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Measurement of Triglyceride-Rich Lipoproteins by Nuclear Magnetic Resonance Spectroscopy

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Summary: Nuclear magnetic resonance (NMR) spectroscopy is being used to determine the concentrations of very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) subclasses of different size. These subclasses have unequal associations with coronary heart disease. Nuclear magnetic resonance distinguishes among the subclasses on the basis of slight differences in the spectral properties of the lipids carried within the particles, which vary according to the diameter of the phospholipid shell. Studies using NMR spectroscopy have shown that individuals with elevated triglycerides are likely to have higher-risk lipoprotein subclass profiles. Triglyceride-rich lipoproteins drive the metabolic reactions that produce LDL of abnormal size and cholesterol content. The quantities of these abnormal LDL particles and the associated risk of coronary heart disease are underestimated by conventional cholesterol measurements. Nuclear magnetic resonance spectroscopy measures lipoprotein subclasses directly and efficiently, and produces information that may improve the assessment and management of cardiovascular disease risk.

Key words: coronary heart disease, very low-density lipoproteins, low-density lipoproteins, high-density lipoproteins, nuclear magnetic resonance spectroscopy, lipoprotein subclasses

Introduction

Historically, inferences about the roles played by lipoproteins in atherogenesis have come from large population studies in which lipids, not lipoproteins, were measured. The focus on lipids was dictated by analytical considerations, as it is much easier to measure the amount of lipid (typically cholesterol or triglyceride) in plasma or in a particular lipoprotein fraction than to measure the lipoprotein particles themselves. All lipoproteins contain cholesterol and triglyceride, but cholesterol is carried mainly in low- and high-density lipoproteins (LDL, HDL), while triglycerides are found predominantly in very low-density lipoproteins (VLDL). Very low-density lipoproteins and LDL are positively associated with coronary heart disease (CHD) (higher levels confer increased risk) and thus are considered atherogenic lipoproteins, whereas HDL is considered antiatherogenic because of its negative association with CHD (higher levels confer protection). Since it is now recognized that patients with widely differing amounts of VLDL, LDL, and HDL (and hence different risks of CHD) can have exactly the same cholesterol level, clinical attention has shifted away from total cholesterol to LDL and HDL cholesterol as the prime risk factors.

What is not widely known is the degree to which LDL and HDL cholesterol levels can fail in many patients to reflect accurately the number of LDL and HDL particles and their atherogenic potentials. There are two sources of this problem and both are related to triglycerides. The first is variability of the lipid composition of LDL and HDL because of a process of cholesterol ester-triglyceride exchange driven by elevated plasma triglyceride (VLDL) levels. When this occurs, HDL and especially LDL become cholesterol-depleted compared with normal. The second is variability in LDL and HDL particle size because of lipoprotein metabolic reactions that are driven in large part by elevated triglycerides. These reactions produce particles that are smaller than normal. By not taking account of the compositional and structural variability of lipoproteins, conventional lipid tests may be failing to provide critical information needed for the accurate diagnosis and management of the CHD risk of many patients.

New technology using nuclear magnetic resonance (NMR) spectroscopy has recently become available to allow lipoprotein particles of different size to be directly quantified. The NMR LipoProfile™ measurement is automated and efficient, and can be performed on either fresh or frozen (−70°C) plasma specimens. Physicians and researchers will now have ready access to information that previously could be obtained only by using time-consuming and laborious laboratory separation procedures.

James Otvos is Chief Scientific Officer and a stockholder at LipoMed, Inc., which is commercializing the NMR LipoProfile technology.

