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Lipoprotein Particle Analysis by Nuclear Magnetic Resonance Spectroscopy

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Laboratory measurements of plasma lipids (principally cholesterol and triglycerides) and lipoprotein lipids (principally low-density lipoprotein [LDL] and high-density lipoprotein [HDL] cholesterol) have been the cornerstone of the clinical assessment and management of atherosclerotic cardiovascular disease (CVD) risk since the Friedewald formula was introduced in 1972 for estimating LDL cholesterol [1]. The lipoprotein particles that carry cholesterol and triglycerides in the bloodstream are the direct mediators of the atherosclerotic process. LDL particles, and to a lesser extent very–low-density lipoprotein [VLDL] particles, cause atherosclerosis by entering the artery wall, becoming oxidized, and subsequently being ingested by macrophages, creating cholesterol-rich foam cells that develop into atherosclerotic plaque. HDL particles entering the artery wall prevent or reverse this process by, among other actions, inhibiting the oxidation of LDL particles and removing cholesterol from the foam cells for delivery back to the liver—a process called *reverse cholesterol transport*. The overall risk for CVD depends on the balance between the ''bad'' LDL (and VLDL) and ''good'' HDL particles.

Traditionally, this lipoprotein balance has been assessed by measuring the cholesterol content of a patient's LDL and HDL particles (LDL-C and HDL-C) rather than the numbers of these particles. The reason is purely analytic; measuring the amounts of cholesterol or triglyceride in plasma or a particular class of lipoprotein (VLDL, LDL, HDL) is straightforward,

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whereas direct assessment of lipoprotein particle numbers was not possible until the advent of nuclear magnetic resonance (NMR) spectroscopic analysis.

The distinction between lipoprotein lipid levels and lipoprotein particle numbers is potentially important clinically, because the two measures are not equivalent. Lipoproteins are the spherical containers of lipid molecules that have a common structure: a shell consisting mainly of phospholipids and specific proteins (apolipoproteins) and a core consisting of a mixture of cholesterol ester and triglyceride molecules. The cholesterol (and triglyceride) content of LDL, HDL, and VLDL particles is not constant, but varies widely among individuals and can change over time as a result of lipid-altering drug treatment or lifestyle changes. Consequently, two patients who have the same measured concentration of LDL-C, for example, can have significantly different numbers of LDL particles and therefore a different risk for CVD [2].

Lipoproteins differ in their cholesterol and triglyceride contents for two reasons [3,4]. First, the sizes of the particles within a given lipoprotein class are not identical (the volumes of the containers are different). Individuals who have elevated triglycerides, for example, are likely to have VLDL particles that are larger and more triglyceride-rich than usual, and LDL and HDL particles that are smaller and more cholesterol-poor. Second, the relative amounts of cholesterol and triglyceride carried in the particle core can differ among individuals. These compositional differences are also related to plasma triglyceride levels, with LDL and HDL particles becoming more cholesterol-depleted and triglyceride-rich as plasma triglyceride levels increase. For these reasons, even the most accurate lipoprotein cholesterol tests will frequently fail to accurately indicate the numbers of atherogenic and antiatherogenic lipoprotein particles in a patient's plasma and the CVD risk these particles confer.

NMR LipoProfile analysis

The concept of using proton NMR spectroscopy to measure plasma lipoprotein particle concentrations in an efficient, reagentless manner was introduced in the early 1990s by researchers at North Carolina State University [5,6]. After the method was further developed and refined to enable simultaneous quantification of multiple lipoprotein subclasses [7,8], the test was commercialized for clinical research in 1997 by LipoScience, Inc. (formerly LipoMed, Inc., Raleigh, North Carolina) and then made available for patient care as the NMR LipoProfile test. Almost 2 million NMR LipoProfile tests have been performed in LipoScience's laboratory in North Carolina, more than 200 clinical studies have been completed, 150 studies are ongoing, and NMR lipoprotein particle data have been reported in more than 110 publications. The NMR LipoProfile test will be decentralized in 2007 when a fully automated, turnkey NMR clinical analyzer, whose operation requires no experience with NMR, will be made available to laboratories worldwide.

Numbers of lipoprotein subclass particles can be quantified by NMR because of two phenomena: (1) VLDL, LDL, and HDL subclasses of different size in plasma simultaneously emit distinctive NMR signals whose individual amplitudes can be accurately and reproducibly measured; and (2) the measured subclass signal amplitudes are directly proportional to the numbers of subclass particles emitting the signal, irrespective of variation in particle lipid composition.

The process begins with automated measurement of the proton NMR spectrum of the patient's plasma or serum sample (approximately 200 μL) using a dedicated 400-MHz NMR analyzer. The digitized spectrum is stored in computer memory and the analysis software then extracts the amplitudes of the individual subclass NMR signals, converts them to concentration units (typically nanomoles of particles per liter, nmol/L), and outputs the data either in spreadsheet form (for clinical research) or in the NMR LipoProfile report format. The overall measurement process requires only approximately 1 minute.

An example of a plasma NMR spectrum is shown in Fig. 1A. Many signals appear in the spectrum from numerous metabolites, but only the composite signal envelope at approximately 0.8 ppm is used for lipoprotein particle quantification. This signal envelope contains the signals emitted by the terminal methyl group protons of the four types of lipid in the lipoprotein particles: phospholipid, unesterified cholesterol, cholesterol ester, and triglyceride. Each lipoprotein subclass signal emanates from 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 three methyl groups, and the phospholipids and unesterified cholesterol in the surface shell each contributing two methyl groups. Because the methyl signals from these lipids are indistinguishable from each other, they overlap to produce a bulk lipid particle signal. The amplitude of each lipoprotein subclass signal serves as a measure of the particle concentration of that subclass.

