The Measurement of Lipids, Lipoproteins, Apolipoproteins, Fatty Acids, and Sterols, and Next Generation Sequencing for the Diagnosis and Treatment of Lipid Disorders

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Received 2 March 2016

ABSTRACT

Standard lipid analysis includes measuring serum or plasma total cholesterol, triglycerides, and high density lipoprotein cholesterol (HDL-C) after an overnight fast. Low density lipoprotein cholesterol (LDL-C) is then calculated. Our own prospective studies from the Framingham Offspring Study indicates that LDL-C, small dense LDL-C (sdLDL-C), lipoprotein(a) or Lp(a),

and HDL particle measurements add significant information about cardiovascular disease (CVD) risk to the standard lipid profile. Common familial lipid disorders associated with premature CVD include Lp(a) excess, combined hyperlipidemia, and dyslipidemia. Plasma fatty acid analysis is important in order to assess adequacy of omega-3 fatty acid intake, and whether there are excess levels of saturated and trans fatty acids for dietary recommendations. Plasma sterol analysis can be helpful for the diagnosis of causes of elevated very low density lipoprotein (VLDL-C > 50 mg/dL) and/or LDL-C (> 160 mg/dL) which includes familial combined hyperlipidemia (elevated lathosterol), familial hypercholesterolemia (normal sterols), dysbetalipoproteinemia (moderate increases in β -sitosterol), phytosterolemia (very high β sitosterol), and cerebrotendinous xanthomatosis (very high cholestanol). The measurement of apolipoprotein (apo) A-I in HDL particles by gel electrophoresis is important in assessing CVD risk, HDL functionality, and for the diagnosis of marked HDL deficiency states (HDL-C < 20 mg/dL due to apoA-I deficiency and variants, Tangier disease and lecithin:cholesteryl acyltransferase (LCAT) deficiency, as well as hepatic lipase and cholesteryl ester transfer protein deficiency. The measurement of apoB is important for the diagnosis of abetalipoproteinemia and hypobetalipoproteinemia. The definitive diagnosis of the above mentioned disorders along with causes of markedly elevated triglycerides (> 1,000 mg/dL) requires next generation DNA sequencing of the appropriate and relevant genes for these disorders in order to provide a definitive molecular diagnosis often necessary to formulate optimal therapy strategies. For complete coverage of this and all related areas of Endocrinology, please visit our FREE web-textbok, www.endotext.org.

INTRODUCTION

The current standard approach for the physician in assessing lipids in patients is to ask the patient to fast overnight for at least eight hours except for water, black coffee, or tea without milk, and then have their blood drawn for a fasting lipid profile. This testing provides a measurement of the serum concentrations of total cholesterol, triglyceride, HDL-C, and calculated LDL-C. A total cholesterol of > 240 mg/dl has been classified as elevated and associated with increased CVD risk, and optimal values are < 200 mg/dL(1). Triglycerides levels > 200 mg/dL have been classified as elevated, with optimal values being < 150 mg/dL. Moreover fasting triglycerides levels > 1,000 mg/dL have been associated with an increased risk of pancreatitus. Low HDL-C has been classified as < 40 mg/dL in men, and < 50 mg/dL in women, while optimal values are > 50 mg/dL in men and > 60 mg/dL in women (1). The Friedewald formula has long been used to calculate LDL-C from total cholesterol, triglycerides, and HDL-C provided the patient was sampled after an overnight fast and the triglyceride values are < 400 mg/dL (2). This formula calculates LDL-C by subtracting the sum of HDL-C and VLDL (triglycerides/5 from total cholesterol). It has been shown that this formula becomes increasingly inaccurate as serum triglyceride levels increase (3). An LDL-C value > 160 mg/dL has been classified as high, and an optimal value as < 100 mg/dL (1). In patients with CVD an optimal value has been classified as < 70 mg/dL (1,4).

