.8	2.7 $\pm$ 3.7	1.1	0 – 7.8
Small HDL	20.5 $\pm$ 5.3	21.6	14.0 – 26.9	21.2 $\pm$ 6.1	21.0	13.7 – 28.8	20.8 $\pm$ 5.7	20.8	13.9 – 27.8
Mean Particle Sizes (nm)									
VLDL size	52.3 $\pm$ 13.2	49.1	41.1 – 65.9	55.2 $\pm$ 16.7	50.5	42.0 – 73.9	53.6 $\pm$ 15.0	49.7	41.5 – 69.4
LDL size	20.4 $\pm$ 0.8	20.3	19.5 – 21.5	21.0 $\pm$ 0.9	21.0	19.9 – 22.3	20.7 $\pm$ 0.9	20.6	19.6 – 21.1
HDL size	8.7 $\pm$ 0.4	8.7	8.3 – 9.3	9.0 $\pm$ 0.4	9.0	8.5 – 9.6	8.9 $\pm$ 0.4	8.8	8.4 – 9.5
Calculated Lipids (mg/dL)									
Total Triglycerides	157 $\pm$ 135	123	57 – 281	134 $\pm$ 104	106	54 – 237	146 $\pm$ 122	115	55 – 261
VLDL Triglycerides	119 $\pm$ 134	84	20 – 241	91 $\pm$ 103	62	13 – 190	106 $\pm$ 122	75	16 – 220
LDL Cholesterol	121 $\pm$ 34	118	81 – 164	132 $\pm$ 35	129	91 – 176	126 $\pm$ 35	123	85 – 170
HDL Cholesterol	40 $\pm$ 14	38	25 – 57	54 $\pm$ 18	52	34 – 79	46 $\pm$ 17	43	27 – 70

Table 4: Inter-correlations Between Parameters in *NMR LipoProfile-II* Report¹

	VLDLP	VL	VM	VS	LDLP	IDL	LL	LS	LMS	LVS	HDLP	HL	HM	HS	VZ	LZ	HZ	NTG	NVTG	NHDLC
VLDLP	---	.5	.9	.6	.4	.4	-.4	.5	.4	.5	-.3	-.5	-.1	.1	-.5	-.5	-.5	.9	.9	-.5
VL	.5	---	.5	.1	.2	.4	-.2	.3	.3	.3	0	-.4	.1	.2	.3	-.3	-.3	.7	.7	-.2
VM	.9	.5	---	.3	.3	.2	-.5	.5	.4	.5	-.3	-.5	-.1	.1	-.4	-.5	-.5	.9	.9	-.4
VS	.6	.1	.3	---	.3	.4	-.1	.2	.2	.3	-.2	-.3	0	.1	-.6	-.2	-.3	.4	.4	-.3
LDLP	.4	.2	.3	.3	---	.3	-.1	.8	.8	.9	-.2	-.3	-.1	.1	-.2	-.5	-.5	.4	.3	-.4
IDL	.4	.4	.2	.4	.3	---	-.2	.4	.3	.4	-.1	-.4	.1	.1	-.1	-.3	-.4	.4	.3	-.3
LL	-.4	-.2	-.5	-.1	-.1	-.2	---	-.6	-.6	-.5	.4	.7	0	-.1	.2	.9	.6	-.4	-.5	.6
LS	.5	.3	.5	.2	.8	.4	-.6	---	1.0	1.0	-.3	-.6	-.1	.1	-.2	-.8	-.7	.5	.5	-.6
LMS	.4	.3	.4	.2	.8	.3	-.6	1.0	---	.9	-.3	-.5	-.1	.1	-.2	-.8	-.6	.5	.5	-.5
LVS	.5	.3	.5	.3	.9	.4	-.5	1.0	.9	---	-.4	-.6	-.2	.1	-.2	-.8	-.7	.5	.5	-.6
HDLP	-.3	0	-.3	-.2	-.2	-.1	.4	-.3	-.3	-.4	---	.6	.3	.6	.2	-.1	-.2	.2	.2	.3
HL	-.5	-.4	-.5	-.3	-.3	-.4	.7	-.6	-.5	-.6	.6	---	0	0	.2	.7	.8	-.5	-.6	.9
HM	-.1	.1	-.1	0	-.1	.1	0	-.1	-.1	-.2	.3	0	---	-.1	.1	.1	0	0	0	.2
HS	.1	.2	.1	.1	.1	.1	-.1	.1	.1	.1	.6	0	-.1	---	0	-.1	-.2	.2	.2	.3
VZ	-.5	.3	-.4	-.6	-.2	-.1	.2	-.2	-.2	-.2	.2	.2	.1	0	---	.2	.3	-.3	-.3	.3
LZ	-.5	-.3	-.5	-.2	-.5	-.3	.9	-.8	-.8	-.8	-.1	.7	.1	-.1	.2	---	.7	-.5	.6	.7
HZ	-.5	-.3	-.5	-.3	-.5	-.4	.6	-.7	-.6	-.7	-.2	.8	0	-.2	.3	.7	---	-.5	-.5	.8
NTG	.9	.7	.9	.4	.4	.4	-.4	.5	.5	.5	.2	-.5	0	.2	-.3	-.5	-.5	---	1.0	-.4
NVTG	.9	.7	.9	.4	.3	.3	-.5	.5	.5	.5	.2	-.6	0	.2	-.3	.6	-.5	1.0	---	-.5
NHDLC	-.5	-.2	-.4	-.3	-.4	-.3	.6	-.6	-.5	-.6	.3	.9	.2	.3	.3	.7	.8	-.4	-.5	---

¹Spearman correlations, based on NMR analysis of 7,371 random fasting patient plasma samples performed at LipoScience in Nov/Dec 2003.

VLDLP = VLDL particles; VL = Large VLDL/Chylos; VM = Medium VLDL; VS = Small VLDL; LDLP = LDL particles; IDL = IDL; LL = Large LDL; LS = Small LDL; LMS = Medium Small LDL; LVS = Very Small LDL; HDLP = HDL Particles; HL = Large HDL; HM = Medium HDL; HS = Small HDL; VZ = VLDL size; LZ = LDL size; HZ = HDL size; NTG = NMR-calculated triglycerides; NVTG = NMR-calculated VLDL triglyceride; NHDLC = NMR-calculated HDL cholesterol

Discussion

The NMR method of quantifying lipids and lipoproteins has been in existence since the early nineties. The *NMR LipoProfile* test has been offered for patient care since 1999. Since its original introduction in 1992 (9, 10) numerous improvements have been made to the methodology. The purpose of this paper is to update the current status of the technology and provide additional validation data for the methodology. As an emerging technology in the coronary heart disease risk assessment arena, the initial focus was on its ability to provide reliable cholesterol and triglyceride information despite the fact that the technique actually measures lipoprotein particles, not lipids. There is no denying that good correlation is observed between NMR-derived lipid values and chemical lipids. These correlations are in the range of 0.91-0.95 for LDL-C, 0.93-0.97 for HDL-C, and >0.97 for TG (10, 11, 14). However, it is becoming increasingly clear that the real strength of the NMR technology in terms of its ability to aid CHD risk assessment derives from its ability to quantify numbers of lipoprotein subclass particles. Evidence has been mounting in recent years that LDL particle concentration predicts CHD risk better than LDL-C (36, 1-6). Subclass particle concentrations of LDL and HDL have also been shown to explain the hitherto unexplained gender differences in CHD risk in the Framingham Offspring Study (37).

Fundamentally, two phenomena make NMR quantification of numbers of lipoprotein subclass particles possible: 1) Lipoprotein subclasses of different size in plasma emit distinctive NMR signals whose individual amplitudes can be accurately and reproducibly measured. 2) Measured subclass signal amplitudes are directly proportional to the numbers of subclass particles emitting the signal, irrespective of variations in particle lipid composition. A natural physical-chemical property of lipoproteins, related to the orientational order of the

phospholipids in the particle shell (12), gives spectroscopic distinctness (ie., a unique frequency and shape) to the NMR signals from the same lipid molecules packaged in larger versus smaller lipoprotein particles. Only the diameter of the particle's lipid shell determines its spectroscopic properties; specifically, signals from larger particles appear downfield (higher frequency) from those of smaller particles, as shown in Figure 3, for the proton NMR signals of purified lipoprotein subfractions. The spectroscopic distinctness of a particular subclass (ie., how much its signal frequency and shape differ from those of its neighboring subclasses) determines how accurately and reproducibly its signal amplitude (and hence its concentration) can be determined via mathematical deconvolution of the measured plasma NMR signal, which is the simple linear sum of the contributing signals from all of the lipoprotein subclasses.

It has already been described how, via plasma spectral deconvolution, the amplitudes of the distinctive NMR signals emitted by different-size lipoprotein subclasses are measured. How and why the amplitudes of these signals enable accurate quantification of numbers of lipoprotein subclass particles will now be explained. The NMR signal used for subclass particle quantification appears at ~0.8 ppm and originates in aggregate from the protons of all of the terminal methyl groups of the triglyceride, cholesterol ester, phospholipid, and unesterified cholesterol molecules in the particle. The cholesterol esters and triglycerides in the particle core each contribute 3 methyl groups and the phospholipids and unesterified cholesterol in the surface shell each contribute 2 methyl groups. A single lipoprotein particle contains many hundreds of these lipid molecules. A large LDL subclass particle, for example, contains >3,000 lipid molecules with >8,000 terminal methyl groups and, thus, >24,000 methyl protons that contribute to the detected NMR signal (33). Because the chemical environments of these methyl groups in the four different types of lipid molecules are very similar, their signals

overlap and each is indistinguishable from the others. As a result, variation of a particle's lipid composition has no discernable effect on the particle's lipid methyl signal. Only the diameter of the particle, as discussed earlier, imparts spectral distinctness to the signal.

The amplitude of the NMR signal emitted by each lipoprotein subclass particle is determined by the total number of lipid methyl groups in the particle. If this number remains effectively invariant in the face of normal variations in particle lipid composition, then the NMR signal amplitude would retain its proportionality to particle number and could be relied upon to provide accurate subclass particle quantification. If, on the other hand, the number of methyl groups in a particle of given size varied substantially from person to person due to variations in particle lipid composition, the amplitude of the methyl NMR signal would also vary and could not be used as the basis for accurate lipoprotein particle measurement.