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Associations between Particle Subclasses and Coronary Heart Disease

Very low-density lipoprotein, LDL, and HDL each comprise a heterogeneous group of particles that differ in size and, in many cases, their associations with CHD (Fig. 1). The best known example is small, dense LDL, which confers at least a 3-fold higher risk compared with large LDL.[1,2] Elevated intermediate-density lipoprotein (IDL), which is included as part of the LDL fraction as measured by standard methods, is also associated with increased risk.[3] Since persons with the same LDL cholesterol level often have very different amounts of small LDL and IDL, it is not surprising that they may differ markedly in their susceptibility to developing CHD. Differing associations of HDL subclasses with CHD have also been noted in several studies. The three largest subclasses separable by gradient gel electrophoresis exhibit the expected inverse correlation with CHD incidence and severity (and therefore are truly antiatherogenic), whereas the two smallest subclasses show the opposite association.[4,5] Thus, the subclasses that contribute to "good" cholesterol (HDL) are, in fact, not all good! In the triglyceride-rich VLDL category, a recent study showed that levels of the largest particles (many of which may be chylomicron remnants) have a strong positive relationship with an arteriographic endpoint, independent of total plasma triglycerides and other lipid risk factors.[6]

Despite research evidence suggesting that clinical decision-making could be improved by direct measurements of lipoprotein subclasses, there has been no practical way to get at this information until now. Gross separation of LDL or HDL from the other lipoproteins is relatively simple and takes only a few minutes using chemical or immunoprecipitation methods. For this reason, LDL and HDL cholesterol measurements have become routine in clinical practice. Further subfractionation to give LDL and HDL subclass distributions requires far more time and effort and is performed in only a limited number of specialized laboratories. Ultracentrifugation and gradient gel electrophoresis are the methods used most often, and several hours to days are required to complete each analysis. Accuracy and precision are unavoidably limited by the many sources of analytical error introduced during the subclass separation process.

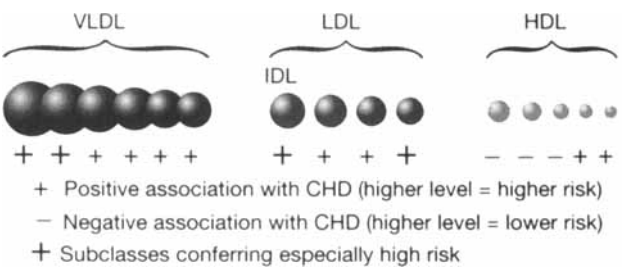

FIG. 1 Lipoprotein subclasses and their associated risk of coronary artery disease. VLDL = very low-density lipoprotein, LDL = low-density lipoprotein, HDL = high-density lipoprotein, CHD = coronary heart disease. Reprinted with permission of the author.

The Value of Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance spectroscopy gathers lipoprotein subclass information in a manner that is completely unique and inherently far more efficient than existing methods.[7,8] The technique relies on the spectroscopic distinctness of lipoprotein particles of a particular size and requires no physical separation of one group of particles from the others. Refined over a 10-year period, the NMR process measures 15 subclasses of VLDL, LDL, and HDL simultaneously. The measurement requires only about 1 min, uses no chemical reagents, and is completely automated. By simply adding up the concentrations of the various subclasses, one obtains all of the information provided in a standard lipid panel (total cholesterol, triglycerides, LDL and HDL cholesterol). In addition, average VLDL, LDL, and HDL particle sizes, as well as the total concentration of LDL particles are calculated. From the LDL particle size value, patients are categorized according to LDL subclass phenotype: pattern A (predominantly large LDL, signifying lower CHD risk), pattern B (predominantly small LDL, signifying higher CHD risk), or pattern AB (intermediate category).