The methyl lipid signal can be used for lipoprotein subclass quantification (without first physically separating the subclasses, as is required by electrophoretic or ultracentrifugal methods) because of a magnetic property specific to lipoproteins that causes the lipids in larger particles to broadcast signals that are characteristically different in shape and higher in frequency than the lipid signals emitted by smaller particles [9]. A clarifying analogy has been drawn between lipoprotein subclasses and bells of varying size. Just as bells of different size produce unique sound "signals," related reasons associated with the physical form of lipoprotein particles allow different-sized subclasses to broadcast distinguishable lipid NMR signals. If a group of bells is struck with equal-force blows (similar to inducing the subclasses to emit their NMR signals on application of a radiofrequency pulse), the amplitude (loudness) of the resultant sound signal is expected to reflect the number of bells struck. By recording the composite signal envelope

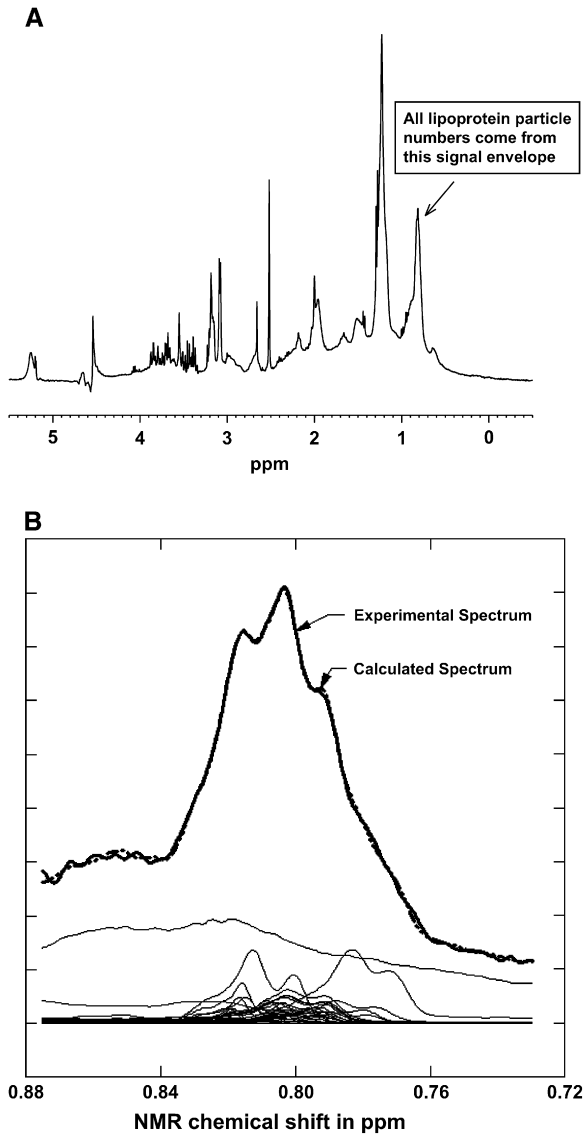

Fig. 1. Proton NMR spectrum of blood plasma. (*A*) A typical proton NMR spectrum of human blood plasma recorded at 47°C on a 400-MHz NMR clinical analyzer. The signal envelope centered at approximately 0.8 ppm arises from the methyl proton signals of the lipoproteins. (*B*) A representative deconvolution of the methyl signal (0.7–0.9 ppm). The solid line represents the experimental spectrum, and the closely matched dotted line is the calculated analytical sum of the lipoprotein subclass signals that are shown at the bottom. The broad peak in the middle accounts for the nonlipoprotein plasma protein signal that also appears in the same region.

produced by the simultaneous ''ringing'' of all of the lipoprotein subclasses in a plasma sample, the concentration of each subclass can be deduced using prior empiric knowledge about the quantitative relationship between the particle concentration of each subclass and its signal amplitude.

The individual subclass signal amplitudes are derived computationally from the recorded plasma methyl signal envelope through a linear least-squares deconvolution process using singular value decomposition [6]. Fig. 1B shows a representative plasma methyl signal and the virtually identical calculated spectrum (dotted line) produced by the linear combination of subclass signal amplitudes derived from the deconvolution. The raw material for each spectral deconvolution is a library of more than 30 methyl signals from every spectrally distinct VLDL, LDL, and HDL subclass likely to be encountered in the plasma of patients who have normolipidemia or dyslipidemia. This library took several years to assemble, requiring preparative subclass isolation from the plasma of a diverse donor population using a combination of ultracentrifugation and agarose gel chromatography. Each of these isolated subclass reference standards was then characterized for particle size distribution using either electron microscopy or gradient gel electrophoresis (GGE) and for lipid composition using chemical analysis. Fig. 2 shows representative spectra of various VLDL, LDL, and HDL subclasses. Each methyl signal has a distinct, complex lineshape, and larger subclass particles produce higher-frequency signals (further to the left) than smaller particles.

Because the NMR signal of each of the more than 30 subclasses in the deconvolution library differs only slightly in frequency and lineshape from the signals of neighboring subclasses, measurement reproducibility of the individual signal amplitudes is inherently limited. To overcome this limitation, and because there is no indication that these numerous subpopulations have unique metabolic relations or clinical usefulness, the neighboring subclasses are grouped empirically into a smaller number of subclass categories (large, medium, and small) so that the summed amplitudes of the individual subpopulation signals provide acceptable measurement precision (coefficients of variation [CVs] $< \sim 10\%$). Table 1 shows the estimated diameter ranges of the particles comprising the subclasses for which the NMR LipoProfile report provides concentrations.

To relate the measured subclass signal amplitudes to subclass particle concentrations, expressed as moles (6.02×10^{23}) of particles per liter, a set of conversion factors was derived for each member of the subclass reference library. Independent determinations were made of the lipid composition and particle size distribution of every isolated subclass reference standard. Particle concentrations (nmol/L for VLDL and LDL; μmol/L for HDL) were calculated for each subclass standard based on existing knowledge about the commonality of lipoprotein structure and the link between particle diameter and total core lipid (cholesterol ester plus triglyceride) content [10,11]. With this information, the relationship was established

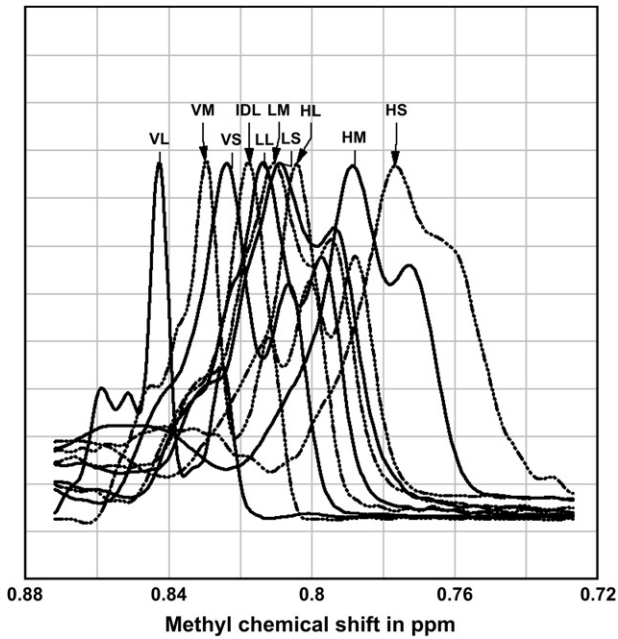

Fig. 2. Spectra of several lipoprotein subclass reference standards in the deconvolution library. The subclass components were isolated by ultracentrifugation and agarose column chromatography. VL, large VLDL; VM, medium VLDL; VS, small VLDL; IDL, intermediate density lipoproteins; LL, large LDL; LM, medium LDL; LS, small LDL; HL, large HDL; HM, medium HDL; HS, small HDL.

between the signal amplitude of each subclass and the concentration of particles giving rise to that amplitude.