CVD risk assessment for many years has been based on the Framingham Heart Study cohorts, consisting of the original cohort and the Framingham Offspring Study cohort (1). Recently in the 2013 cholesterol guidelines this risk assessment has been replaced by a new risk algorithm that, in addition to using both Framingham cohorts, also incorporates data from the three additional cohorts, namely those of the Atherosclerosis Risk in Communities (ARIC) study, the Cardiovascular Health Study (CHS), and the Coronary Artery Risk Development in Young Adults (CARDIA) study. Therefore the new risk algorithm has the benefit of using data from a much larger group of subjects including many more women, the young and the elderly, and significant numbers of African-Americans and Hispanic subjects, more reflective of the United States population (5). The risk factors included in this new algorithm are: gender, age, race, total cholesterol, HDL-C, systolic blood pressure, treatment for blood pressure, history of smoking, and diabetes, and can be accessed at www.myamericanheart.org/CVriskcalcular. An increased CVD risk has been defined as > 7.5% (5). Moreover according to the 2013 cholesterol guidelines if the patient has any evidence of CVD, diabetes and is over age 40 years, an LDL-C > 190 mg/dL, or has a 10 year CVD risk > 7.5%, he/she is a candidate for lifestyle and statin therapy (6).

The guidelines panel recommended ruling out secondary causes of elevated LDL-C, which include increased dietary intake of saturated fat, trans fats, weight gain, hypothyroidism, biliary obstruction, nephrotic syndrome, obesity, as well as of diuretics, cyclosporin, glucocorticoids, and amiodorone. They also recommended ruling out secondary causes of elevated TG and decreased HDL-C which include obesity, diabetes, hypothyroidism, kidney disease, high sugar intake, excessive alcohol intake, oral estrogens, glucocorticoids, protease inhibitors, retinoic acid, anabolic steroids, sirolimus, raloxifene, tamoxifen, beta blockers (except carvedilol), or thiazides (6).

Before consideration of drug therapy, the panel recommended that patients be placed on a lowsaturated fat (< 7% of calories), low cholesterol (< 200 mg/day), low trans fat, low sugar diet. Calorie restriction in overweight and obese patients (BMI > 30 kg/m², waist size > 40 inches in men and > 35 inches in women) and a regular exercise program (> 40 minutes 4 times/week) was recommended. They indicated that replacement of beef, pork, and lamb, with poultry and fish, and replacement of butter with soft margarine would be beneficial for LDL-C lowering. For cooking oil use the panel recommended canola oil or olive oil, and for salads they recommended use of canola, soybean, or olive oil and vinegar. Vegetables, fruits, and whole grains were also recommended, as was avoidance of foods and beverages high in sugar or high fructose corn syrup. The panel also recommended that men consume \leq 2 alcoholic drinks/day and women \leq 1 drink per day (7). Moreover the cornerstone of CVD prevention is lifestyle modification (7).

It should be pointed out that the above standard guidelines were not designed to diagnose and treat lipid disorders, but to make recommendations for the use of lifestyle modification and cholesterol modifying medications for overall CVD risk reduction. Moreover the current guidelines have omitted prior recommendations for target LDL-C levels of < 70 mg/dL in CVD patients and < 100 mg/dL in patients at high risk of developing CVD (6). These

recommendations are not consistent with the prior recommendations (1,4), or current recommendations of the National Lipid Association, the European Atherosclerosis Society, or the International Atherosclerosis which do include the targets of therapy for LDL-C as per the prior recommendations (8-11). The current guidelines are likely to be amended soon to again include target LDL-C values. Another clear weakness of the current cholesterol guidelines is the lack of recommendations with regard to type and frequency of laboratory testing. Our purpose in this chapter is to review the current status of the measurements of lipids, lipoproteins, apolipoproteins, fatty acids, and sterols, as well as the use of next generation DNA sequencing for the diagnosis and management of lipid disorders.