When used for clinical decision-making purposes, the NMR data are presented in a 2-page NMR LipoProfile report (Fig. 2). This format simplifies interpretation of the new NMR variables by relating them in percentile terms to values observed in the general population (based on soon-to-be published data from 3,437 participants in the Framingham Offspring Study). The names of individual subclasses (V3, L2, H4, etc.) are based on a simple nomenclature scheme that assigns a larger number to a larger particle. Thus, V3 refers to a VLDL subclass that is smaller than V4, L2 to an LDL subclass smaller than L3, H4 to an HDL subclass smaller than H5, and so forth. Check boxes on page 2 of the NMR LipoProfile report help identify patients who are at higher risk than would be inferred from a standard lipid panel, as a result of a clustering of metabolic abnormalities associated with insulin resistance: the so-called *metabolic syndrome*. This clustering of nontraditional risk factors includes a predominance of small LDL, elevated numbers of LDL particles, low levels of large HDL particles, and elevated levels of triglycerides (carried in large VLDL particles, as will be described later). Among the terms used to refer to the clustering of these variables are *the atherogenic lipoprotein phenotype, atherogenic dyslipidemia*, or *the lipid triad*. The unique clinical value of NMR spectroscopy is its ability with a single, low-cost test to identify those patients who do (and do not) have an underlying lipid metabolism that confers higher CHD risk, and for whom different therapeutic choices might be indicated.

Origin of the Nuclear Magnetic Resonance Subclass Data

A detailed description of how the NMR lipoprotein measurement process works has been published[7,8] and will not be

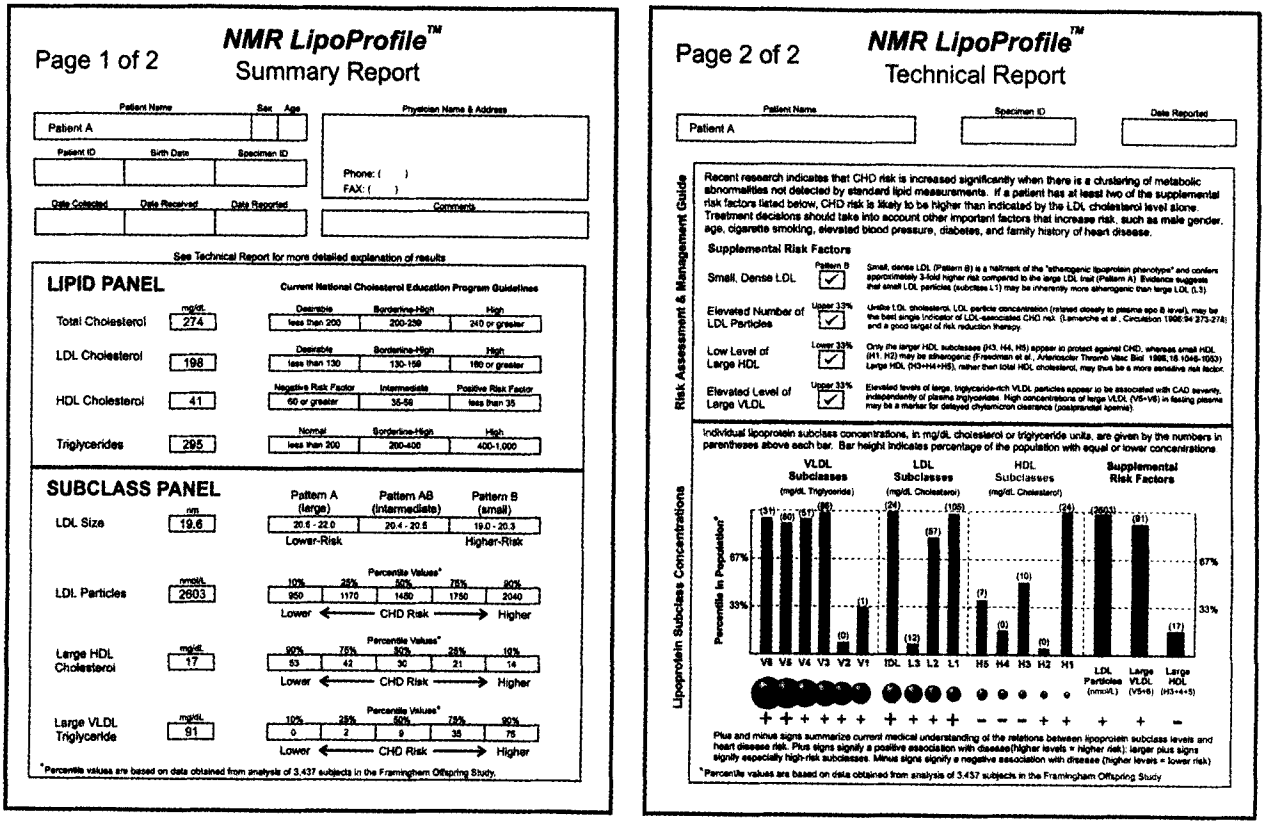

FIG. 2 Example of a patient's NMR LipoProfile. Reprinted with permission of the author.