Among sources of lipid compositional variability that would be expected to have little or no effect on the total number of terminal methyl groups contributing to the particle NMR signal are differences in fatty acid unsaturation, phospholipid chemical heterogeneity, and variations in the relative amounts of shell phospholipid and unesterified cholesterol. The greatest source of lipoprotein lipid compositional variability is the CETP-mediated exchange of core cholesterol ester for triglyceride in VLDL, LDL, and HDL. As a result of this process, LDL (mainly from those with hypertriglyceridemia) can be substantially cholesterol-depleted and triglyceride-enriched. To a close approximation, however, the number of methyl groups in the particle remains constant, since a one-to-one exchange of a cholesterol ester molecule for a triglyceride molecule removes 3 methyl groups and replaces these with 3 new (indistinguishable) methyl groups. In reality, an exact one-to-one exchange does not occur, since a triglyceride molecule occupies a larger volume than a cholesterol ester molecule (33).

Taking this volume difference into account, it can be calculated that there is at most only a 3% difference in the number of methyl groups present in a cholesterol-rich compared to cholesterol-poor LDL particle. Thus, the detected NMR signal amplitude of a lipoprotein subclass remains effectively invariant in the face of lipid compositional variability, which makes it a reliable and unique source of subclass particle quantification.

The original NMR spectral deconvolution incorporated a standard linear least-squares analysis routine with a simple non-negative constraint, in which negative concentrations were set to zero and each component with a negative contribution was removed from the model. This resulted in a discontinuity in adjacent subclasses with several subclass concentrations being reported as zero. The newer method uses a more sophisticated non-negative least squares regression with singular value decomposition (30). This avoids setting subclasses with low concentrations to zero and reflects known lipoprotein heterogeneity. The data gathered from a multitude of clinical studies with disease end points and a large number of normal samples have helped us to group the subcomponents of each major lipoprotein class more logically, so that their concentrations have clinical meaning. The current output of the *NMR LipoProfile* test includes particle concentrations of 10 lipoprotein subclasses, 3 each for VLDL, LDL and HDL, and one for IDL, all of which can now be measured with a higher degree of analytical precision.

Many lipoprotein subclasses are metabolically interrelated, and we used that knowledge to appropriately group the more than 30 subcomponents. For example, in agreement with results obtained using other subclass fractionation methods, the intercorrelation data in Table 4 show that LDL and HDL size are strongly correlated ($r=0.7$) and both are inversely related to triglyceride level ($r=-0.5$). Of special note is the very strong

correlation ($r=0.9$) observed between the two smallest LDL subclasses, labeled LMS and LVS, and the fact that each has very similar associations with all of the other lipoprotein parameters. For this reason, it was considered appropriate to label both of these subclasses “Small”: Medium Small LDL (formerly labeled Intermediate LDL) and Very Small LDL (formerly labeled Small LDL). Grouping these two subclasses together into a single subclass called “Small LDL” may simplify future outcome association studies and possibly provide stronger relations with CVD. Another reason for providing the table is to help investigators using NMR data for clinical trials to assess the data quality. Sample integrity is paramount for the *NMR LipoProfile* test to work well and produce meaningful results. Improper storage conditions (freezer temperatures warmer than -70°C , multiple freeze/thaw cycles etc.) can deteriorate the physical properties of lipoproteins, resulting in breakdown of the expected intercorrelations.

Normal ranges are provided from measurements conducted on over 7300 plasma samples, again to establish reference ranges for lipoprotein subclass concentrations expressed as particle concentrations. The separate normal range data for men and women reported in Table 3 conforms to known male/female differences. For example, the median values for small LDL particles are 1122 nmol/L for men, and 870 nmol/L for women. The median value for the cardioprotective large HDL particles for men is nearly 45% lower than that of women (4.6 vs 8.3 $\mu\text{mol/L}$).

We have once again established that LDL and HDL particle sizes determined by *NMR LipoProfile* analysis closely match those assessed by gradient gel electrophoresis. For 27 samples analyzed by GGE by Quest Diagnostics (San Juan Capistrano, CA) the correlation for LDL size with NMR was 0.87 (data not shown). The split samples ($n=15$)

analyzed by Rainwater's lab (figure 10) showed a linear correlation ($r=0.95$) with NMR results for both LDL and HDL size. This excellent correlation is probably due to the fact that Rainwater's laboratory provides a median particle diameter, similar to the average particle size provided by NMR, as opposed to the peak particle diameter provided by Quest.

The validity of LDL particle concentrations determined by NMR was established by data showing a linear relationship with LDL apoB levels (figure 12B) with $r=0.93$. The correlation plots separated by LDL size (figure 12C) where better correlations (0.96 for pattern A, 0.95 for pattern B) were observed are worth a closer look. It is intriguing that the regression line for small LDL (pattern B) samples is parallel to the pattern A samples, but with uniformly lower numbers. It appears that compared to the particle concentration (LDL-P) measured by NMR, the LDL apoB is under-estimated by the apoB assay for small LDL samples. This may explain why LDL-P in several clinical trials gave better prediction of CHD risk than plasma apoB. LDL subspecies differences resulting in apoB conformational changes have been noted (33), which may affect the specificity of the immunoassay for large versus small LDL particles, or compositionally different LDL particles.

Intraassay and interassay precision studies reported here showing 2-6% CVs for difficult-to-measure subclasses elevates the NMR lipoprotein technology to the realm of clinical assays. The excellent linearity and specificity data presented here substantiates the accuracy and robustness of the technique.

Acknowledgements: We thank Qun Zhou for excellent technical help. Ms. Valerie Knowlton's assistance with electron microscopy and Dr. Martha Wilson's help with GGE and compositional analysis are greatly appreciated.

References

1. Blake GJ, Otvos JD, Rifai N, Ridker PM. LDL particle concentration and size as determined by NMR spectroscopy as predictors of cardiovascular disease in women. *Circulation* 2002;106:1930-37.
2. Kuller L, Arnold A, Tracy R, et al. Nuclear magnetic resonance spectroscopy of lipoproteins and risk of coronary heart disease in the cardiovascular health study. *Arterioscler Thromb Vasc Biol* 2002;22:1175-80.
3. Rosenson RS, Otvos JD, Freedman DS. Relations of lipoprotein subclass levels and low-density lipoprotein size to progression of coronary artery disease in the Pravastatin Limitation of Atherosclerosis in the Coronary Arteries (PLAC-I) Trial. *Am J Cardiol* 2002;90:89-94.
4. Mackey RH, Kuller LH, Sutton-Tyrrell K, Evans RW, Holubkov R, Matthews KA. Lipoprotein subclasses and coronary artery calcium in postmenopausal women from the healthy women study. *Am J Cardiol* 2002;90:71i-76i.
5. Otvos JD, Jeyarajah EJ, Cromwell WC. Measurement Issues Related to Lipoprotein Heterogeneity. *Am J Cardiol* 2002;90:22i-29i.
6. Freedman,DS, Otvos,JD, Jeyarajah,EJ, Barboriak,JJ, Anderson,AJ, Walker,JA: Relation of lipoprotein subclasses as measured by proton nuclear magnetic resonance spectroscopy to coronary artery disease. *Arterioscler Thromb Vasc Biol* 1998;18:1046-1053.
7. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 1972;18:499-502.
8. Executive Summary of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). *JAMA* 2001;285:2486-2497.
9. J. D. Otvos, E.J. Jeyarajah, D.W. Bennett. Quantification of Plasma Lipoproteins by Proton Nuclear Magnetic Resonance Spectroscopy. *Clin Chem* 1991;37:377-386.
10. Otvos JD, Jeyarajah EJ, Bennett DW, Krauss RM. Development of a proton nuclear magnetic resonance spectroscopic method for determining plasma lipoprotein concentrations and subspecies distributions from a single, rapid measurement. *Clin Chem* 1992;38:1632-8.
11. James Otvos, Elias Jeyarajah, Dennis Bennett A Spectroscopic Approach to Lipoprotein Subclass Analysis. *Journal of Clinical Ligand Assay* 1996;19:184-189.
12. Lounila J, Ala-Korpela M, Jokisaari J. Effects of Orientational Order and Particle Size on the NMR Line Positions of Lipoproteins. *Phys Rev Lett* 1994;72:4049-4052.

13. Redgrave TG, Carlson LA. Changes in plasma very low density and low density lipoprotein content, composition, and size after a fatty meal in normo- and hypertriglyceridemic men. *J Lipid Res* 1979;20:217-34.
14. Otvos JD. Measurement of lipoprotein subclass profiles by nuclear magnetic resonance spectroscopy. In: Rifai N, Warnick GR, Dominiczak MH, eds. *Handbook of Lipoprotein Testing*. Washington, DC: AACC Press; 2000:609-23.
15. Otvos JD, Jeyarajah EJ, Hayes LW, Freedman DS, Janjan NA, Anderson T. Relationships between the proton nuclear magnetic resonance properties of plasma lipoproteins and cancer. *Clin Chem* 1991;37:369-76.
16. Hatch FT, Lees RS. Practical Method for Plasma Lipoprotein Analysis. *Adv Lipid Res* 1968;6:1-68.
17. Rudel LL, Marzetta CA, Johnson FL. Separation and Analysis of Lipoproteins by Gel Filtration. *Methods Enzymol* 1986;129:45-56.
18. Sata T, Havel RJ, Jones AL. Characterization of subfractions of triglyceride-rich lipoproteins separated by gel chromatography from blood plasma of normolipemic and hyperlipemic humans. *J Lip Res* 1972; 13:757-768.
19. Contois JH, McNamara JR, Lammi-Keefe CJ, Wilson PWF, Massov T, Schaefer EJ. Reference intervals for plasma apolipoprotein B determined with a standardized commercial immunoturbidimetric assay: results from the Framingham Offspring Study. *Clin Chem* 1996;42:515-23.
20. Nichols AV, Krauss RM, Musliner TA. Nondenaturing polyacrylamide gradient gel electrophoresis. In: Segrest JP, Albers JJ, eds. *Plasma Lipoproteins*. *Methods Enzymol* 1986;128:417-31.
21. Rainwater DL, Moore PH, Shelledy WR, Dyer TD. Characterization of a composite gradient gel for the electrophoretic separation of lipoproteins. *J Lipid Res* 1997;38:1261-6.
22. Rainwater DL. Lipoprotein correlates of LDL particle size. *Atherosclerosis* 2000;148:151-8.
23. Rainwater DL, Moore PH, Gamboa IO. Improved method for making nondenaturing composite gradient gels for the electrophoretic separation of lipoproteins. *J Lipid Res* 2004;45:773-5.
24. Forte TM, Nordhausen RW. Electron Microscopy of Negatively Stained Lipoproteins. In: Segrest JP, Albers JJ, eds. *Structure of Plasma Lipoproteins*. *Methods Enzymol* 1986;128:442-57.