MEASUREMENT OF LIPIDS

The measurement of plasma or serum cholesterol and triglycerides was greatly improved with the development of standardized automated high throughput enzymatic analyses, which achieved within and between run coefficients of variation of < 5% (12). These assays have been widely used since the 1980s, and have been standardized via the Center for Disease Control's Lipid Standardization program originally initiated by the National Heart, Lung, and Blood Institute for the Lipid Research Clinics Primary Prevention Trial. Our assays are standardized through the Centers for Disease Control Lipid Standardization Program. In a population analysis of 235,347 subjects from Boston Heart Diagnostics (52% female, median age 58 years) sampled in the fasting state without known CVD and not on lipid lowering medication the 1st, 5th, 25th, 50th, 75th, 95th, and 99th percentile values for total cholesterol in mg/dL were 104, 124, 158, 186, 217, 268, and 313, while for fasting triglycerides these values in mg/dL were 40, 52, 80, 111, 160, 285, and 477, respectively. Therefore in our population about 1/3 have total cholesterol values > 200 mg/dL and about 15% have total triglyceride values > 200 mg/dL.

LIPOPROTEIN MEASUREMENTS

Early studies separated lipoproteins by electrophoresis, with chylomicrons migrating at the origin, LDL migrating at the β position, very low density lipoproteins (VLDL) at the pre β position, remnant lipoproteins migrating between the pre β and β position, and HDL migrating at the α position. Barr, Russ, and Eder of New York were the first to document that CHD patients had increases in β and pre β migrating lipoproteins, and decreases in α migrating lipoproteins as compared to control subjects (13). Fredrickson, Levy, and Lees at the National Institutes of Health developed an entire diagnosis and treatment system for lipoprotein disorders based on the measurement of cholesterol and triglyceride and the semi-quantitative determination of plasma lipoproteinemia: type I (elevated chylomicrons), type IIA (elevated LDL), type IIB (elevations of both LDL and VLDL), type III (elevations of lipoproteins migrating between VLDL and LDL, dysbetalipoproteinemia or elevated remnant lipoproteins), type IV (elevated VLDL), and type V (elevated chylomicrons and VLDL) (13). It was recognized that patients with type II, III, and IV hyperlipoproteinemia were at increased risk of developing CVD, while those with

types I and V were at increased risk of developing pancreatitus (14). This methodology had the disadvantage of being semi-quantitative, and ignoring HDL and Lp(a). This terminology is no longer used, and was discarded in favor of direct assessment of lipoproteins, and because of the observation that elevated VLDL, LDL or both could be observed in the same kindred as in familial combined hyperlipidemia (15,16). Goldstein and colleagues at the University of Washington studied 500 survivors of myocardial infarction and their families and documented that about 15% had familial combined hyperlipidemia (elevations of either total cholesterol, total triglycerides, or both), about 15% had familial hypertriglyceridemia, and about 1% had familial hypercholesterolemia (15,16). At that time neither HDL-C or Lp(a) were measured.