repeated here. In brief, though, it is useful to draw an analogy between the different NMR signals emitted by lipoprotein particles of different size and the ringing sounds produced by bells of different size. The actual source of the NMR signals used for subclass quantification are the protons of the terminal methyl groups of the various types of lipid carried in the particles (mainly the cholesterol ester and triglycerides of the particle core, and the phospholipid of the particle shell). The signals from all these different lipids combine to produce a "bulk lipid" signal that has a characteristic frequency and shape that is directly dependent on the size of the particle (specifically, the diameter of the phospholipid shell, excluding the influence of the apolipoproteins attached to the particle). Obtaining the NMR spectrum of a plasma sample is analogous to simultaneously ringing a collection of bells of varying size and recording the composite sound output. With sufficient prior knowledge of the exact sound expected to be produced by each of the different size bells in the collection, one could imagine it might be possible to work backward from the composite sound signal to deduce how big was the signal coming from each set of bells of a given size. In this way, the number of bells (lipoprotein particles) of each size could be determined, since there is a direct proportionality between the amplitude of sound (bulk lipid signal) emitted by each set of bells of a given size (particles of a given diameter) and the number of bells (subclass particles). The great efficiency of NMR lipoprotein analysis derives from the fact that it takes less than a minute to collect the plasma NMR spectrum and only seconds to perform the linear least-squares deconvolution that produces the subclass concentrations. Results are accurate and precise because no particle separation steps are involved and the automated process includes built-in quality control checks.

There is one analytical point that needs to be stressed because of its relation to the topic of triglycerides. Nuclear magnetic resonance directly quantifies the lipoprotein particles themselves (on the basis of their bulk lipid mass), whereas traditional methods measure the amount of one particular type of lipid in the particles (usually cholesterol). This distinction is very important, since the cholesterol content of a particular lipoprotein can vary substantially from person to person. If, for example, two people have the same number of LDL particles, but one has particles containing less cholesterol than normal, their chemically measured LDL cholesterol levels will differ. In contrast, an NMR measurement will show them to have the same LDL concentration. The chief source of cholesterol compositional variability is a metabolic reaction catalyzed by cholesterol ester transfer protein (CETP), 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 and HDL. The result, in people with elevated VLDL levels, is the production of LDL and HDL that are cholesterol-deplet-

ed and triglyceride-enriched compared with normal. In such circumstances, traditional cholesterol measurements (but not NMR) will underestimate the true concentrations of circulating LDL and HDL.

Influence of Triglycerides on Coronary Heart Disease Risk

Several suggestions have been made to explain the observed association between plasma triglyceride levels and CHD. The one most frequently cited is the inverse relation between triglycerides and HDL cholesterol levels. Another is the association of high fasting triglyceride levels with delayed postprandial clearance of chylomicrons and their remnants, which in several studies is an independent predictor of CHD. A relationship to be explored here is that between plasma triglyceride level and the levels of individual VLDL, LDL, and HDL subclasses, which themselves appear to have different strengths of association with CHD. The other relationship to be discussed—which has been cited by many investigators but is still largely unappreciated—is that between triglyceride level and the content of cholesterol in LDL. When the ratio of the concentration of LDL particles (approximated by a plasma apoB measurement) to LDL cholesterol is higher than normal (termed hyperapobetalipoproteinemia), it can be for two reasons. First, the core lipid content of the LDL is relatively enriched in triglyceride and depleted of cholesterol ester. This condition was described in the previous paragraph and might be termed "LDL masking syndrome" since standard LDL cholesterol measurements will give a falsely low impression of the actual amount of LDL present in the patient's circulation. The second reason for hyperapobetalipoproteinemia is the presence of LDL particles that are smaller than usual (LDL subclass pattern B). There is less cholesterol per particle in this case simply because the volume of each particle is smaller. Since NMR measures the whole spectrum of subclass particles directly and efficiently, it is particularly well suited to provide insights into the origin(s) of the relationship between triglycerides and CHD risk.