The constancy of the relationship between subclass signal amplitude and particle concentration is what gives NMR its unique ability to quantify lipoprotein particle numbers, even in the face of significant variation in the cholesterol composition of subclass particles among individuals. The major source of subclass lipid compositional variability is cholesterol ester–triglyceride exchange mediated by cholesterol ester transport protein (CETP) [3]. Because (to a close approximation) the number of methyl groups in a particle of given size remains constant despite the mutual exchange of cholesterol ester for triglyceride (because both triglyceride and cholesterol ester contribute three methyl groups to the overall subclass methyl signal), the relationship between subclass signal amplitude and particle concentration is maintained although the relationship between subclass cholesterol (or triglyceride) and signal amplitude may vary.

Despite the lack of an invariant relationship between subclass signal amplitudes and subclass cholesterol or triglyceride concentrations, estimates of lipoprotein lipid levels can be derived from the NMR particle measurements using conversion factors that assume that the various subclass

Table 1
Diameter ranges of lipoprotein subclasses measured by nuclear magnetic resonance

NMR lipoprotein parameter	Diameter range (nm)
VLDL	
Large VLDL/chylomicrons	>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

particles have the lipid content of an individual who is normolipidemic. Because subclass lipid compositional variability is restricted for VLDL and HDL particles, NMR-estimated and chemically measured triglyceride and HDL-C levels generally agree ($r > 0.9$). NMR-estimated values for LDL-C less closely correlate with chemical LDL-C measurements ($r = \sim 0.8$–0.9) [8].

Finally, weighted average VLDL, LDL and HDL particle sizes (in nm diameter units) can be calculated from the various subclass concentrations by summing the known diameter of each subclass multiplied by its relative mass percentage as estimated from the intensity of its methyl NMR signal. However, to be consistent with particle sizes estimated by GGE, these are mass-weighted, not particle number–weighted average diameters. For this reason, reported diameters may often seem inconsistent with the relative numbers of large and small particle subclasses.

NMR LipoProfile specimen requirements

Preferred specimens

NMR LipoProfile analyses can be performed on plasma and serum samples. Fasting blood samples are required if VLDL subclass concentrations or fasting triglyceride levels are needed. Otherwise, either fasting or nonfasting specimens are suitable for analysis, because LDL and HDL subclass concentrations are minimally altered in the postprandial state. Blood should be drawn into lavender-top EDTA collection tubes or plain red-top serum tubes. Plasma from heparin tubes is also acceptable. Serum separator tubes are not suitable for NMR specimen collection because they introduce substances producing NMR signals that interfere with the analysis. Blood samples should be promptly centrifuged (at 3000 rpm for 10 to 15 minutes at

room temperature) and the separated plasma or serum refrigerated immediately. If immediate centrifugation is not possible, specimens may be refrigerated and centrifuged within 24 hours. Refrigerated plasma or serum specimens may be stored up to 7 days without affecting NMR results.

Frozen sample stability

Refrigerated specimens that are unable to be analyzed within 7 days of blood collection should be promptly frozen at −70°C, after which they are stable indefinitely (for at least 10 years). If a −70°C freezer is unavailable, samples may be stored at −20°C and subsequently transferred to a −70°C freezer within 1 month. Storage for longer than 1 month at −20°C is likely to alter NMR LipoProfile results. The only specimens for which freezing may adversely affect NMR lipoprotein results are postprandial samples or samples with fasting triglyceride values greater than approximately 300 mg/dL. Freezing these samples may alter (lower) chylomicron and VLDL subclass concentrations, but LDL and HDL determinations are not significantly affected. Accurate measurement of chylomicrons and VLDL subclasses in postprandial or hypertriglyceridemic samples can be assured only if the samples have never been frozen. Data from a split-sample comparison study of 397 pairs of fresh and −70°C frozen plasma samples gave correlations for all parameters that were comparable to those obtained for duplicate analyses of fresh samples. Subjecting samples to multiple freeze–thaw cycles is more likely to alter NMR results, so this procedure should be avoided.

Hemolyzed specimens

The NMR LipoProfile assay is virtually unaffected by hemolysis. In a study in which increasing amounts of hemolysate were added to pooled serum, the NMR lipoprotein results were not significantly affected up to a hemoglobin concentration of 500 mg/dL, which is considered gross hemolysis. Therefore, NMR LipoProfile analysis may be performed successfully on samples from patients undergoing dialysis treatment, because they are prone to gross hemolysis.

NMR LipoProfile measurement precision

Because the NMR assay is fully automated and does not require physical separation of the lipoprotein subclasses, the measurement reproducibility of NMR lipoprotein profiles is very good. Tables 2 and 3 display the results of a precision study to estimate intra-assay (within-run) and inter-assay precision (or total imprecision) of the various NMR-measured lipoprotein parameters. Two plasma pools were prepared, one with nominally high triglycerides and low HDL (pool A), and the other with low triglycerides

Table 2
Intra-assay and inter-assay measurement precision: pool A

NMR lipoprotein	Intra-assay precision[a]			Inter-assay precision[b]		
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/chylomicrons	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

Abbreviation: CV, coefficient of variation.

[a] Intra-assay measurement precision was based on analysis of 20 replicates of each of two plasma pools (A & B).

[b] Inter-assay precision (measure of total imprecision) was derived from the analysis of frozen aliquots of each of two plasma pools for 20 days across six instruments.

** %CV not reported because mean values for these parameters for the samples analyzed are very low and similar to SD.

and high HDL (pool B). The pools were aliquoted and frozen. Intra-assay precision was determined by thawing and analyzing 20 replicates of each of the two pools on one NMR analyzer, following standard protocols. Inter-assay precision was evaluated by analyzing a frozen aliquot of each of the two pools for 20 consecutive days across six different NMR analyzers.