Lipoprotein Assessment by Ultracentrifugation

Cholesterol, triglyceride, phospholipids, and proteins (known as apolipoproteins) are carried in plasma on generally spherical lipoprotein particles. Lipoproteins carry phospholipid, free cholesterol, and apolipoproteins on the surface, and triglyceride and cholsteryl ester in their core. Lipoproteins were originally characterized by DeLalla and Gofman of the University of California, Berkeley using an analytical ultracentrifuge, and later by Havel, Bragdon, and Eder of the National Institutes of Health using a preparative ultracentrifuge (17,18). Triglyceride-rich lipoproteins of intestinal origin or chylomicrons were classified as having a density of < 0.94 g/ml, and were subsequently found to be very triglyceride-rich, with some phospholipid and cholesterol, and to contain apolipoprotein (apo) B-48. This protein comprises the first 48% of the form of apoB-100 made in the liver. ApoB-48 is produced only in the intestine at a rate of 1-2 mg/kg/day. It is produced by novel mRNA editing. In normal subjects chylomicrons rapidly undergo lipolysis to form chylomicron remnants which remain in the bloodstream for about 5 hours, before being taken up by a specific liver receptor that binds apoE. In the fasting state the intestine makes chylomicron particles that have a density > 0.94 g/mL, since cholesterol and phospholipids are entering the intestine from the bile at all times even after prolonged fasting. VLDL particles of liver origin were defined as having a density of 0.94-1.006 g/ml. These particles are the major triglyceride carrying lipoproteins in the fasting state, and contain apoB-100 as their major apolipoprotein. VLDL apoB-100 is produced at a rate of about 15 mg/kg/day. In the fasting state all of VLDL apoB-100 is converted to LDL apoB-100 over about 5 hours. However, in the fed state about 1/3 of VLDL apoB-100 is cleared directly from the circulation and taken up by a specific liver receptor that binds apoE. In the fed state about 1/2 of the remainder of VLDL apoB-100 is converted to intermediate density lipoproteins (IDL), which were classified as having a density of 1.006 – 1.019 g/ml, and further to LDL which has a density of of 1.019-1.063 g/ml. LDL is generally the major cholesterol carrying lipoprotein in plasma. Another 1/3 of VLDL apoB-100 in the fed state is directed converted to small dense LDL apoB-100, and this proportion increases in the setting of hypertriglyceridemia. The LDL contains apoB-100 as its major protein. LDL is cleared from the plasma with a residence time of about 2.0 days. LDL has been further divided into large buoyant LDL (density 1.019-1.044 g/ml) and small dense LDL (density 1.044-1.063 g/ml). The apoB-100 in this particle has a plasma residence time of about 1.5 days, and this particle is converted to small dense LDL, whose apoB-100 molety is cleared more slowly with a plasma residence time of about 3 days.

HDL has a density of 1.063-1.21 g/ml (17). It is the major phospholipid carrying lipoprotein in plasma, and has apoA-I and apoA-II as its major apolipoproteins. ApoA-I has a production rate of about 12 mg/kg/day, while for apoA-II this value is about 3 mg/kg/day. ApoA-I has a residence time of about 4 days while for apoA-II this value is about 4.5 days. The catabolism of HDL proteins is enhanced in the setting of hypertriglyceridemia. HDL was further divided into large HDL also known as HDL₂ (density 1.063-1.125 g/ml) and small HDL or HDL₃ (density 1.125-1.21 g/ml). HDL₁ turned out to be Lp(a), whose density is intermediate between small dense LDL and HDL₂. Gofman and colleagues in Berkeley documented that CHD patients had significantly higher LDL and small dense LDL and significantly lower HDL and large HDL than controls based on the use of the analytical ultracentrifuge (19).

The isolation of lipoprotein by sequential ultracentrifugation is cumbersome and labor-intensive, and was found to be associated with substantial particle alteration and loss of lipoprotein constituents. Therefore this method is currently only used for research purposes. Isolation of plasma lipoproteins using single spin gradient ultracentrifugation was developed by Chapman and colleagues in Paris (20). This methodology was further refined by Patsch and colleagues and by Chung and Segrest at the University of Alabama (21-23). The latter method using vertical rotor ultracentrifugation was optimized to allow for the measurement of cholesterol concentrations by automated enzymatic methods across the entire lipoprotein spectrum, and this is known as the vertical auto profile or (VAP). It provides a measure of cholesterol concentrations in the twenty lipoprotein fractions in the VLDL, LDL, and HDL density regions, as well as an estimate of Lp(a) cholesterol (24-26). However this latter estimate is not very precise. Doctors until recently could order this VAP profile from Atherotech, a laboratory in Alabama; however this laboratory recently terminated its business. Moreover since this methodology was introduced, very little research has been done with this method to document its clinical utility versus standard methods.