Subclass Concentrations and Triglyceride Levels

Nuclear magnetic resonance spectroscopy has been used for the past 2 years to examine large numbers of frozen plasma specimens from various clinical trials and observational studies. Some of the most useful data have come from analyses of over 3,400 samples from men and women participating in the Framingham Offspring Study (Cycle 4). These data, which are not yet published, show the powerful influence exerted by triglycerides on lipoprotein subclass levels and average lipoprotein particle size. For LDL, it is well established using methods other than NMR that, as triglyceride levels increase in the plasma, LDL size decreases. What NMR shows is that this reduction in particle size is the result of a steady decrease in the level of large LDL (L3) as triglyceride levels rise above 100 mg/dl. Concomitantly, the levels of the more atherogenic small LDL particles (L1) increase. Concentrations of IDL also are observed to be generally higher as triglyceride levels increase. The very high prevalence of the lower-risk LDL pattern A phenotype when triglycerides are < 100 mg/dl and the correspondingly high prevalence of the higher-risk pattern B phenotype when triglycerides are > 250 mg/dl are explained quantitatively by changes in the individual subfractions.

There are also triglyceride-related changes in HDL subclass levels. As triglyceride levels increase, average HDL particle size decreases. This change is explained almost entirely by the response to triglyceride levels of the largest HDL subclasses, H4 and H5. Above a triglyceride level of 200 mg/dl, the H4 and H5 subclasses are no longer the predominant species. These subclasses are thought to be primarily responsible for protection against CHD. Thus, at high triglyceride levels much of the protection afforded by HDL is absent.

Average VLDL particle size is also strongly dependent on plasma triglyceride levels. Above a triglyceride level of about 100 mg/dl, the levels of the largest subclasses (V5 and V6) increase more steeply than those of intermediate-size VLDL (V3 and V4). Virtually the entire increment of triglyceride beyond a level of 300 mg/dl is carried in V5 and V6. It is not clear how many of these largest triglyceride-rich species are chylomicron remnants as opposed to true VLDL, since NMR does not differentiate between liver- and intestinally-derived particles.

Low-Density Lipoprotein Composition and Triglyceride Levels

As mentioned earlier, elevated concentrations of triglyceride-rich lipoproteins (VLDL) drive the production of cholesterol-depleted, triglyceride-enriched LDL particles by promoting cholesterol ester-triglyceride exchange. When large LDL becomes enriched in triglyceride, it becomes a good substrate for hepatic lipase (HL) and may, as a result of core triglyceride hydrolysis and structural remodeling, become transformed into small, dense LDL. Depending on the triglyceride level and CETP activity, the small LDL particles may end up with a normal cholesterol content (cholesterol/triglyceride > 4) or become significantly cholesterol-depleted (cholesterol/triglyceride < 4). Four different types of LDL particles will therefore be encountered depending on the patient's lipid metabolic circumstances (Fig. 3): large LDL with a normal lipid content, small LDL with a normal lipid content, and abnormal large and small LDL with cholesterol-poor and triglyceride-rich cores. Based on a detailed compositional analysis of LDL isolated from 118 healthy subjects, approximately 65% had large LDL with a normal lipid content. Virtually every subject with a plasma triglyceride level < 100 mg/dl fell into this category (Fig. 4). The other 35% of subjects were fairly equally divided among the other 3 LDL categories. For these individuals, a standard LDL cholesterol measurement does not accurately reflect the true amount of LDL present, at least when compared with individuals with large LDL of normal composition.