25. Bax A. A spatially selective composite 90^0 radiofrequency pulse. *J Magn Reson* 1985;65:142-5.
26. Westgard JO, Klee GG. Quality Management. Chapter 16 in *Fundamentals of Clinical Chemistry*, 4th edition. Burtis C, ed., WB Saunders Company, Philadelphia, 1996;211-23.
27. Westgard JO, Barry PL, Hunt MR, Groth T. A multi-rule Shewhart chart for quality control in clinical chemistry. *Clin Chem* 1981;27:493-501.
28. Lawson, C. L., Hanson, R. J. Solving Least Squares problems. Philadelphia, PA: SIAM, 1995, pp. 160-165.
29. Hager, W.W. *Applied Numerical Linear Algebra*. Englewood Cliffs, NJ: Prentice Hall, 1988, pp 294-302.
30. Tony F. Chan. An improved algorithm for computing the Singular Value Decomposition. *ACM Trans Math Softw* 1982;8:72-83.
31. Groszek E, Grundy SM. Electron-microscopic evidence for particles smaller than 250 Å in very-low-density lipoproteins of human plasma. *Atherosclerosis* 1978;31:241-50.
32. Rumsey SC, Galeano NF, Arad Y, Deckelbaum RJ. Cryopreservation with sucrose maintains normal physical and biological properties of human plasma low-density lipoproteins. *J Lipid Res* 1992;33:1551-61.
33. McNamara JR, Small DM, Li Z, Schaefer EJ. Differences in LDL subspecies involve alterations in lipid composition and conformational changes in apolipoprotein B. *J Lipid Res* 1996;37:1924-35.
34. Sniderman AD, Furberg CD, Keech A, Roeters, van Lennep JE, et al. Apolipoproteins versus lipids as indices of coronary risk and as targets for statin therapy treatment. *Lancet* 2003;361:777-80.
35. Talmud PJ, Hawe E, Miller GJ, Humphries SE. Non-fasting apolipoprotein B and triglyceride levels as a useful predictor of coronary heart disease risk in middle-aged UK men. *Arterioscler Thromb Vasc Biol* 2002;22:1918-23.
36. Lamarche B, Tchernof A, Moorjani S, Cantin B, Dagenais GR, Lupien PJ, Després J-P. Small, dense, low-density lipoprotein particles as a predictor of the risk of ischemic heart disease in men: prospective results from the Québec Cardiovascular Study. *Circulation* 1997;95:69-75.
37. Freedman DS, Otvos JD, Jeyarajah EJ, Shalauova I, Cupples LA, Parise H, D'Agostino RB, Wilson PW, Schaefer EJ. Sex and age differences in lipoprotein subclasses measured by Nuclear Magnetic Resonance spectroscopy: The Framingham Study. *Clin Chem* 2004;50:1189-1200.

Chapter 4

Measurement Issues Related to Lipoprotein Heterogeneity

James D. Otvos, Ph.D., Elias J. Jeyarajah, M.S. and William C. Cromwell, M.D.

From LipoScience, Inc., Raleigh, North Carolina, USA.

American Journal of Cardiology 2002;90(suppl):22i-29i

Measurement Issues Related to Lipoprotein Heterogeneity

James D. Otvos, Ph.D., Elias J. Jeyarajah, M.S. and William C. Cromwell, M.D.
LipoScience, Inc., Raleigh, North Carolina, USA.

Abstract: In clinical practice, the cardiovascular disease (CVD) risk associated with high levels of LDL or low levels of HDL is assessed not by measuring LDL and HDL particles directly, but by measuring the amount of cholesterol carried by these lipoproteins. It is not generally appreciated how much the amount of cholesterol per particle varies from person to person, especially for LDL, due to differences in the relative amounts of cholesterol ester and triglycerides in the particle core as well as differences in particle diameter. As a consequence of the magnitude and prevalence of this lipid compositional variability, even the most accurate lipoprotein cholesterol measurements will, for many individuals, provide an inaccurate measure of the number of circulating lipoprotein particles and the CVD risk they confer. Nuclear magnetic resonance (NMR) spectroscopy offers an efficient new means of measuring lipoprotein levels in plasma, with quantification based not on cholesterol content, but the amplitudes of spectral signals emitted by lipoprotein subclasses of different size. Since the subclass signal amplitudes are not influenced by cholesterol compositional variability, they provide a direct measure of lipoprotein particle concentrations. NMR data from the Framingham Offspring Study demonstrate a significant "disconnect" between LDL cholesterol and LDL particle concentrations in patients with low levels of HDL cholesterol. The results imply that a substantial portion of the excess CVD risk of patients with low HDL stems from an unrecognized excess of LDL particles containing less cholesterol than normal. Patients with this abnormality would benefit from LDL lowering therapy, but are not identified as candidates for such treatment on the basis of traditional LDL cholesterol tests.

Am J Cardiol 2002;90(suppl):22i-29i

Lipids and Lipoproteins

Assessment of cardiovascular disease (CVD) risk has traditionally been framed as a cholesterol issue, not a lipoprotein issue. People are taught that there are two forms of serum cholesterol, "bad" and "good," and it is healthier to have less of the former and more of the latter. Some people may know that the bad and good forms refer to LDL and HDL respectively, but few understand that it is actually LDL and HDL particles that are functionally "bad" and "good", with the cholesterol carried within the particles serving merely as a convenient surrogate marker for the concentrations of these lipoproteins.

Thirty-five years ago, the surrogate relationship of lipids (cholesterol and triglycerides) to lipoproteins was described in the landmark writings of Fredrickson, Levy, and Lees,¹ who noted that "... all abnormalities in plasma lipid concentrations, or dyslipidemia, can be translated into dyslipoproteinemia," and "the shift of emphasis to lipoproteins offers distinct advantages in the recognition and management of such disorders." The reason that lipids, rather than lipoproteins, are the traditional focus of clinical attention was also discussed: "...there is no single test that infallibly separates all those who have dyslipoproteinemia from those who do not....the majority of laboratories still employ a combination of chemical measurements of plasma lipids for this purpose."

Laboratory management of dyslipoproteinemias has changed surprisingly little in the intervening 35 years, despite even better understanding of the key roles in atherogenesis played by lipoprotein particles interacting with the arterial wall.^{2,3} For reasons that are related primarily to the difficulty of measuring lipoprotein particles directly, triglycerides continue to serve as a

surrogate measure of VLDL levels, and LDL and HDL cholesterol values as indicators of the concentrations of LDL and HDL particles. To be sure, few people regard the surrogate relationship of lipids to lipoproteins as a clinical limitation because of the extensive body of data showing that abnormal lipid levels are strongly related to atherosclerosis and CVD events.^{4,5} The question to be asked, however, is whether the disease risk of individual patients might be assessed and managed more effectively by measuring the lipoprotein mediators of the disease process, rather than the lipids carried within them.

Although in the past it was difficult to quantify lipoprotein particles, we will describe later in this article an efficient new method of lipoprotein analysis that uses the nuclear magnetic resonance (NMR) signals emitted by lipoprotein particles of different size as the basis of their quantification. Data will be presented showing that for many individuals LDL cholesterol values do not accurately reflect the number of circulating LDL particles and the CVD risk associated with them. This is the same problem described over 20 years ago by Drs. Sniderman, Kwiterovich and coworkers based on measured differences in the ratio of apolipoprotein B (apoB) to LDL cholesterol.⁶ Coronary atherosclerosis was associated with elevated apoB levels in individuals with normal LDL cholesterol, a condition termed hyperapobetalipoproteinemia. The metabolic origins of the LDL compositional heterogeneity that produces this condition are now well understood, and will be outlined below. Results from several recent clinical trials in both primary and secondary prevention populations⁷⁻¹⁰ support the contention that plasma apoB, which provides a good estimate of LDL particle number, is a better index of atherosclerotic risk than LDL cholesterol.¹¹⁻¹³ Since other articles in this volume are devoted to the subject of apoB,

we will focus here on new insights provided by NMR spectroscopy into lipoprotein particle heterogeneity as it relates to cardiovascular disease risk prediction.

Prevalence and Metabolic Origins of LDL Cholesterol Compositional Variability

LDL cholesterol has been used for so long as the basis of quantifying LDL that physicians and patients rarely bother to note the distinction. For example, it is common for someone to express concern about "my LDL of 160 mg/dL" without worrying about the fact that it is a cholesterol concentration they are talking about, not an LDL concentration. Why is this distinction important? Because LDL particles from different individuals vary tremendously in the amount of cholesterol they contain. As a result, a measured LDL cholesterol level of 160 mg/dL may or may not be a valid reason for clinical concern, depending on whether the number of LDL particles carrying the cholesterol is markedly elevated or not.

There are two independent sources of LDL cholesterol compositional variability and both are related to plasma triglyceride (or VLDL) levels.^{14,15} The first is variability in *core lipid composition*, driven by reactions that modulate the relative amounts of cholesterol ester and triglyceride contained within the neutral lipid interior of the particles. The second is variability in *particle size*, since smaller particles contain less cholesterol than larger ones simply because of the smaller physical volume of the lipid core.

The metabolic reactions responsible for producing cholesterol-deficient LDL particles are outlined in Fig.1. When plasma triglyceride levels are elevated, even modestly, a reaction

catalyzed by cholesterol ester transfer protein (CETP) becomes important, in which triglyceride molecules from the core of triglyceride-rich lipoproteins (mainly VLDL) exchange one-for-one with cholesterol ester molecules in the core of LDL. When large LDL thus becomes depleted in cholesterol and enriched in triglycerides, the particle becomes a substrate for hepatic lipase (HL) and may, as a result of core triglyceride hydrolysis and structural remodeling, become transformed into a smaller, denser LDL particle.

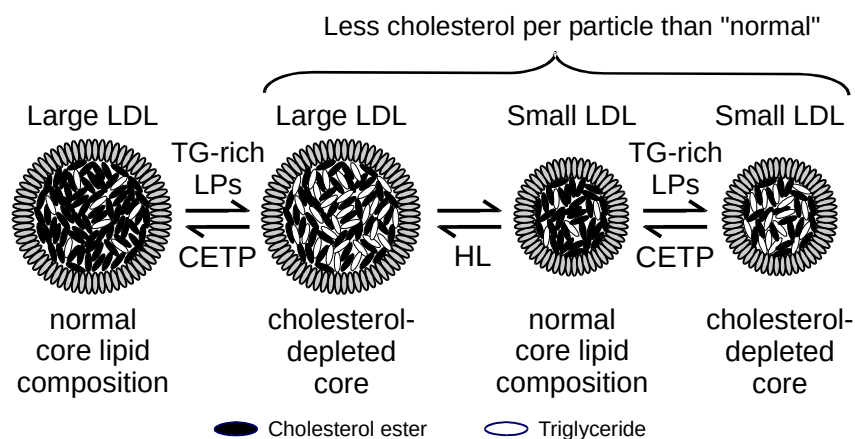


FIGURE 1. Schematic representation of the metabolic origins of LDL particles containing less cholesterol than normal. TG = triglyceride, LPs = lipoproteins, CETP = cholesterol ester transfer protein, HL = hepatic lipase.