Lipoprotein Assessment by Gradient Gel Electrophoresis and Ion Mobility

Another method of assessing lipoproteins has been through the use of gradient gel electrophoresis followed by specific staining to identify LDL and HDL particles of various sizes. These methods were pioneered by Anderson, Krauss, Austin and colleagues of the Donner Laboratory at the University of California, Berkeley (27,28). Patients with CHD were shown to have increased amounts of small dense LDL (28). Physicians were able to order this testing on their patients in the past through Berkeley Heart Laboratory of California; however this laboratory was acquired by Quest Diagnostics, and they no longer offer this testing. Patients with a preponderance of small dense LDL and small dense HDL often have elevated triglycerides, and their levels can be reduced by triglyceride lowering (29-31).

An ion mobility method using gas phase differential electrophoretic macromolecular separation was developed for assessing LDL and HDL particle size over a range of 17 to 540 angstroms (32). However according to Krauss neither the ion mobility or the gradient gel methods have clearly been shown to add information above and beyond standard measurements with regard to LDL particles and CVD risk assessment in large prospective studies (33). Despite these

findings, the ion mobility method is offered to healthcare providers by Quest Diagnostics. In addition a one dimensional high resolution gradient gel electrophoresis method was also developed by Li and Schaefer for examining HDL particles, but this method was never applied to large scale studies (34).

Two-dimensional gel electrophoresis separates lipoprotein particles by size in the vertical dimension and by charge in the horizontal dimension. This method was first developed to assess HDL particles in the laboratory of Christopher Fielding at the University of California, San Francisco. (35,). They were the first to report the presence of very small preβ migrating HDL particles in human plasma. Subsequently Kane and colleagues, along with other laboratories, noted that these particles were increased in patients with CVD (35,). Two dimensional gel electrophoresis of HDL particle was further optimized by Asztalos and Roheim at Louisiana State University in New Orleans, and later by Asztalos and Schaefer at Tufts University in Boston (37-46). Like the prior methods the isolation of HDL particles does not require ultracentrifugation or column chromatography, and this method had the benefit of using specific apoA-I antibodies for immunoblotting to detect the individual HDL particles and assess their relative concentration.

Moreover Asztalos and colleagues defined the functions of these particles. They showed that the very small discoidal pre β -1 HDL particles pick up free cholesterol from cells via the ATP binding cassette A1 (ABCA1) transporter to become small discoidal α -4 HDL, and potentially larger discoidal HDL particles by particle fusion (38). Discoidal HDL particles are converted to medium spherical α -3 HDL and larger particles by the esterification of free cholesterol via the enzyme lecithin:cholesterol acyltransferase (LCAT) and the addition of apoA-II. These particles are further converted to large and very large spherical α -2 and α -1 HDL by the actions of cholesteryl ester transfer protein (CETP). CETP transfers cholesteryl ester from HDL to triglyceride-rich lipoproteins in exchange for triglyceride. Very large α -1 HDL particles are preferential donors of cholesterol to the liver, and the constituents of these particles can recycle back to form very small discoidal particles and can re-enter the HDL cycle, or be catabolized directly by the kidney or liver (38,39).

The concentrations of apoA-I in HDL particles in the general population and in CHD patients have been extensively assessed by Asztalos and colleagues at Tufts University in Boston (40-42). CVD patients have significant increases in apoA-I levels in very small pre β -1 HDL and significant decreases in apoA-I in large and very large α -2 and α -1 HDL (40-42). In the Framingham Offspring Study it was documented that for every 1 mg/dL increase in the apoA-I level in very large α -1 HDL there was a 26% reduction in CVD risk (40). These data indicate that they often have decreased conversion of the smaller particles to the larger particles, as well as excess removal of cholesteryl ester from the larger particles to triglyceride-rich lipoproteins via CETP (39-43). Moreover in the prospective Veterans Affairs HDL Intervention Trial (VA HIT) decreased apoA-I levels in large α migrating HDL particles and increased apoA-I levels in very small pre β -1 HDL were associated with an increased CHD risk (42). Optimization of these HDL particles with the simvastatin/niacin combination was associated with regression of coronary atherosclerosis (44). Moreover HDL particle analysis can be used for the diagnosis of homozygous and heterozygous apoA-I deficiency (lack of apoA-I containing HDL particles),