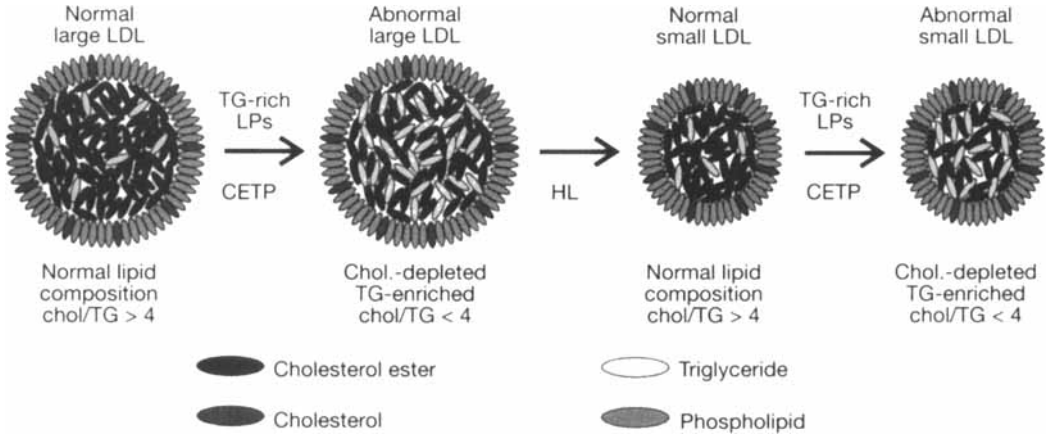

FIG. 3 Schematic representation of the triglyceride-driven metabolic reactions that lead to production of small low-density lipoprotein (LDL) and cholesterol-depleted LDL. TG = triglyceride, LP = lipoprotein, CETP = cholesterol ester transfer protein, HL = hepatic lipase, chol = cholesterol. Reprinted with permission of the author.

To illustrate the disconnect between LDL cholesterol levels and LDL particle concentrations observed in individuals with elevated triglyceride levels, the 118 subjects were grouped according to triglyceride ranges. Low-density lipoprotein cholesterol was measured by beta-quantification (ultracentrifuge) and LDL particle concentration by NMR. At relatively low triglyceride levels, LDL cholesterol levels rise in step with LDL particle numbers (Fig. 5). At higher triglyceride levels, the LDL particle concentrations are shown to be much higher than would be inferred from the LDL cholesterol values. With triglycerides > 250 mg/dl, there is a very large discrepancy between the cholesterol and particle concentrations. For these subjects, standard measurements of LDL cholesterol will seriously underestimate the number of circulating LDL particles, most of which are of the more atherogenic small variety (Fig. 4), and thereby seriously underestimate their risk of developing CHD.

Conclusion

Individuals with elevated triglycerides are observed to have higher-risk lipoprotein subclass profiles. They have a dis-

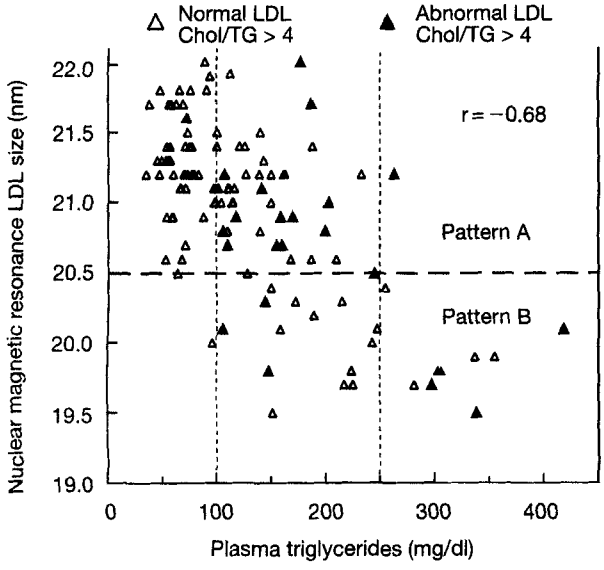

FIG. 4 Relationship between plasma triglyceride level and LDL particle size and lipid composition. Samples containing LDL with a normal lipid content (cholesterol/triglyceride > 4) are represented by open triangles, and those with abnormal lipid content (cholesterol/triglyceride < 4) by filled triangles. Abbreviations as in Figure 3. Reprinted with permission of the author.