Depending on the triglyceride level and CETP activity, the small LDL particles may end up with a normal core lipid composition or become significantly cholesterol-depleted. There are thus four different types of LDL particles likely to be seen in individuals depending on their lipid metabolic circumstances (Fig. 1): large LDL with a normal core lipid content, small LDL with a normal lipid content, and large and small LDL with relatively cholesterol-deficient, triglyceride-rich lipid cores. It should be noted that the reactions leading to the production of these different forms of LDL are fully reversible, so that a patient with high triglycerides and cholesterol-deficient LDL who is placed on successful triglyceride-lowering therapy may exhibit an LDL

cholesterol increase even though the number of LDL particles has decreased, simply because the particles have become more cholesterol-rich.

To gain some understanding of the prevalence of LDL particles (large and small) containing cholesterol-depleted, triglyceride-rich cores, we isolated LDL from 118 healthy men and women by preparative ultracentrifugation and conducted detailed lipid compositional analyses as well as LDL size measurement by NMR. Mean ($\pm$ SD) plasma triglyceride levels were 135 ($\pm$ 89) mg/dL, representative of levels found in the general population. 11 subjects (9%) had values exceeding 250 mg/dL and only 2 had levels greater than 400 mg/dL. The measured ratios of cholesterol/triglyceride in the LDL of these subjects ranged from 1.8 to 11.5 and the distribution is shown in Fig. 2. The majority (65%) of the study population had large LDL particles of "normal" composition (cholesterol/triglyceride ratio >4)¹⁴. However, a surprisingly large

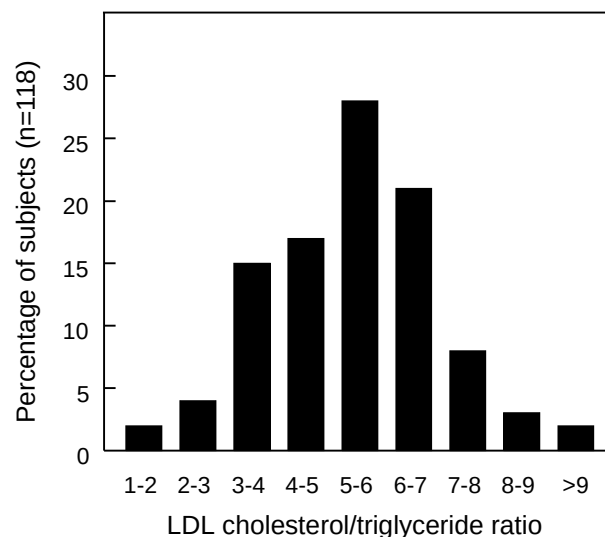


FIGURE 2. Distribution of the measured ratios of cholesterol/triglyceride in the LDL fraction ($d=1.006-1.063$ kg/L) isolated by ultracentrifugation from 118 healthy subjects.

percentage of subjects (21%) had LDL particles that were compositionally cholesterol-depleted (cholesterol/triglyceride ratio <4) compared to normal. Even the most accurate LDL cholesterol measurement will underestimate by about 10-25% the actual amounts of LDL these individuals have in their bloodstream, compared to those with LDL particles containing a normal amount of cholesterol. Or put another way, measured LDL cholesterol values even for people with LDL particles of the same size can easily vary by 10 to 40 mg/dL without there being any difference in LDL particle concentration (or CVD risk, we would argue).

LDL size differences, as noted earlier, make an independent contribution to LDL cholesterol compositional variability. Although LDL diameters differ by what seems to be only a small amount, typically up to about 3 nm (~12%), the volume differences of the spherical lipid core are substantial because they scale according to the third power of the radius. For LDL particles differing by 3 nm in diameter, there is approximately 40% less core cholesterol in the smaller particle. On this basis alone, the person with the smaller LDL particles will require almost 70% more particles to carry the same amount of LDL cholesterol as the person with the larger particles. A not insignificant number of people (12 of the 118 subjects in our survey) have the double whammy of small LDL particles that are also compositionally cholesterol-poor and triglyceride-rich (far right illustration in Fig. 1). With equal numbers of LDL particles, these individuals can easily have LDL cholesterol values that are 50 mg/dL lower than people who have large LDL particles of normal lipid composition. In the face of LDL cholesterol compositional variability of this magnitude, it is not difficult to understand why LDL cholesterol measurements may not always accurately reflect a patient's cardiovascular disease risk.

Lipoprotein Quantification by NMR Spectroscopy

NMR spectroscopic analysis of lipoprotein subclasses is considerably more efficient than traditional electrophoretic or ultracentrifugal methods, since no physical fractionation of the lipoproteins is required. But potentially more important in terms of clinical utility is the fact that NMR provides direct access for the first time to lipoprotein particle data. Instead of basing lipoprotein quantification on cholesterol or apolipoprotein measurements, the NMR method employs the characteristic signals broadcast by lipoprotein subclasses of different size as the basis of their quantification.^{16,17} Each subclass signal is contributed to by the aggregate number of terminal methyl groups on the lipids contained within the particle, with the cholesterol esters and triglycerides in the particle core each contributing 3 methyl groups and the phospholipids and unesterified cholesterol in the surface shell each contributing 2 methyl groups. The total number of these methyl groups contained within a subclass particle is, to a close approximation, dependent only on the particle's diameter and is not affected by differences in lipid composition arising from such sources as variability in the relative amounts of cholesterol ester and triglycerides in the particle core, varying degrees of unsaturation of the lipid fatty acyl chains, or varying phospholipid composition. For this reason, the methyl NMR signal emitted by each subclass serves as a direct measure of the concentration of that subclass.

Each NMR measurement of fasting plasma produces the signal amplitudes of 15 lipoprotein subclasses (Fig. 3). Using reference data on isolated subclasses of independently-measured diameter and lipid composition, this information is then transformed into concentrations expressed either in particle concentration units (nanomoles of particles per liter, nmol/L) or,

alternatively, in cholesterol or triglyceride mass concentration units (mg/dL).¹⁷ In addition, weighted average VLDL, LDL, and HDL particle sizes are computed. To simplify clinical utilization of the NMR information, several of the subclass levels are grouped to give concentrations of large, intermediate, and small VLDL, LDL and HDL (Fig. 3). Further

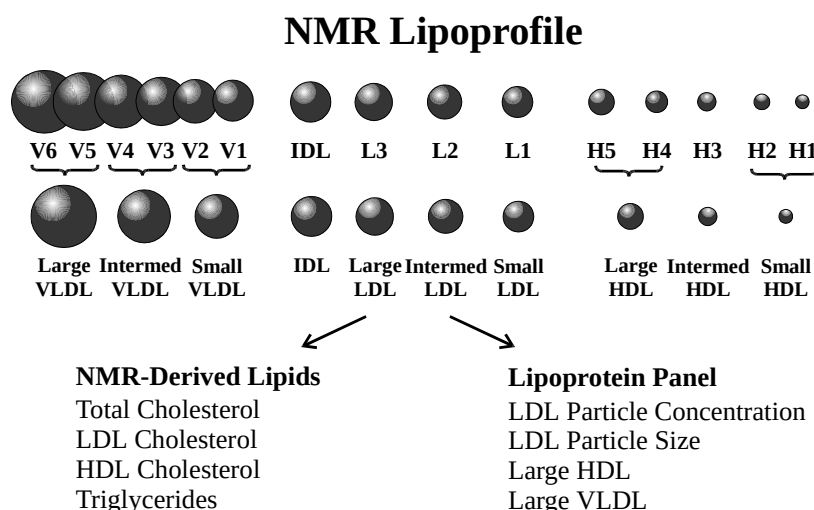


FIGURE 3. Representation of the lipoprotein subclasses quantified by NMR and the information reported in the *NMR LipoProfile*.

summing the relevant subclass levels permits NMR-derived estimates to be made of total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides.

Although there is a high degree of correlation between chemically-measured and NMR-derived lipid levels, it must be stressed that the NMR values come from direct measurement of the lipoprotein particles carrying the lipids, not from an actual lipid measurement. NMR-derived levels are, in effect, the lipid values a person would have with lipoprotein particles of normal composition. When a person's LDL particles are "abnormal" due to cholesterol depletion and triglyceride enrichment, as previously discussed and depicted in Fig. 1, the NMR-derived values will be higher than those measured chemically. The NMR levels will, however, reflect the true

mass concentration of LDL and, as a result, should give a better indication of CVD risk. Recent results from the Cardiovascular Health Study support this expectation by showing that NMR-derived LDL cholesterol levels in women are more predictive of future cardiovascular events (MI and angina) than chemically-measured LDL cholesterol.¹⁸

The four NMR risk factors included in the Lipoprotein Panel (Fig. 3) have potential clinical diagnostic advantages over those in the traditional lipid panel. Based on results from the Quebec Cardiovascular Study and other trials that examined the comparative disease prediction of lipid versus lipoprotein data,^{7-10,19-23} it might be anticipated that LDL particle concentration and LDL particle size will prove to be more informative than total or LDL cholesterol. Although less data are available, several studies support the potential clinical utility of large HDL and large VLDL subclass concentrations as alternatives for the traditional HDL cholesterol and triglyceride risk factors.²²⁻²⁵ To document the extent to which Lipoprotein Panel parameters may improve prediction of cardiovascular disease outcomes, results will soon be available from studies of over 60,000 frozen plasma samples analyzed by NMR during the past two years from many ongoing or completed clinical trials. Included among these are the Framingham Offspring Study, Women's Health Study, Heart and Estrogen Replacement Study (HERS), Honolulu Heart Study, Cardiovascular Health Study, Rancho Bernardo Study, Diabetes Atherosclerosis Intervention Study (DAIS), HDL Atherosclerosis Treatment Study (HATS), Insulin Resistance Atherosclerosis Study (IRAS), Diabetes Control and Complications Trial (DCCT), Fenofibrate Intervention and Event Lowering in Diabetes (FIELD), and the VA-HDL Intervention Trial (VA-HIT).