Tangier disease (usually only pre β -1 HDL particles), lecithin:cholesterol acyltransferase deficiency (mainly only pre β -1 and α -4 HDL particles), hepatic lipase deficiency (increased α -1 HDL and decreased α -2 HDL) and cholesteryl ester transfer protein deficency (presence of very large abnormal α migrating HDL particles) (45-48). HDL particle analysis by high resolution gel electrophoresis can be obtained commercially through Boston Heart Laboratory of Framingham, Massachusetts.

In a recent population analysis of 35,794 subjects at Boston Heart Diagnostics using newer automated methods of assessing the apoA-I content of HDL particles the 25th, 50th, and 75th percentile values for apoA-I in mg/dL in very large α-1 HDL in men were 23, 30, and 39, and in women were 34, 45, and 60, respectively. These values in large α -2 HDL in men were was 47, 54, and 63, and in women were 57, 66, and 76, respectively. Women have significantly higher level of apoA-I in very large α -1 HDL and large α -2 HDL than do men. High risk apoA-I values in mg/dL for these particles in men are < 25 and < 45 in men, and < 35 and < 55 in women, respectively. There was no significant gender difference in values for apoA-I in medium α -3 HDL, small α -4 HDL and very small pre β -1 HDL. The 25th, 50th, and 75th percentile values for apoA-I in mg/dL in medium α -3 HDL were 18, 21, and 24, respectively. These values for apoA-I in mg/dL in small α -4 HDL were 13, 16, and 19, respectively. These values for apoA-I in very small preß-1 HDL was 10, 14, and 18, respectively. High risk values for apoA-I in all three smaller HDL particles are > 25 mg/dL in our population, indicating a decreased ability to convert smaller HDL particles to larger HDL particles associated with protection from CVD and efficacy in delivering cholesterol back to the liver. Moreover the data indicate that with this new method that the percent of plasma apoA-I in men in HDL particles is about 11% for pre β -1, 12% for α -4, 16% for α -3, 40% for α -2, and 21% for α -1, while for for women these values are about 9%, 10%, 13%, 41%, and 28%, respectively. The apoA-I concentration in very large α -1 HDL is significantly and inversely correlated with serum triglyceride levels.

Lipoprotein Assessment by Nuclear Magnetic Resonance (NMR)

Otvos and colleagues in Raleigh, North Carolina have pioneered the NMR technique which measures plasma liproteins using nuclear magnetic resonance signals, triggered by the terminal methyl groups of lipids on lipoprotein particles. This method provides for an assessment of the concentration of VLDL, LDL, and HDL particles in nmoles/L. Currently the method is most widely used to assess total LDL particle number, and patients with CHD have elevated levels of this parameter as compared to control subjects (49-51). This parameter can be used to predict CVD, and can be favorably altered by a variety of lipid lowering agents, especially statins. It has been documented that this analysis is superior to LDL-C (49-51). This testing in the past could be obtained commercially through Liposcience Laboratories of Raleigh, North Carolina, and can now be obtained through LabCorp, a large United States reference laboratory. In our population based on LDL-P measurements in 51,158 subjects sampled in the fasting state and without CVD or on lipid lowering medications the 1st, 5th, 25th, 50th, 75th, 95th, and 99th percentile values in nmoles/L were 376, 596, 947, 1247, 1591, 2195, and 2711. A high risk value has been classified as > 1600 nmoles/L and an optimal value < 1000 nmoles/L, corresponding

approximately to the 75th and 25th percentile values in our population, as well as in other populations (49-51).