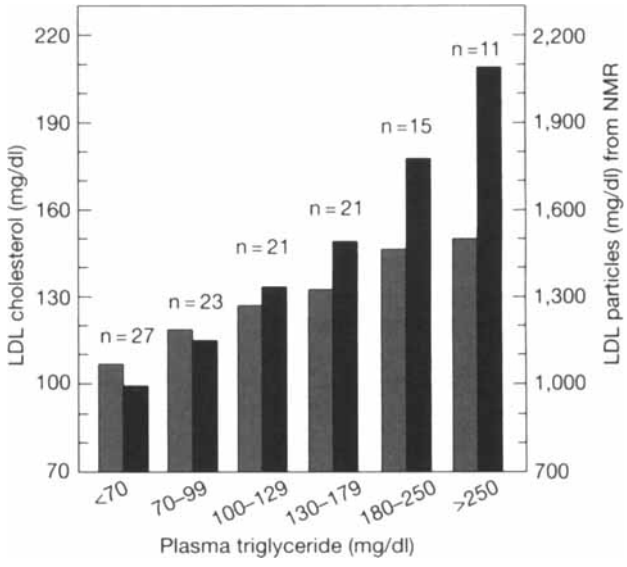

FIG. 5 Comparisons of LDL cholesterol and LDL particle concentrations in 118 subjects grouped according to plasma triglyceride levels. LDL = low-density lipoprotein, NMR = nuclear magnetic resonance. Reprinted with permission of the author.

proportionate amount of large VLDL, higher levels of small, dense LDL, and small, even more dense HDL. The triglyceride-rich lipoproteins drive the metabolic reactions that produce LDL particles with abnormal composition. As a result, the quantities of these particles and their associated CHD risk are underestimated by conventional cholesterol measurements. Direct measurement of lipoprotein particles by NMR or other methods will help to clarify the roles played by triglycerides in atherogenesis.

Discussion

Bachorik: There are a few circumstances where conventional beta quantification should be used. One is the detection of Type III hyperlipoproteinemia. How are the patterns affected by the presence of floating beta-lipoprotein? Another risk factor in certain populations is Lp(a). Where does this risk factor fall into these subclass patterns? If NMR patterning were substituted for conventional beta quantification, is there any way to flag that the patient might be a Type 3 or is less responsive to conventional LDL-lowering agents since their Lp(a), not their LDL, is elevated?

Otvos: We don't yet have enough experience with the Type III situation to know whether NMR can detect it well. As for Lp(a), remember that NMR differentiates particles on the basis of differences in the diameter of the phospholipid shell. What distinguishes Lp(a) from LDL is the extra apo(a) protein molecule covalently attached to the apoB protein, which makes Lp(a) particles physically bigger and their density greater than normal LDL. However, the diameter of the phospholipid shell is unchanged when the apo(a) protein is attached, so NMR is unable to distinguish Lp(a) from LDL. We wondered whether this might impair our ability to measure LDL size accurately. To investigate this question, we examined plasma samples containing both large and small LDL from which Lp(a) was selectively removed by a lectin binding process. It was shown that when LDL is small, the phospholipid shell diameter of the Lp(a) particles in that sample were also small. The same thing was shown for samples containing large LDL; the diameter of the Lp(a) phospholipid shell was also large. We therefore believe that even high concentrations of Lp(a) do not impair the ability of NMR to assess LDL size accurately. However, it is also true that Lp(a) information is important to have in certain clinical situations, and NMR is unable to supply it.