Clinical Implications of the Disconnect Between LDL Cholesterol and LDL Particles in Patients with Low HDL Cholesterol– Insights from the Framingham Offspring Study

NMR analyses conducted on >3400 frozen plasma samples from the Framingham Offspring Study cohort ²⁶ provide an opportunity to take a fresh look at the reasons for the enhanced cardiovascular disease risk of patients whose primary lipid abnormality is not high LDL cholesterol, but low HDL cholesterol. Prospective epidemiological studies have consistently shown low HDL cholesterol to be a strong CVD risk factor, independent of the level of LDL cholesterol. ⁴ With clinical trial support coming from the recent Veterans Affairs High-Density Lipoprotein Intervention Trial (VA-HIT), ²⁷ raising the level of low HDL is becoming a focus of therapeutic intervention. What still requires further examination, however, is the extent to which the CVD risk associated with low HDL cholesterol and the clinical benefit achieved in VA-HIT can be attributed causally to HDL, *per se*, as opposed to interrelated factors for which low HDL is simply a marker. NMR data from the Framingham Offspring Study, shown below, suggest that a substantial portion of the excess CVD risk experienced by patients with low HDL stems from an unrecognized LDL abnormality, that of excessive numbers of LDL particles containing less cholesterol than normal. High-risk patients with this abnormality, who would be expected to benefit from LDL-lowering therapy, are not now considered candidates for such treatment because they do not have a recognized LDL cholesterol “problem”.

Numerous studies have documented the association of low HDL cholesterol levels with small LDL and HDL particle sizes. ^{28,29} An explanation at the lipoprotein subclass level is provided by smoothed NMR data from the Framingham Offspring Study showing variations in the

concentrations of large, intermediate, and small HDL and LDL particles as a function of HDL cholesterol level (Fig. 4, top panels). In the upper left panel, increasing levels of HDL cholesterol in the population are shown to be accounted for almost entirely by increases in the large HDL subclass. This is the subclass suggested to be the most cardioprotective HDL component in many studies.²²⁻²⁵ It is noteworthy that concentrations of this species are negligible below HDL cholesterol levels of about 40 mg/dL.

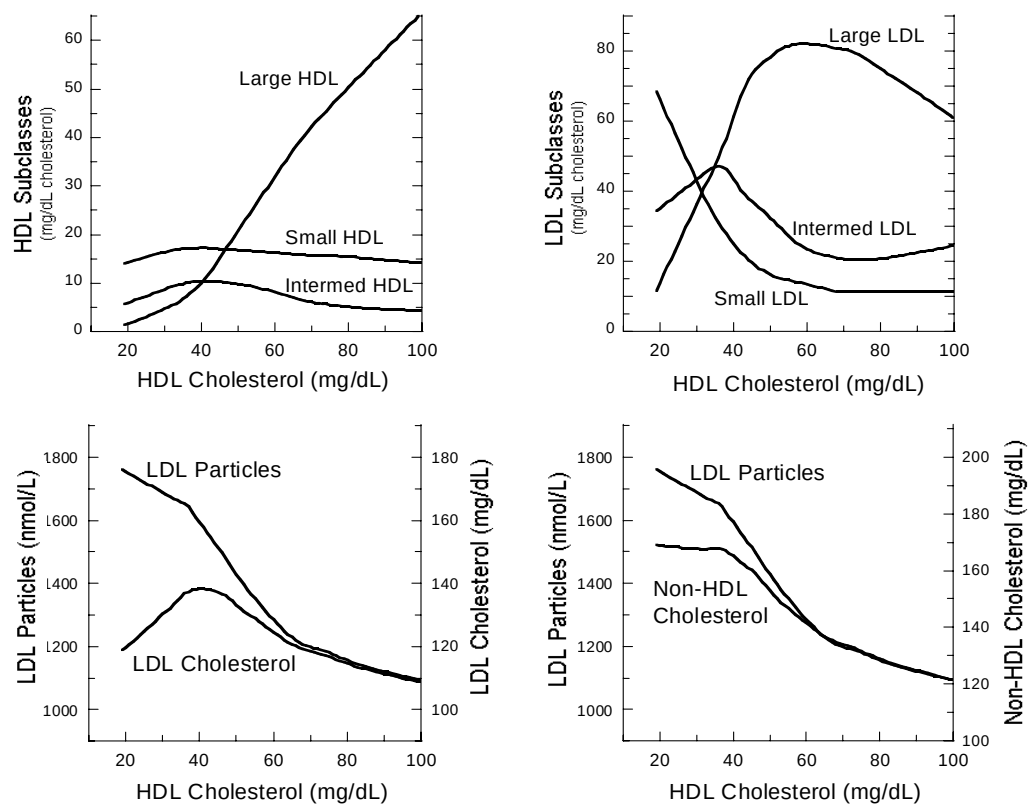


FIGURE 4. Relations in the Framingham Offspring cohort (n=3,437) of HDL cholesterol levels (x-axis) to levels of HDL subclasses (top left panel), LDL subclasses (top right panel), LDL particles and cholesterol (bottom left panel), and LDL particles and non-HDL cholesterol (bottom right panel). HDL and LDL subclasses and LDL particle concentrations were measured by NMR and HDL, LDL (Friedewald estimate), and non-HDL cholesterol was determined by standard chemical methods. Smoothed lines were fitted using LOWESS (locally weighted scatterplot smoother).

As seen in the upper right panel of Fig. 4, concentrations of the large LDL subclass greatly exceed those of small LDL when HDL cholesterol levels are greater than 50 mg/dL. However, as

HDL cholesterol decreases below 50 mg/dL there is a progressive shift in LDL subclass distribution with increases in small LDL accompanied by decreases in large LDL. The average LDL size and amount of cholesterol per LDL particle is therefore significantly lower in populations with low HDL cholesterol compared to those with higher HDL. The consequence of this strong association of HDL level with the cholesterol content of LDL particles is the strikingly large "disconnect" observed between LDL cholesterol and LDL particle concentrations in individuals with relatively low HDL cholesterol values (Fig. 4, bottom left). In those with HDL cholesterol levels less than about 40 mg/dL, the data indicate that LDL cholesterol values are likely to considerably underestimate LDL particle concentrations and, by inference, the risk of CVD.

The current Adult Treatment Panel III (ATP III) guidelines of the NCEP discuss the limitations of LDL cholesterol as an indicator of the number of atherogenic lipoprotein particles, but only in hypertriglyceridemic patients.³⁰ Citing apoB assay deficiencies and their lack of universal availability, the guidelines do not advise that apoB be used as an alternative to LDL cholesterol as a target of therapy. Instead, non-HDL cholesterol was recommended as a secondary target of therapy, since this routinely-measurable parameter has a high correlation with apoB. In the bottom right panel of Fig. 4, relations between non-HDL cholesterol and LDL particles are compared as a function of HDL cholesterol levels. Although there is still a "disconnect" between NMR-measured LDL particle concentrations and non-HDL cholesterol at low HDL cholesterol levels, the magnitude is less than with LDL cholesterol. Clinical trial data must be awaited to establish the relative strengths of association of LDL cholesterol, LDL particles, apo B, and non-HDL cholesterol with cardiovascular disease outcomes.

Prevalence of the Disconnect Between LDL Cholesterol and LDL Particles

The ATP III guidelines recommend LDL cholesterol targets of < 100 mg/dL for patients with CHD or CHD risk equivalents, < 130 mg/dL for patients with ≥ 2 risk factors and a 10-year Framingham risk ≤ 20 percent, and < 160 mg/dL for patients with 0-1 risk factors.³⁰ As shown in Fig. 5, these targets correspond to the 20th, 50th, and 80th percentile values in the Framingham Offspring population. The LDL particle concentration distribution, also given in Fig. 5, shows that the corresponding 20th, 50th, and 80th percentile values for this parameter are 1100 nmol/L, 1400 nmol/L, and 1800 nmol/L, respectively.

To gain some feeling for the prevalence of clinically relevant discrepancies between LDL cholesterol and LDL particle concentrations, we used the Framingham Offspring data to compare the numbers and characteristics of people who might be classified as “high-risk” or “low-risk” on the basis of having LDL cholesterol or LDL particle concentrations above the 80th percentile or below the 20th percentile, respectively. In Table 1, it is seen that only 419 (62%) of the 681 individuals with high-risk LDL cholesterol levels >80th percentile (>160 mg/dL) also have high-risk LDL particle concentrations >80th percentile (>1800 nmol/L). There are thus 262 individuals (38%) classified as high-risk using LDL cholesterol criteria who have a lower-risk status according to their LDL particle concentrations, and a different group of 262 people classified as high-risk based on having elevated numbers of LDL particles that would fall in a lower-risk category using LDL cholesterol stratification.

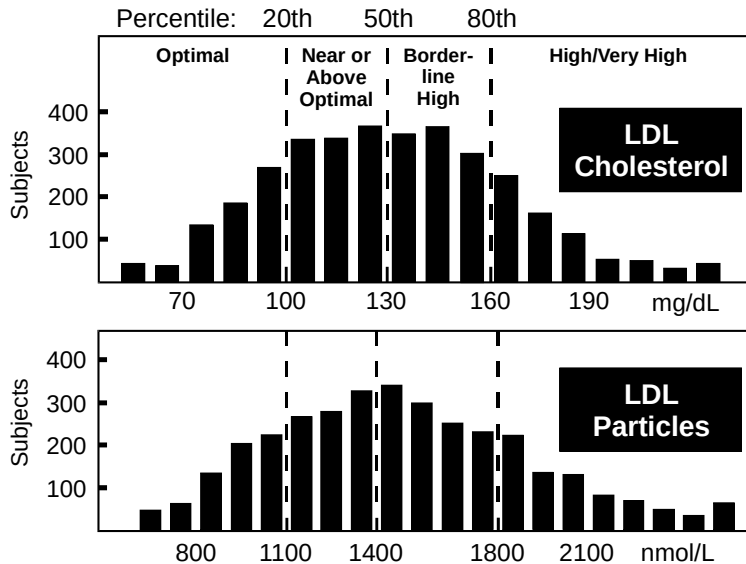


FIGURE 5. Histograms for LDL cholesterol (Friedewald estimate) and NMR-measured LDL particle concentration from the Framingham Offspring Study (n=3,437).