Total inter-assay imprecision was only slightly worse than the intra-assay imprecision. The CVs for the particle concentrations of VLDL, LDL, and HDL classes were 4% or less, and approximately 2% in most cases. The CVs for the individual subclasses (large, medium, and small) that made appreciable contributions to these totals were generally less than 6%. NMR estimates of total and VLDL triglycerides and HDL-C ranged from 1% to 2%. LDL and HDL particle sizes had CVs of approximately 0.5%.

Table 3
Intra-assay and inter-assay measurement precision: pool B

NMR lipoprotein	Intra-assay precision[a]			Inter-assay precision[b]		
Parameter (Units)	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/chylomicrons	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	7.0	6.8	**	6.0	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

Abbreviation: CV, coefficient of variation.

[a] Intra-assay measurement precision was based on analysis of 20 replicates of each of two plasma pools (A & B).

[b] Inter-assay precision (measure of total imprecision) was derived from the analysis of frozen aliquots of each of two plasma pools for 20 days across 6 instruments.

** %CV not reported because mean values for these parameters for the samples analyzed are very low and similar to SD.

Relationship between NMR–derived and chemically measured lipid levels

As described earlier, NMR estimates of lipoprotein lipid levels may be made by converting NMR subclass particle numbers to lipid mass concentration units by assuming that the various subclass particles have a normal lipid content. Previously published data showed that NMR-derived total triglycerides and HDL-C values correlate well with chemically measured values using samples from healthy volunteers [7,8]. Shown in Fig. 3 are additional data from a randomly selected group of 255 patient samples sent by physicians to LipoScience for analysis. NMR LipoProfile measurements were conducted in a single day using 11 different NMR analyzers. Aliquots of the same samples were used for chemical determinations of triglycerides

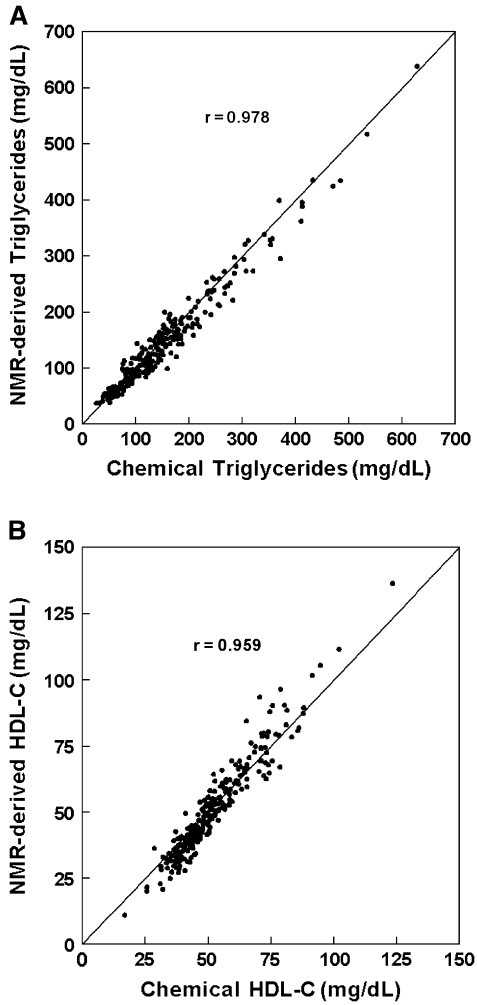

Fig. 3. Comparison of NMR-derived and chemically measured triglycerides and HDL cholesterol. Line drawn is the line of identity (n = 251). (*A*) $r = 0.978$ for triglycerides; regression line $y = 0.94x + 2.7$. (*B*) $r = 0.959$ for HDL-C; regression line $y = 1.16x - 9.3$.

and HDL-C (homogeneous assay). Strong correlations were observed between chemical and NMR measures of triglycerides ($r = 0.978$) and HDL-C ($r = 0.959$).

Relationship between low-density lipoprotein particle number and apolipoprotein B

Before the NMR LipoProfile assay was introduced, apolipoprotein B (apo B) assays were the only way to access information about plasma

concentrations of LDL or VLDL particles. Because one molecule of apo B is present on every VLDL and LDL particle, measuring plasma apo B provides the total numbers of VLDL and LDL particles. If the VLDL fraction (density <1.006 g/L) is removed from plasma through ultracentrifugation, an apo B assay on the remaining material provides a measure of LDL apo B, which should be proportional to the number of LDL particles.

To establish the relationship between NMR-measured LDL particle concentration (LDL-P) and LDL apo B, 29 fasting plasma samples were analyzed by NMR to obtain values for LDL-P. Another aliquot of the plasma samples was subjected to ultracentrifugal separation to remove VLDL. The apo B of the bottom fraction was measured using a nephelometric apo B immunoassay to provide values for LDL apo B. The correspondence between these two measures of LDL particle concentration is shown in Fig. 4. As expected, a strong correlation was observed ($r = 0.928$).

Comparison of low-density and high-density lipoprotein particle sizes measured by NMR spectroscopy and gradient gel electrophoresis

LDL and HDL particle sizes determined by NMR LipoProfile analysis and GGE were compared in a split-sample analysis of 15 fasting plasma samples. GGE analyses were performed in Dr. Rainwater's laboratory at the Southwest Foundation for Biomedical Research [12]. Fig. 5A, B show the correlations between NMR-measured average particle sizes and GGE-determined median particle diameters for LDL and HDL, respectively.

LDL particle sizes determined by the two methods were highly correlated ($r = 0.946$). NMR-derived LDL sizes are uniformly smaller by

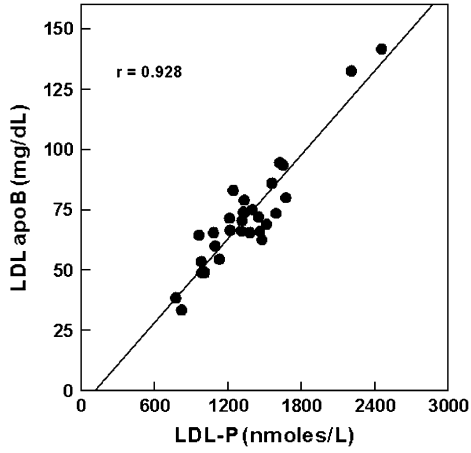

Fig. 4. Correlation of NMR LDL particle number (LDL-P) with LDL apolipoprotein B (apo B). Apo B measurements were conducted on a Beckman Synchron CX-7 analyzer using a commercially available turbidimetric immunoassay (Wako Chemicals, Osaka, Japan).