Glueck: Antiphospholipid antibodies, anticardiolipin antibody, and lipid anticoagulant are known to bind to LDL among other things. Do these affect the NMR signal in any way since this signal is dependent on the phospholipids to which these antibodies bind? In addition, how much does the equipment cost to perform these NMR procedures?

Otvos: Although the equipment is not being made available commercially, the test is being made available. Currently, it is available for clinical research studies. In early 1999, it will become available for patient care. There is no direct evidence that suggests these antibodies and other factors interfere with the NMR signal.

Krauss: For clinicians, a measurement of apo B gives an estimate of particle number within the whole atherogenic range. While it does not provide the specificity across the subfractions, it does unmask some of the risk masked by LDL cholesterol. A VLDL subfractionation was used to document the size distribution across V1 through V6, with V1 being the smallest. It appears to be relatively flat and at low concentration across the triglyceride range. When mass is measured using analytical ultracentrifugation and other techniques, first smaller and then larger particles are measured. It appears that nothing was happening to V1. Is the VLDL subfractionation measuring V1 triglyceride? If so, is it a function of the composition of those particles?

Otvos: V1 is really a very minor constituent; it is very close to IDL. The concentration is expressed in triglyceride units since it is the most abundant lipid in the particles. What is really being measured is the signal intensity that comes from the particle, which is then converted to triglyceride equivalents. Large triglyceride-rich particles are the most sensitive to change at higher triglyceride concentrations.

Krauss: The signal on the phospholipids is the index of particle size. Can the procedure be sensitive to alterations in phospholipid composition or to pharmacologically induced changes in surface lipid or proteins that might affect this signal?

Otvos: We have not tried to generate a defined population where something like that existed, though it might be worthwhile. In these particles, the bulk of the signal amplitude comes from what is in the core and not what is found in the shell. The shell signal becomes more important in the HDL regime, where the core-to-shell ratio is very different. There is no modulation due to the tremendous compositional heterogeneity. The methyl group does not feel this chemical heterogeneity.

Pek: In diabetic patients with hyperlipidemia, when blood sugar goes up, many proteins get glycated, including LDL. Does this glycated LDL influence particle size?

Otvos: It does not influence particle size as far as we know. Glycated LDL does not have a spectral perturbation and cannot be seen. This is occurring on the protein moiety to which the NMR is not sensitive.

Abrams: This is a small particle size group for the most part. There has been controversy over whether it is necessary to interrogate the issue of pattern A versus pattern B. Some clinicians claim one only needs to look at triglycerides and HDL in order to make assumptions about particle size. Can this same assumption be made with respect to the total number of LDL particles in anyone with a high triglyceride and a low HDL? Can one assume that the LDL measurement, whether it is done by the direct or conventional subtraction, is likely to be wrong?

Otvos: It is likely to be wrong reproducibly when the triglyceride levels are quite high, such as above 250 to 300 mg/dl. Most people, however, have triglyceride levels lower than this. There is really no good ability to predict, on the basis of triglyceride level, the extent to which a particle becomes triglyceride enriched and cholesterol depleted. Enzyme activity levels have a lot to do with this. It is a very complex meta-

bolic milieu. The efficiency of NMR spectroscopy will, hopefully, allow it to substitute at approximately the same price for the standard lipid panel.

Abrams: In a patient with a triglyceride level of >350 mg/dl and an abnormal HDL, can one conclude that the particle size will be small (pattern B) and that the particle number will be greater than one might anticipate from the LDL concentration?

Otvos: This can be concluded with reasonably good certainty. If the particles are small, and the frame of reference is the large particle with normal lipid composition, the particle number is greater than if the particles were large. This is why the apo B to LDL cholesterol ratio is useful in this regard. If one is only interested in the number of LDL particles, then a plasma apo B measurement is a useful parameter. If small particles on a per-particle basis are believed to be inherently more atherogenic, then it would be good to know both the number of particles and how big these particles are. Both pieces of information are desired. The Quebec Cardiovascular Study has shown that both apo B and LDL size, when taken together, help to discriminate the at-risk population much better than either one alone.[2]

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