Insight into which of these two groups actually has the higher risk comes from an examination of mean values of several non-LDL risk factors. Comparing the last two columns of Table 1, it can be seen that those in the high-risk LDL particle group have a considerably worse risk factor profile consisting of higher levels of triglycerides, lower HDL cholesterol, and smaller LDL particles. Such a profile is characteristic of persons with the metabolic syndrome.³¹ ATP III recognized the need to invoke the metabolic syndrome as a major contributor to CVD risk *beyond LDL cholesterol* precisely because the risk of these individuals exceeds that which can be accounted for by LDL cholesterol. It remains to be established, however, how much the metabolic syndrome contributes to CVD risk *beyond LDL particles*. From the data presented in Fig. 4 and Table 1, it seems likely that LDL particles make a much greater contribution to the risk of patients with the metabolic syndrome than has generally been recognized. Aggressive LDL lowering therapy may be at least as beneficial to these patients as treating the underlying causes of the syndrome and the accompanying atherogenic dyslipidemia as recommended by ATP III.

TABLE 1**High-Risk Subjects in the Framingham Offspring Study According to LDL Particle Concentration and LDL Cholesterol**

	LDL Particles >80 th percentile	LDL Particles >80 th percentile	LDL particles <80 th percentile
	LDL Cholesterol >80 th percentile	LDL Cholesterol <80 th percentile	LDL Cholesterol >80 th percentile
Number of subjects (%)	419 (62%)	262 (38%)	262 (38%)
LDL cholesterol (mg/dL)	189	142	172
LDL particles (nmol/L)	2160	2000	1620
Triglycerides (mg/dL)	157	192	104
HDL cholesterol (mg/dL)	43	39	52
LDL size (nm)	20.6	20.3	21.1
LDL pattern B	43%	61%	8%
Men	56%	66%	48%

TABLE 2**Low-Risk Subjects in the Framingham Offspring Study According to LDL Particle Concentration and LDL Cholesterol**

	LDL Particles <20 th percentile	LDL Particles <20 th percentile	LDL particles >20 th percentile
	LDL Cholesterol <20 th percentile	LDL Cholesterol >20 th percentile	LDL Cholesterol <20 th percentile
Number of subjects (%)	421 (66%)	218 (34%)	218 (34%)
LDL cholesterol (mg/dL)	82	113	90
LDL particles (nmol/L)	890	990	1310
Triglycerides (mg/dL)	76	70	146
HDL cholesterol (mg/dL)	58	62	45
LDL size (nm)	21.1	21.2	20.6
LDL pattern B	11%	6%	42%
Men	34%	28%	50%

At the low-risk end of the spectrum, a similar case can be made that LDL particle concentration <1100 nmol/L (<20th percentile) may be a more appropriate target of therapy than LDL cholesterol <100 mg/dL (<20th percentile) for patients with CHD or CHD risk equivalents. As shown in Table 2, 34% of Framingham Offspring subjects with "optimal" LDL cholesterol <100 mg/dL have sub-optimal LDL particle concentrations >1100 nmol/L. This group, compared to that with the opposite phenotype ("optimal" LDL particles <20th percentile and sub-optimal LDL cholesterol >20th percentile), has higher triglycerides, lower HDL cholesterol, smaller LDL size, and contains more men, all of which are characteristics associated with higher risk. By recognizing that a major source of this higher risk is a higher-than-desirable number of LDL particles, despite LDL cholesterol being "optimal," physicians treating patients with CHD or CHD risk equivalents would have justification for targeting selected individuals for more aggressive LDL lowering therapy than would be called for by ATP III guidelines.

References

1. Fredrickson DS, Levy RI, Lees RS. Fat transport in lipoproteins - an integrated approach to mechanisms and disorders. *N Engl J Med* 1967;276:148-156.
2. Beisiegel U, St. Clair RW. An emerging understanding of the interactions of plasma lipoproteins with the arterial wall that leads to the development of atherosclerosis. *Curr Opin Lipidol* 1996;7:265-268.
3. Williams KJ, Tabas I. The response-to-retention hypothesis of early atherogenesis. *Arterioscler Thromb Vasc Biol* 1995;15:551-561.
4. Castelli WP, Garrison RJ, Wilson PWF, Abbott RD, Kalousdian S, Kannel WB. Incidence of coronary heart disease and lipoprotein cholesterol levels: The Framingham Study. *JAMA* 1986;256:2835-2838.
5. Austin MA, Hokanson JE, Edwards KL. Hypertriglyceridemia as a cardiovascular risk factor. *Am J Cardiol* 1998;81:7B-12B.
6. Sniderman A, Shapiro S, Marpole D, Malcolm I, Skinner B, Kwiterovich PO. The association of coronary atherosclerosis and hyperapobetalipoproteinemia (increased protein but normal cholesterol content in human plasma low density lipoprotein). *Proc Natl Acad Sci USA* 1980;97:604-608.

7. van Lennep JE, Westerveld HT, van Lennep HW, Zwinderman AH, Erkelens DW, van der Waal EE. Apolipoprotein concentrations during treatment and recurrent coronary artery disease events. *Arterioscler Thromb Vasc Biol* 2000;20:2408-2413.
8. Gotto AM, Whitney E, Stein EA, Shapiro DR, Clearfield M, Weis S, Jou JY, Langendorfer A, Beere PA, Watson DJ, Downs JR, de Cani JS. Relation between baseline and on-treatment lipid parameters and first acute major coronary events in the Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS). *Circulation* 2000;101:477-484.
9. Lamarche B, Despres JP, Moorjani S, Cantin B, Dagenais GR, Lupien PJ. Prevalence of dyslipidemic phenotypes in ischemic heart disease (Prospective results from the Quebec Cardiovascular Study). *Am J Cardiol* 1995;75:1189-1195.
10. Waldius G, Jungner I, Holme I, Aastveit AH, Kolar W, Steiner E. High apolipoprotein B, low apolipoprotein A-1, and improvement in the prediction of fatal myocardial infarction (AMORIS study): a prospective study. *Lancet* 2001;358:2026-2033.
11. Sniderman AD, Cianflone K. Measurement of apoproteins: time to improve the diagnosis and treatment of the atherogenic dyslipoproteinemias. *Clin Chem* 1996;42:489-491.
12. Sniderman AD, Pedersen T, Kjekshus J. Putting low-density lipoproteins at center stage in atherogenesis. *Am J Cardiol* 1997;79:64-67.
13. Sniderman AD, Scantlebury T, Cianflone K. Hypertriglyceridemic hyperapoB: the unappreciated atherogenic dyslipoproteinemia in type 2 diabetes mellitus. *Ann Intern Med* 2001;135:447-459.
14. Otvos J. Measurement of triglyceride-rich lipoproteins by nuclear magnetic resonance spectroscopy. *Clin Cardiol* 1999;22(6 Suppl):II21-27.
15. Packard CJ. Understanding coronary heart disease as a consequence of defective regulation of apolipoprotein B metabolism. *Curr Opin Lipidol* 1999;10:237-244.
16. Otvos JD, Jeyarajah EJ, Bennett DW, Krauss RM. Development of a proton NMR spectroscopic method for determining plasma lipoprotein concentrations and subspecies distribution from a single, rapid measurement. *Clin Chem* 1992;38:1632-1638.
17. Otvos JD. Measurement of lipoprotein subclass profiles by nuclear magnetic resonance spectroscopy. IN: Handbook of Lipoprotein Testing (Rifai N, Warnick GR, Dominiczak MH, eds), AACC Press, Washington DC, 2000, pp 609-623.
18. Kuller LH, Tracy R, Arnold A, Otvos JD, Burke G, Psaty B, Siscovick D, Kronmal R. (Abstract) Relationship between LDL size, number of particles and risk of coronary heart disease in the Cardiovascular Health Study. *Circulation* 2001;(suppl II) 104:II-826.
19. Austin,MA, Breslow,JL, Hennekens,CH, Buring,JE, Willett,WC, Krauss,RM: Low-density lipoprotein subclass patterns and risk of myocardial infarction. *JAMA* 1988;260:1917-1921.
20. Stampfer MJ, Krauss RM, Ma J, Blanche PJ, Holl LG, Sacks FM, Hennekens CH. A prospective study of triglyceride level, low-density lipoprotein particle diameter, and risk of myocardial infarction. *JAMA* 1996;276: 882-888.
21. Griffin BA, Freeman DJ, Tait GW, Thomson J, Caslake MJ, Packard CJ, Shapard J. Role of plasma triglyceride in the regulation of plasma low density lipoprotein (LDL) subfractions: relative contribution of small, dense LDL to coronary heart disease risk. *Atherosclerosis* 1994;106:241-253.
22. Freedman,DS, Otvos,JD, Jeyarajah,EJ, Barboriak,JJ, Anderson,AJ, Walker,JA: Relation of lipoprotein subclasses as measured by proton nuclear magnetic resonance spectroscopy to coronary artery disease. *Arterioscler Thromb Vasc Biol* 1998;18:1046-1053.

23. Rosenson RS, Freedman DS, Otvos JD. Relations of lipoprotein subclass levels and LDL size to progression of coronary artery disease in the PLAC I trial. *Amer J Cardiol*. In press.
24. Wilson HM, Patel JC, Russe D, Skinner ER. Alterations in the concentration of an apolipoprotein-E-containing subfraction of plasma high-density lipoprotein in coronary heart disease. *Clin Chim Acta* 1993;220:175-187.
25. Johansson J, Carlson LA, Landou C, Hamsten A. High-density lipoproteins and coronary atherosclerosis: a strong inverse relation with the largest particles is confined to normotriglyceridemic patients. *Arterioscler Thromb* 1991;11:174-182.
26. Couture P, Otvos JD, Cupples LA, Wilson PWF, Schaefer EJ, Ordovas JM. Association of the A-204C polymorphism in the cholesterol 7 α -hydroxylase gene with variations in plasma low density lipoprotein cholesterol levels in the Framingham Offspring Study. *J Lipid Res* 1999;40:1883-1889.
27. Robins SJ, Collins D, Wittes JT, Papademetriou V, Deedwania PC, Schaefer EJ, McNamara JR, Kashyap ML, Hershman JM, Wexler LF, Rubins HB. Relation of gemfibrozil treatment and lipid levels with major coronary events. *JAMA* 2001;285:1585-1591.
28. Williams PT, Krauss RM, Vranizan KM, Stefanick ML, Wood PDS, Lindgren FT. Associations of lipoproteins and apolipoproteins with gradient gel electrophoresis estimates of high density lipoprotein subfractions in men and women. *Arterioscler Thromb* 1992;12:332-340.
29. Rainwater DL. Lipoprotein correlates with LDL particle size. *Atherosclerosis* 2000;148:151-158.
30. Executive Summary of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). *JAMA* 2001;285:2486-2497.
31. Grundy SM. Hypertriglyceridemia, insulin resistance, and the metabolic syndrome. *Am J Cardiol* 1999;83:25F-29F.