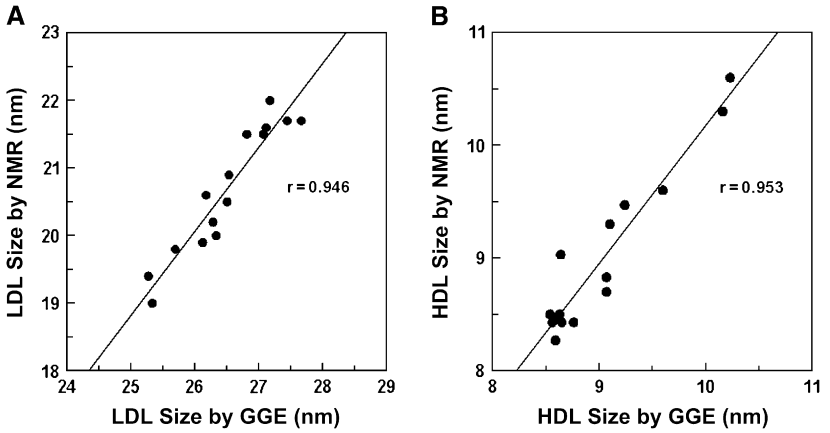

Fig. 5. Comparison of (*A*) LDL and (*B*) HDL particle sizes determined by NMR and gradient gel electrophoresis (GGE). GGE particle sizes are the median particle diameter [12].

approximately 5 to 6 nm compared with those determined by GGE [13]. The mean values for the 15 samples were 20.69 nm for NMR and 26.50 nm for GGE. This difference simply reflects the fact that NMR LDL particle sizes are referenced to diameters measured by electron microscopy, which are consistently smaller than those estimated by the GGE referencing method [11,14,15]. The NMR-determined HDL sizes also correlated extremely well with GGE sizes ($r = 0.953$). The mean values for the HDL sizes were nearly identical: 8.99 nm with NMR and 9.03 nm with GGE.

Normal ranges for the NMR LipoProfile parameters

Table 4 provides the normal ranges for the parameters measured in the NMR LipoProfile assay. The data were compiled from more than 7300 randomly selected fasting plasma samples analyzed at LipoScience in November and December, 2003. The patients were mainly from the Southeastern United States and were a mix of those who did not have coronary disease and were undergoing primary prevention and those who had coronary disease and were undergoing secondary prevention. Ages ranged from 20 to 94 years, with a median age of 58 years. Data are provided separately for men (n = 4054), women (n = 3317), and the total combined population (n = 7371). Mean and median values are reported for each parameter along with ranges defined by the 10th to 90th percentile values.

Correlations among NMR LipoProfile parameters

Many lipoprotein subclasses are metabolically interrelated and therefore their concentrations are not independent. To help evaluate and interpret

Table 4
Normal ranges (10th–90th percentile) for NMR LipoProfile parameters

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

NMR-derived lipoprotein subclass particle data, Spearman correlations among the 20 reported NMR LipoProfile parameters are provided in Table 5. These correlations were determined using the same 7371 patient samples used to generate the normal range data in Table 5.

Consistent with results obtained using other subclass fractionation methods, the correlation data in Table 5 indicate that LDL and HDL particle sizes are strongly correlated ($r = 0.7$) and are inversely related to triglyceride level ($r = -0.5$). Of particular note is the very strong correlation ($r = 0.9$) between the two subsets of small LDL particles, labeled LMS (medium small) and LVS (very small). Because these subsets have virtually identical associations with all other lipoprotein parameters, categorizing them as *small*, rather than the previous designations of *intermediate* and *small*, was considered appropriate. Combining the two subclasses into a single subclass called *small LDL* may not only provide simplification, but possibly also stronger relations with CVD.

The correlations in Table 5 can also help in assessing the quality of frozen specimens from clinical trials. If sample integrity was compromised by improper storage conditions (eg, storage warmer than $-70^{\circ}C$, multiple freeze–thaw cycles), it will be reflected by a weakening of the expected subclass correlations.

Clinical use of the NMR lipoprotein particle assay

NMR LipoProfile test results

To simplify and enhance clinical use of NMR lipoprotein particle information, test results are supplied to clinicians in a two-page NMR LipoProfile report, which is organized into four sections:

1. LDL Particle Numbers
2. Lipids
3. Metabolic Syndrome Markers
4. Subclass Particle Numbers

The ''LDL Particle Numbers'' section is the main focus of clinical decision-making about a patient's LDL-based CVD risk, and is shown in Fig. 6. In prospective epidemiologic and clinical intervention trials, LDL-P values have been shown consistently to have stronger associations with CVD risk than LDL-C (representative data are summarized in the following section). Population data have been obtained on more than 6800 participants in the National Institutes of Health (NIH)-sponsored Multi-Ethnic Study of Atherosclerosis (MESA) to relate levels of LDL-P to LDL-C and provide a basis for suggested LDL-P treatment targets equivalent to those of the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) for LDL-C [16]. A patient's LDL-P concentration is reported along with a highlighted LDL risk category that corresponds to the ATP III risk

Table 5
Correlations between NMR LipoProfile parameters[a]