Chapter 5: Discussion

5.1 Advantages of NMR lipoprotein testing

The *NMR LipoProfile* lipoprotein particle assay presented in this work has several advantages over traditional methods of lipoprotein subclass measurements. It is very rapid and efficient, taking less than a minute to collect the NMR data, with another minute or two required for sample delivery to and removal from the measurement cell. The entire process is automatable. The fundamental information accessed by this technology, namely concentrations of lipoprotein subclass particle numbers, appears to give superior prediction of atherosclerotic risk compared to alternate methods that rely on lipid markers as surrogate measures of lipoprotein levels. There is no need for any reagents and the calibration of the instrumentation is achieved simply and inexpensively with a stable standard such as trimethyl acetic acid (TMA). The validation studies have established that the NMR method has excellent measurement precision, with the CVs of important clinical variables in the 2-4% range. The standard addition studies established that the method provides accurate results and that the dynamic range of the assay is very large. The LDL particle number has important clinical use as a target for risk reduction therapy in those being treated with diet, exercise, or lipid lowering drugs. The efficiency with which the automated analysis can be performed makes it possible in a short period of time to analyze thousands of archived frozen plasma samples from observational and drug intervention studies addressing the risk of CHD, diabetes, insulin resistance and the metabolic syndrome.

5.2 Interferences

There are very few interferences with the *NMR LipoProfile* assay. The free glycerol (in disease states, dialysis, and exercise) that interferes with standard chemical analysis for TG is not an issue, since the NMR assay measures only intact lipoprotein particles. Low to moderate hemolysis has been shown not to affect the NMR results. Samples affected by gross hemolysis, however, are not suitable for NMR or chemical analyses. Certain gel barrier blood collection tubes contribute a contaminating substance that gives rise to an NMR signal that appears in the methyl lipid region of the plasma spectrum. Depending on the type of gel and manufacturer, the interfering signal can be a singlet or triplet. The magnitude of the interfering signal depends on the contact time with the gel and the volume of sample drawn. Low draw volumes and longer contact with the gel increase the amplitude of these signals. We have developed computational methods to circumvent this problem by including the spectrum of the interfering substance(s) as one of the components in the basis set used for the lineshape deconvolution.

Samples with highly abnormal composition, like those from patients with Type III hyperlipidemia, are not fit adequately with the normal analysis model and are flagged on that basis by the analysis software. Over 99% of plasma samples encountered give a “good” lineshape fit (correlation coefficient >0.999 between calculated and experimental methyl signal) and are thus considered successfully analyzed. Other samples consistently flagged on the basis of giving relatively poor lineshape fits are those containing the abnormal lipoprotein called Lp-X. Patients with obstructive jaundice and liver cirrhosis often have Lp-X in their plasma. Lp-X has a density similar to LDL, but has very high percentage of unesterified cholesterol and phospholipid. Special analysis models have been constructed to analyze

samples containing Lp-X and physicians are notified about the possible clinical implications of the presence of Lp-X.

5.3 Stability of plasma for NMR analysis

Accuracy of the *NMR LipoProfile* assay requires that the native structure of the lipoprotein particles be maintained during storage and transport of the plasma samples. Exposure of plasma to ambient temperature for longer than 8 hours, or even short-term exposure to higher temperatures (for example, samples without cold packs in an over-heated car trunk) can affect the *LipoProfile* results. Blood samples should always be spun and refrigerated promptly. Refrigerated samples are stable for up to 10 days. The need for refrigeration is a limitation of the technique, but one shared by other lipid assays. It is possible that this limitation may be at least partially overcome by the addition of lipoprotein stabilizers to the blood collection tubes.

NMR data are not appreciably affected by freezing of the samples at -70°C or below. On 200 pairs of fresh and frozen samples it was found that the LDL particle number and size correlations are >0.95 . Samples with TG levels above about 400 mg/dL show significant reductions in NMR-derived TG levels after a single freeze/thaw cycle. The problem is exacerbated by multiple freeze thaw cycles. There is also some evidence that post-prandial samples containing chylomicrons and remnant particles are more susceptible to these freeze/thaw-related reductions in TG levels. In certain cases, aggregation of VLDL/chylomicron particles generated by freeze/thaw appears to produce a broad NMR signal and the quality of lineshape fit is affected. Removal of VLDL from such samples by

fast ultracentrifugation has been shown to overcome the problem and give reliable LDL and HDL subclass information.

5.4 Miscellaneous

Plasma and serum samples from various animal models of atherosclerosis and diabetes have been tested for their ability to be analyzed by NMR using either the unmodified human lipoprotein subclass analysis model or models containing lipoprotein species unique to that particular species. For example, normal mice have very high levels of HDL-C and larger HDL subclasses than usually found in humans. Samples from monkeys, mice, rats, hamsters, dogs and pigs have all been successfully analyzed by NMR.

We have also demonstrated that NMR lipoprotein analysis may be successfully carried out on the NMR spectrometer platforms of both major NMR manufacturers, Bruker and Varian. There is excellent inter-machine reproducibility of *NMR LipoProfile* analyses that are conducted using 15 different NMR instruments. Most of the data presented in this work were acquired with a presaturation water suppression scheme. Gradient water suppression using the WET technique (74) works equally well. The presaturation process reduces the intensity of the plasma protein signal by a factor of approximately two (via saturation transfer), thereby making the lipoprotein peaks in the methyl region more prominent. The WET technique does not affect the signals from either the plasma proteins or lipoproteins, and therefore the methyl signal is contributed to relatively less by the lipoproteins and more by the plasma proteins. There is no evidence that the accuracy or reproducibility of the NMR LipoProfile assay is any different using one or the other water suppression methods.

Conclusion

Development of a nuclear magnetic resonance-based lipoprotein particle assay is presented. The validation studies document the accuracy, precision and robustness of the *NMR LipoProfile* assay.

References[†]

[†] for chapters 1, 2 and 5.

1. Stamper J. Population Studies. In: Levy RI, Rifkind BM, Dennis BH, Ernst N. (Eds.), Nutrition. Lipids and coronary diseases: A global view, New York: Raven, 1979:25-88.
2. Keys AB. Seven Counties: A multivariate analysis of death and coronary heart disease. Harvard University Press 1980:1-381.
3. Kannel WB, Castelli WP, Gordon T. Cholesterol in the prediction of atherosclerotic disease. New perspectives based on the Framingham Study. *Ann Intern Med* 1979;90:85-91.
4. Study group, European Atherosclerosis Society. Strategies for the prevention of coronary heart disease: A policy statement of the European Atherosclerosis Society. *Eur Heart J* 1987;8:77-88.
5. Castelli W, Doyle JT, Gordon T, et al. HDL cholesterol and other lipids in coronary heart disease. The Cooperative Lipoprotein Phenotyping Study. *Circulation* 1977;55:767-772.
6. Gordon DJ, Rifkind BM. High-density lipoproteins: the clinical implications of recent studies. *N Engl J Med* 1989;321:1311-1316.
7. Report of the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults – National Cholesterol Education Program, National Heart, Lung, and Blood Institute. *Arch Intern Med* 1988;145:36-69.
8. Hegsted DM, Nicholosi RJ. Individual variation in serum cholesterol levels. *Proc Natl Acad Sci USA* 1987;84:6259-61.
9. Naito HK. Reliability of lipid, lipoprotein, and apolipoprotein measurements. *Clin Chem* 1988;34(Suppl):B84-94.
10. Executive Summary of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). *JAMA* 2001;285:2486-2497.
11. Grundy SM, Becker D, Clark LT, et al. Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation and Treatment of high blood cholesterol in adults (Adult Treatment Panel III) Final Report. *Circulation* 2002;106:3143-3421.
12. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 1972;18:499-502.

13. N. Rifai, GR Warnick, MH Dominiczak (eds.). Handbook of lipoprotein testing, 2nd edition, Washington, AACC Press, 2000.
14. N. Rifai, GR Warnick, (eds.). Methods for clinical laboratory measurement of lipid and lipoprotein risk factors. Washington, AACC Press, 1991.
15. Zak B. Cholesterol methodologies: A review. Clin Chem 1977;23:1201-14.
16. Hassemer DJ, Koch DD, Wiebe DA. Four commercially-available cholesterol methods meet 1992 NCEP guidelines. Clin Chem 1988;34:1231.
17. Bachorick PS, Virgil DG, Derby C, et al. Enzymatic analysis of total- and HDL-cholesterol: Comparison with the standardized Lieberman-Burchard method used by the lipid research clinics program. Clin Chem Acta 1988;174:307-14.
18. Klotzsch SG, McNamara JR. Triglyceride measurements: A review of methods and interferences. Clin Chem 1990;36:1605-13.
19. Warnick GR, Cheung MC, Albers JJ. Comparison of current methods for high density lipoprotein cholesterol quantitation. Clin Chem 1979;25:596-604.
20. Fredrickson DS, Levy RI, Lees RS. Fat transport in lipoproteins - an integrated approach to mechanisms and disorders. N Engl J Med 1967;276:148-156.
21. Beisiegel U, St. Clair RW. An emerging understanding of the interactions of plasma lipoproteins with the arterial wall that leads to the development of atherosclerosis. Curr Opin Lipidol 1996;7:265-268.
22. Sniderman AD, St-Pierre AC, Cantin B, Dagenais GR, Després, JP, Lamarche B. Concordance/Discordance between plasma apolipoprotein B levels and the cholesterol indexes of atherosclerotic risk. Am J Cardiol 2003;91:1173-7.
23. Austin MA, Breslow JL, Hennekens CH, Buring GE, Willet WC, Krauss RM. Low-density lipoprotein subclass patterns and risk of myocardial infarction. JAMA 1988;260:1917-21.
24. Gardner CD, Fortman SP, Krauss RM. Association of small low-density lipoprotein particles with the incidence of coronary artery disease in men and women. JAMA 1996;276:875-81.
25. Lamarche B, Tchernof A, Moorjani S, Cantin B, Dagenais GR, Després, J-P. Small, dense, low-density lipoprotein particles as a predictor of the risk of ischemic heart disease in men: prospective results from Québec Cardiovascular Study. Circulation 1997;95:69-75.