	VLDLP	VL	VM	VS	LDLP	IDL	LL	LS	LMS	LVS	HDLP	HL	HM	HS	VZ	LZ	HZ	NTG	NVTG	NHDLC
VLDLP	—	0.5	0.9	0.6	0.4	0.4	−0.4	0.5	0.4	0.5	−0.3	−0.5	−0.1	0.1	−0.5	−0.5	−0.5	0.9	0.9	−0.5
VL	0.5	—	0.5	0.1	0.2	0.4	−0.2	0.3	0.3	0.3	0	−0.4	0.1	0.2	0.3	−0.3	−0.3	0.7	0.7	−0.2
VM	0.9	0.5	—	0.3	0.3	0.2	−0.5	0.5	0.4	0.5	−0.3	−0.5	−0.1	0.1	−0.4	−0.5	−0.5	0.9	0.9	−0.4
VS	0.6	0.1	0.3	—	0.3	0.4	−0.1	0.2	0.2	0.3	−0.2	−0.3	0	0.1	−0.6	−0.2	−0.3	0.4	0.4	−0.3
LDLP	0.4	0.2	0.3	0.3	—	0.3	−0.1	0.8	0.8	0.9	−0.2	−0.3	−0.1	0.1	−0.2	−0.5	−0.5	0.4	0.3	−0.4
IDL	0.4	0.4	0.2	0.4	0.3	—	−0.2	0.4	0.3	0.4	−0.1	−0.4	0.1	0.1	−0.1	−0.3	−0.4	0.4	0.3	−0.3
LL	−0.4	−2	−0.5	−0.1	−0.1	−0.2	—	−0.6	−0.6	−0.5	0.4	0.7	0	−0.1	0.2	0.9	0.6	−0.4	−0.5	0.6
LS	0.5	0.3	0.5	0.2	0.8	0.4	−0.6	—	1.0	1.0	−0.3	−0.6	−0.1	0.1	−0.2	−0.8	−0.7	0.5	0.5	−0.6
LMS	0.4	0.3	0.4	0.2	0.8	0.3	−0.6	1.0	—	0.9	−0.3	−0.5	−0.1	0.1	−0.2	−0.8	−0.6	0.5	0.5	−0.5
LVS	0.5	0.3	0.5	0.3	0.9	0.4	−0.5	1.0	0.9	—	−0.4	−0.6	−0.2	0.1	−0.2	−0.8	−0.7	0.5	0.5	−0.6
HDLP	−0.3	0	−0.3	−0.2	−0.2	−0.1	0.4	−0.3	−0.3	−0.4	—	0.6	0.3	0.6	0.2	−0.1	−0.2	0.2	0.2	0.3
HL	−0.5	−0.4	−0.5	−0.3	−0.3	−0.4	0.7	−0.6	−0.5	−0.6	0.6	—	0	0	0.2	0.7	0.8	−0.5	−0.6	0.9
HM	−0.1	0.1	−0.1	0	−0.1	0.1	0	−0.1	−0.1	−0.2	0.3	0	—	−0.1	0.1	0.1	0	0	0	0.2
HS	0.1	0.2	0.1	0.1	0.1	0.1	−0.1	0.1	0.1	0.1	0.6	0	−0.1	—	0	−0.1	−0.2	0.2	0.2	0.3
VZ	−0.5	0.3	−0.4	−0.6	−0.2	−0.1	0.2	−0.2	−0.2	−0.2	0.2	0.2	0.1	0	—	0.2	0.3	−0.3	−0.3	0.3
LZ	−0.5	−0.3	−0.5	−0.2	−0.5	−0.3	0.9	−0.8	−0.8	−0.8	−0.1	0.7	0.1	−0.1	0.2	—	0.7	−0.5	0.6	0.7
HZ	−0.5	−0.3	−0.5	−0.3	−0.5	−0.4	0.6	−0.7	−0.6	−0.7	−0.2	0.8	0	−0.2	0.3	0.7	—	−0.5	−0.5	0.8
NTG	0.9	0.7	0.9	0.4	0.4	0.4	−0.4	0.5	0.5	0.5	0.2	−0.5	0	0.2	−0.3	−0.5	−0.5	—	1.0	−0.4
NVTG	0.9	0.7	0.9	0.4	0.3	0.3	−0.5	0.5	0.5	0.5	0.2	−0.6	0	0.2	−0.3	0.6	−0.5	1.0	—	−0.5
NHDLC	−0.5	−0.2	−0.4	−0.3	−0.4	−0.3	0.6	−0.6	−0.5	−0.6	0.3	0.9	0.2	0.3	0.3	0.7	0.8	−0.4	−0.5	—

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

[a] Spearman correlations, based on NMR analysis of 7371 random fasting patient plasma samples performed at LipoScience in November/December, 2003.

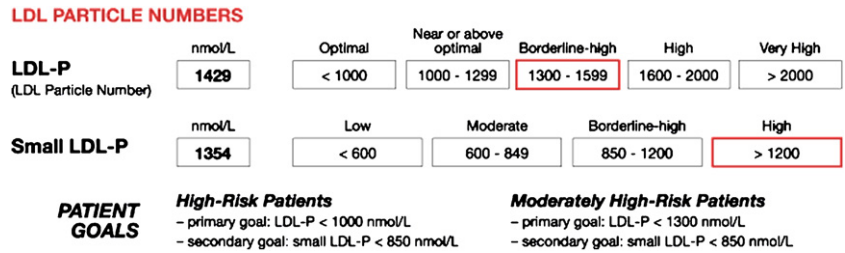

Fig. 6. LDL Particle Numbers section of NMR LipoProfile report.

categories for LDL-C based on cut points of 100, 130, 160, and 190 mg/dL. Optimal LDL-P is less than 1000 nmol/L (<20th percentile in MESA), analogous to the NCEP optimal LDL-C of less than 100 mg/dL. Near or above optimal LDL-P values are 1000 to 1299 nmol/L (20th–50th percentile), borderline-high values are 1300 to 1599 nmol/L (50th–80th percentile), high values are 1600 to 2000 nmol/L (80th–95th percentile), and very high values are more than 2000 nmol/L (>95th percentile).

For patients deemed to be at high risk for CVD based on current ATP III guidelines, the LDL-P goal of less than 1000 nmol/L (<20th percentile) is a reasonable alternative to the LDL-C goal of less than 100 mg/dL and optional goal of less than 70 mg/dL for patients at very high risk. For patients at moderately high risk, LDL-P less than 1300 nmol/L (<50th percentile) is an alternative to the LDL-C goal of less than 130 mg/dL. LDL-P, like LDL-C, can be lowered effectively through statin therapy in conjunction with diet and exercise.

High levels of the small LDL subclass (small LDL-P) are also associated strongly with CVD risk. Small LDL-P is often elevated in individuals who have high triglycerides or low HDL and is a significant source of CVD risk in patients who have diabetes mellitus or metabolic syndrome. Small LDL-P less than 850 nmol/L (<50th percentile) is suggested to be a reasonable secondary treatment goal for high-risk and moderately high-risk patients. Small LDL-P is lowered effectively through diet and exercise and by combining agents such as niacin or a fibrate with LDL-lowering drugs.

The "Lipids" section, shown in Fig. 7, reports all of the information contained in a traditional lipid panel. Values for triglycerides and HDL-C are

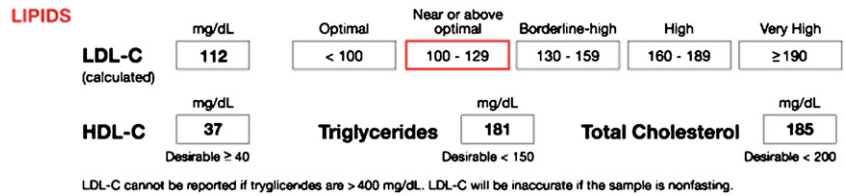

Fig. 7. Lipids section of NMR LipoProfile report.

NMR-derived, total cholesterol is measured by a conventional chemistry assay, and LDL-C is calculated using the Friedewald formula.

When a patient's LDL-C and LDL-P values are discrepant (as in the example shown, with LDL-C near or above optimal and LDL-P borderline-high), clinical outcomes data indicate that LDL-P levels are more indicative of the patient's LDL-based risk.

The "Metabolic Syndrome Markers" section, depicted in Fig. 8, reports key traits associated with metabolic syndrome and increased risk for type 2 diabetes mellitus.

LDL particle size is the estimated average diameter (in nm) of the patient's LDL particles, with values ranging from 18 to 23 nm. Patients who have small average LDL size (18 to 20.5 nm; pattern B) are more likely to be insulin resistant and have metabolic syndrome, and are at increased risk for developing type 2 diabetes mellitus [17–20]. Large HDL-P is the particle concentration of the large HDL subclass, which has an inverse association with insulin resistance and metabolic syndrome [17,18]. Low concentrations are defined as less than 4 μmol/L (<25th percentile in MESA) and confer an increased risk for developing type 2 diabetes mellitus. Large VLDL-P is the particle concentration of the large VLDL subclass, which is strongly associated with triglycerides, insulin resistance, and metabolic syndrome [17,18,21]. High concentrations are defined as more than 5 nmol/L (>75th percentile in MESA) and confer an increased risk for developing type 2 diabetes mellitus.

The "Subclass Particle Numbers" section, presented in Fig. 9, displays the particle concentrations of the individual VLDL, LDL, and HDL subclasses and indicates whether these values are high or low relative to values observed in a contemporary, ethnically diverse reference population in the United States (MESA).

The numbers in parentheses above each bar indicate the subclass particle numbers. The height of each bar indicates, in percentile units, whether the subclass levels are high or low.

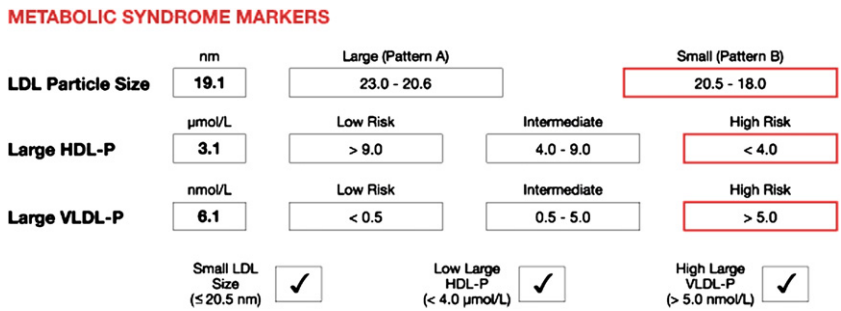

Fig. 8. Metabolic Syndrome Markers section of NMR LipoProfile report.

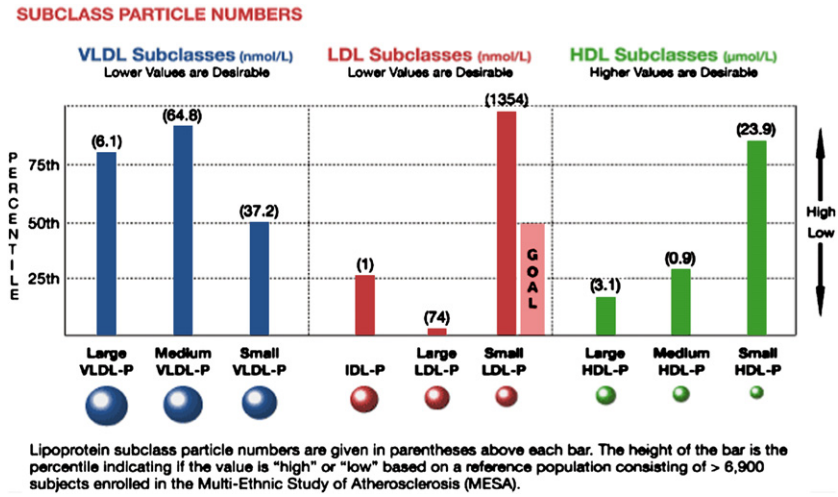

Fig. 9. Subclass Particle Numbers section of NMR LipoProfile report.

Relationship between NMR LipoProfile parameters and disease outcomes

Because the NMR LipoProfile assay can successfully analyze plasma stored for long periods at −70°C, archived baseline samples from numerous completed observational and intervention studies have been analyzed to establish relationships between the various NMR lipoprotein measures, particularly LDL particle number and size; "hard" clinical and "soft" subclinical CVD outcomes; and end points such as insulin resistance, diabetes, and metabolic syndrome.

Relationship with insulin resistance, diabetes, and metabolic syndrome

Garvey and colleagues [17] measured the NMR lipoprotein profile in patients who had type 2 diabetes mellitus and patients who were not diabetic, but had a wide range of insulin sensitivity as defined by the hyperinsulinemic-euglycemic clamp. The strongest relationships with insulin resistance and diabetes were found for large HDL-P (inversely), large VLDL-P, and total and small LDL-P. LDL-P differed significantly between individuals who were insulin sensitive and those who were insulin resistant, but LDL-C did not. In the much larger Insulin Resistance Atherosclerosis Study (IRAS), very similar strong subclass associations were found with insulin resistance [19] and incident type 2 diabetes mellitus [20]. In the latter study, VLDL particle size and small HDL-P predicted incident diabetes independently of lipids and insulin sensitivity measured with frequently sampled intravenous glucose tolerance testing. More recently, LDL-P and small LDL-P, but not LDL-C, were shown to be strongly associated with metabolic syndrome

in the Framingham Heart Study [18]. In a study comparing the effects of two insulin sensitizers, pioglitazone and rosiglitazone, on lipids and lipoproteins in patients who had type 2 diabetes, pioglitazone lowered LDL-P despite significantly raising LDL-C, because of a large increase in the size (and cholesterol content) of the patients' LDL particles [22].