26. Austin MA, King M-C, Vranizan KM, Krauss RM. Atherogenic lipoprotein phenotype: A proposed genetic marker for coronary heart disease risk. *Circulation* 1990;82:495-506.
27. Johansson J, Carlson LA, Landou C, Hamsten A. High-density lipoproteins and coronary atherosclerosis: a strong inverse relation with the largest particles is confined to normotriglyceridemic patients. *Arterioscler Thromb* 1991;11:174-82.
28. Wilson HM, Patel JC, Russe D, Skinner ER. Alterations in the concentration of an apolipoprotein-E-containing subfraction of plasma high-density lipoprotein in coronary heart disease. *Clin Chim Acta* 1993;220:175-87.
29. Lindgren FT, Elliot HA, Gofman JW. The ultracentrifugal characterization and isolation of human blood lipids and lipoproteins, with applications to the study of atherosclerosis. *J Phys Colloid Chem* 1951;55:80-93.
30. Schumaker VN, Puppione DL. Sequential flotation ultracentrifugation. *Methods Enzymol* 1986;128:155-70.
31. Lee DM, Downs D. A quick and large-scale density gradient subfractionation method for low density lipoproteins. *J lipid Res* 1982;23:14-27.
32. Chung BH, Segrest JP, Marjory JR, et al. Single vertical spin density gradient ultracentrifugation. In: Segrest JP, Albers JJ, eds. *Methods in Enzymol. Vol 128. Plasma Lipoproteins*. Academic Press, Orlando, FL, 1986;128:181-209.
33. Chapman MJ, Laplaud PM, Luc G, et al. Further resolution of low density lipoprotein spectrum in normal human plasma: physicochemical characteristics of discrete subspecies separated by density gradient ultracentrifugation. *J Lipid Res* 1988;29:442-58.
34. Rudel LL, Marzetta CA, Johnson FL. Separation and Analysis of Lipoproteins by Gel Filtration. *Methods Enzymol* 1986;129:45-56.
35. Sata T, Havel RJ, Jones AL. Characterization of subfractions of triglyceride-rich lipoproteins separated by gel chromatography from blood plasma of normolipemic and hyperlipemic humans. *J Lip Res* 1972; 13:757-768.
36. Nichols AV, Krauss RM, Musliner TA. Nondenaturing polyacrylamide gradient gel electrophoresis. In: Segrest JP, Albers JJ, eds. *Plasma Lipoproteins. Methods Enzymol* 1986;128:417-31.
37. Rainwater DL, Moore PH, Shelledy WR, Dyer TD. Characterization of a composite gradient gel for the electrophoretic separation of lipoproteins. *J Lipid Res* 1997;38:1261-6.
38. Cheung MC, Wolf AC. Differential effect of ultracentrifugation on apolipoprotein A-I containing lipoprotein subpopulations. *J Lipid Res* 1988;29:15-26.

39. J. D. Otvos, E.J. Jeyarajah, D.W. Bennett. Quantification of Plasma Lipoproteins by Proton Nuclear Magnetic Resonance Spectroscopy. *Clin Chem* 1991;37:377-386.
40. Otvos JD, Jeyarajah EJ, Bennett DW, Krauss RM. Development of a proton nuclear magnetic resonance spectroscopic method for determining plasma lipoprotein concentrations and subspecies distributions from a single, rapid measurement. *Clin Chem* 1992;38:1632-8.
41. James Otvos, Elias Jeyarajah, Dennis Bennett. A Spectroscopic Approach to Lipoprotein Subclass Analysis. *Journal of Clinical Ligand Assay* 1996;19:184-189.
42. Steim JM, Edner OJ, Bargoot FG. Structure of human lipoproteins: nuclear magnetic resonance supports a micellar model. *Science* 1968;162:909-911.
43. Bell JD, Sadler PJ, Macleod AF, Turner PR, La Ville A. ^1H NMR studies of human blood plasma. *FEBS Lett* 1987;219:239-43.
44. Otvos JD, Coffey MC, Chen S-M, Wehrli S. An improved method for the detection of malignancy by proton NMR spectroscopy of plasma. *Biochem Biophys Res Commun* 1987;145:1397-403.
45. Chmurny GN, Hilton BD, Halverson, D, et al. An NMR blood test for cancer: a critical assessment. *NMR Biomed* 1988;1:136-50.
46. Herring FG, Phillips PS, Pritchard PH. Proton magnetic resonance spectroscopy of plasma from patients with dyslipoproteinemia: identification of factors governing methyl and methylene proton line widths. *J Lipid Res* 1989;30:521-8.
47. Mims MP, Morrisett JD, Mattioli CA, Gotto AM Jr. Effect of triglyceride levels on methyl and methylene envelope linewidths in proton magnetic resonance spectroscopy of human plasma. *N Engl J Med* 1989;320:1452-7.
48. Otvos JD, Jeyarajah EJ, Hayes LW, Freedman DS, Janjan NA, Anderson T. Relationships between the proton nuclear magnetic resonance properties of plasma lipoproteins and cancer. *Clin Chem* 1991;37:369-76.
49. Mika Ala-Korpela. ^1H NMR spectroscopy of human blood plasma. *Prog NMR Spec* 1995;27:475-554.
50. Lounila J, Ala-Korpela M, Jokisaari J. Effects of Orientational Order and Particle Size on the NMR Line Positions of Lipoproteins. *Phys Rev Lett* 1994;72:4049-4052.
51. Otvos JD. Measurement of lipoprotein subclass profiles by nuclear magnetic resonance spectroscopy. In: Rifai N, Warnick GR, Dominiczak MH, eds. *Handbook of Lipoprotein Testing*. Washington, DC: AACC Press; 2000:609-23.

52. Freedman DS, Otvos JD, Jeyarajah EJ, Shalaurova I, Cupples LA, Parise H, D'Agastino RB, Wilson PW, Schaefer EJ. Sex and age differences in lipoprotein subclasses measured by Nuclear Magnetic Resonance spectroscopy: The Framingham Study. *Clin Chem* 2004;50:1189-1200.
53. Hiltunen Y, Ala-Korpela M, Jokisaari J, Eskelinen S, Kivinitty K, Savolainen M, Kesaniemi YA. A lineshape fitting model for ^1H NMR spectra of human blood plasma. *Magn Reson Med* 1991;21:222-32.
54. Hiltunen Y, Ala-Korpela M, Jokisaari J, Eskelinen S, Kivinitty K. Proton nuclear magnetic resonance lineshape studies on human blood plasma lipids from newborn infants, healthy adults, and adults with tumors. *Magn Reson Med* 1992;26:89-99.
55. Ala-Korpela M, Korhonen A, Keisala J, Horkko S, Korpi P, Ingman LP, Jokisaari J, Savolainen M, Kesaniemi YA. ^1H NMR-based absolute quantitation of human lipoproteins and their lipid contents directly from plasma. *J Lipid Res* 1994;35:2292-2304.
56. Hiltunen Y, Heinieme E, Ala-Korpela M. Lipoprotein-lipid quantification by neural network analysis of ^1H NMR data from human blood plasma. *J Magn Reson B* 1995;106:191-4.
57. Ala-Korpela M, Hiltunen Y, Bell JD. Quantification of biomedical NMR data using artificial neural network analysis: Lipoprotein lipid profiles from ^1H NMR data of human plasma. *NMR Biomed* 1995;8:235-44.
58. Bathen TF, Engan T, Krane J. Principal component analysis of proton nuclear magnetic resonance spectra of lipoprotein fractions from patients with coronary heart disease and healthy subjects. *Scand J Clin Lab Invest* 1999;59:349-60.
59. Bathen TF, Krane J, Engan T, Bjerve KS, Axelson D. Quantification of plasma lipids and apolipoproteins by use of proton NMR spectroscopy, multivariate and neural network analysis. *NMR Biomed*. 2000;13:271-88.
60. Bathen TF, Engan T, Krane J, Axelson D. Analysis and classification of proton NMR spectra of lipoprotein fractions from healthy volunteers and patients with cancer or CHD. *Anticancer Res*. 2000;20:2393-408.
61. Freedman DS, Otvos JD, Jeyarajah EJ, Barboriak JJ, Anderson AJ, Walker JA. Relation of lipoprotein subclasses as measured by proton nuclear magnetic resonance spectroscopy to coronary artery disease. *Arterioscler Thromb Vasc Biol* 1998;18:1046-1053.
62. Hatch FT, Lees RS. Practical Method for Plasma Lipoprotein Analysis. *Adv Lipid Res* 1968;6:1-68.
63. Caslake MJ, Packard CJ. The use of ultracentrifugation for the separation of lipoproteins. In: *Handbook of lipoprotein testing*. 2nd ed. AACC Press, Washington, DC 2000;625-46.

64. Contois JH, McNamara JR, Lammi-Keefe CJ, Wilson PWF, Massov T, Schaefer EJ. Reference intervals for plasma apolipoprotein B determined with a standardized commercial immunoturbidimetric assay: results from the Framingham Offspring Study. *Clin Chem* 1996;42:515-23.
65. Forte TM, Nordhausen RW. Electron Microscopy of Negatively Stained Lipoproteins. In: Segrest JP, Albers JJ, eds. *Structure of Plasma Lipoproteins*. *Methods Enzymol* 1986;128:442-57.
66. Rainwater DL. Lipoprotein correlates of LDL particle size. *Atherosclerosis* 2000;148:151-8.
67. Rainwater DL, Moore PH, Gamboa IO. Improved method for making nondenaturing composite gradient gels for the electrophoretic separation of lipoproteins. *J Lipid Res* 2004;45:773-5.
68. Bax A. A spatially selective composite 90^0 radiofrequency pulse. *J Magn Reson* 1985;65:142-5.
69. Westgard JO, Klee GG. Quality Management. Chapter 16 in *Fundamentals of Clinical Chemistry*, 4th edition. Burtis C, ed., WB Saunders Company, Philadelphia, 1996;211-23.
70. Westgard JO, Barry PL, Hunt MR, Groth T. A multi-rule Shewhart chart for quality control in clinical chemistry. *Clin Chem* 1981;27:493-501.
71. Lawson, C. L., Hanson, R. J. *Solving Least Squares problems*. Philadelphia, PA: SIAM, 1995, pp. 160-165.
72. Hager, W.W. *Applied Numerical Linear Algebra*. Englewood Cliffs, NJ: Prentice Hall, 1988, pp 294-302.
73. Tony F. Chan. An improved algorithm for computing the Singular Value Decomposition. *ACM Trans Math Softw* 1982;8:72-83.
74. Zhang S, Yang X, Gorenstein DG. Suppression of linearly responsive residual water. *Concepts in Mag Res* 2002;14:102-111.