Relationship of high-density lipoprotein particle subclass with cardiovascular disease outcomes

In the Pravastatin Limitation of Atherosclerosis in the Coronary Arteries (PLAC-I) statin intervention trial, on-trial levels of large and small HDL particle subclasses were associated (oppositely) with progression of angiographically documented coronary artery disease, independent of HDL-C and other lipids [23]. In the Veterans Affairs HDL Intervention Trial (VA-HIT), NMR-measured total HDL-P and small HDL-P were strong, independent predictors of recurrent CVD events, whereas levels of HDL-C were not [24]. A recent study showed that among 5538 subjects in the MESA trial who were not taking lipid-lowering medication, total HDL particle number was more strongly associated with carotid atherosclerosis compared with HDL cholesterol [16].

Relations of low-density lipoprotein particle size and low-density lipoprotein particle number with cardiovascular disease outcomes

One report recently reviewed the associations of CVD risk with LDL particle size and LDL particle number in more than 70 cross-sectional and prospective epidemiologic and clinical intervention trials [25]. With few exceptions, small LDL particle size (pattern B) was found to be significantly associated with CVD risk in univariate analyses. However, the origin of this risk association remains controversial. Many authors cite indirect lines of evidence implicating atherogenic properties of small-sized LDL particles. Various data indicate that small LDL more easily enters the arterial wall, undergoes localized retention caused by binding with arterial wall proteoglycans, exhibits enhanced oxidizability in several in vitro models, and directly participates in the production of subendothelial macrophage foam cells [26]. Collectively, these findings imply that small LDL is a potent atherogenic lipoprotein, and that its measurement may be useful for enhancing CVD risk prediction and better evaluating response to lipid therapy [27–29].

However, small-sized LDL particles are most commonly present as a component of a broader pathophysiology characterized by high triglycerides, low HDL-C, increased LDL particle number, obesity, insulin resistance, diabetes, and metabolic syndrome [18,30–32]. As a result, it is unclear whether the increased risk associated with small LDL size in univariate analyses reflects an increased atherogenic potential of small LDL

particles or is simply a consequence of the broader pathophysiology of which small LDL is a part. After multivariate adjustment for these confounding risk factors, LDL size was rarely found to be a significant, independent predictor of CVD risk.

An alternative explanation for the higher CVD risk observed among individuals who have pattern B LDL is their increased quantity (numerically) of LDL particles. Total plasma apo B has been used historically to estimate numbers of circulating LDL particles, because each LDL, VLDL, and IDL particle contains one apo B molecule, and approximately 95% of plasma apo B is bound to LDL. Many prospective epidemiologic and clinical intervention trials have documented that cardiovascular events are significantly more strongly associated with apo B than with LDL-C [25,33]. Using NMR spectroscopy to quantify LDL particle subclasses has provided further insight into the quantitative relationships of LDL particles with CVD risk. Data from six recently published or presented outcome studies (Table 6) indicate that NMR-measured LDL-P is a significantly stronger predictor of incident CVD events or disease progression compared with LDL-C [13,23,24,34–36]. In all of these studies, CVD associations with LDL-P and small LDL-P were independent of the standard lipid variables. Although LDL particle size was associated in univariate analyses with CVD risk in three of six studies, it failed to retain significant prediction after multivariate adjustment for lipids or LDL particle number.

Table 6
Associations of nuclear magnetic resonance–measured lipoprotein particle concentrations with cardiovascular disease outcomes in recent clinical trials

Study	Cardiovascular disease status	Atherosclerotic end point	NMR particle number associations[a]
Cardiovascular Health Study [35]	Primary prevention	Incident MI or angina	↑ LDL-P ↑ Small LDL-P
Women's Health Study [13]	Primary prevention	Incident MI, CHD death, stroke	↑ LDL-P ↑ Small LDL-P
Framingham Heart Study [36]	Primary prevention	Incident MI or angina	↑ LDL-P ↑ Small LDL-P
VA-HIT [24]	Secondary prevention	Nonfatal MI or CHD death	↑ LDL-P ↑ Small LDL-P ↓ HDL-P ↓ Small HDL-P
PLAC-I [23]	Secondary prevention	Angiographic stenosis	↑ LDL-P ↑ Small LDL-P ↑ Small HDL-P
Healthy Women Study [34]	Primary prevention	EBCT coronary calcium score	↑ LDL-P ↑ Small LDL-P

Abbreviations: CHD, coronary heart disease; MI, myocardial infarction; PLAC–I, Pravastatin Limitation of Atherosclerosis in the Coronary Arteries; VA-HIT, Veteran's Affairs High-Density Lipoprotein Intervention Trial; ↑, positive association; ↓, negative association.

[a] Significant and independent after multivariate modeling.

Comparative relations of apolipoprotein B and low-density lipoprotein particle concentration with cardiovascular disease outcomes

The close correlation documented earlier between LDL apo B measured by immunoassay and LDL-P measured by NMR leads to the expectation that the two measures of LDL particle number would show similar relationships with CVD outcomes. No recent CVD outcome studies have measured LDL apo B (because a time-consuming ultracentrifugal separation step is required). However, four studies have included measures of plasma apo B (highly correlated with LDL apo B) and NMR LDL-P, allowing comparison of the strengths of their disease associations. In all four studies, apo B was related less strongly to CVD than to LDL-P. In VA-HIT, on-trial levels of LDL-P predicted recurrent CVD events, but apo B did not [24]. In the Women's Health Study [13], LDL-P was the best lipid or lipoprotein predictor of incident CVD events and stroke, and was much more strongly related to these outcomes compared with apo B. A similar observation was made in the Johns Hopkins Sibling Study, with carotid atherosclerosis as the outcome [37], and in a recent study with a venous thrombosis end point [38].

The reasons why apo B has so far exhibited a weaker relationship with CVD outcomes compared with LDL-P are not understood and deserve further investigation. Speculation has centered on the apparently better measurement precision of the NMR assay and the fact that plasma apo B is only a surrogate for LDL particle number because of the inclusion of variable numbers of VLDL particles. Another possibility is that at least some apo B immunoassays may not be as accurate as assumed. Data from IRAS indicate that many associated lipid and insulin resistance variables are significantly more strongly correlated with LDL-P than with apo B [39], lending some support to this idea. Preliminary data from the authors' investigations suggest that apo B, relative to LDL-P, "undervalues" small LDL particles compared with large LDL particles. A possible reason is that apo B adopts a substantially different conformation on small LDL than it does on large LDL, potentially causing differential exposure of the epitopes and differential antibody binding. Finally, measured ratios of apo B:LDL cholesterol, which should always be greater for small versus large LDL particles, do not always show the expected consistency of association with LDL size [40,41].

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