Lipoprotein Assessment Using Precipitation Methods

The previously described methods are all carried out in advanced lipid testing laboratories. Laboratories worldwide precipitate and remove lipoproteins containing apoB (chylomicrons, chylomicron remnants, VLDL, LDL, and Lp(a), leaving HDL behind, so that its cholesterol content can be measured by automated enzymatic analysis. This technique was first developed by Burstein and colleagues in Paris and then in New York in 1969 and 1970 using a variety of polyanions including heparin sodium and magnesium chloride (52-54). The methodology was standardized by the Lipid Research Clinics program supported by the National Institutes of Heath using heparin magnesium chloride, and also was evaluated by Warnick and Albers of the University of Washington (55,56). Warnick and colleagues also optimized the dextran sulfate magnesium precipitation technique (57). Miller and Miller of London were among the first to document that CHD patients had significantly lower HDL-C levels than controls (58). This finding was subsequently verified in many prospective studies including the Framingham Heart Study (59). Schaefer and colleagues at the National Institutes of Health documented that subjects with significant hypertriglyceridemia (types I, IIB, III, IV, and V hyperlipoproteinemia), had marked decreases in HDL-C (60). Genest, Schaefer and colleagues at Tufts University in Boston studied the families of 500 patients with premature CHD and documented that 19% of families had elevated Lp(a), 15% had dyslipidemia (elevated triglycerides and decreased HDL cholesterol), 14% had combined hyperlipidemia, 4% had isolated low HDL, and 1% had familial hypercholesterolemia (61).

Beginning in the 1980s, laboratories began developing methods for the direct measurement of LDL-C and HDL-C. McNamara and Schaefer developed an immunoseparation method for measuring direct LDL-C requiring sample pre-treatment with antibodies in collaboration with the Genzyme Corporation of Cambridge, MA (62). However this method was cumbersome and expensive. Laboratories in Japan then developed direct homogeneous LDL-C and HDL-C assays using a variety of precipitation techniques that did not require sample pre-treatment (63,64). These newer direct assays are now widely used throughout the world. We have tested both the direct LDL-C and HDL-C assays being marketed by Roche in the United States and developed by Kyowa Medex of Japan (65). Other Japanese assay companies have also developed direct online homogeneous LDL-C and HDL-C assays. We have compared the results obtained with the Roche assays and those obtained by the dextran method for HDL-C and the calculated LDL-C method in participants in the Framingham Offspring Study, and have noted very comparable results (correlation r^2 values of = 0.97 for both methods) with similar absolute values (65). The value of the newer methodology is that it does not require manual pretreatment of the sample, and allows for the immediate direct measurement of HDL-C and LDL-C on a high throughput analyzer.

Recently we have compared calculated LDL-C with direct LDL-C in 228,142 subjects using samples obtained after an overnight fast from subjects not on lipid lowering medication and

without CVD. Median direct LDL C values in mg/dL were 111, while median calculated LDL-C values in these same subjects was 105 mg/dL. Results from the two methods were highly correlated (r=0.98), but calculated LDL-C values were significantly lower, representing a 5% underestimation bias. These data indicate that calculated values for LDL-C are not an accurate measure of true values. Moreover calculated LDL-C becomes increasingly inaccurate as serum triglyceride levels rise, especially over 250 mg/dL. For direct LDL-C in mg/dL the 1st, 5th, 25th, 50th, 75th, 95th, and 99th percentile values in our population were 44, 60, 87, 111, 139, 184, and 222. About 10% of our population had direct LDL-C values of < 70 mg/dL, about 40% have optimal values of < 100 mg/dL, about 15% have high risk values > 160 mg/dL, and about 4% have very high risk values > 190 mg/dL.

With regard to HDL-C values in this same population, in men in mg/dL the 1st, 5th, 25th, 50th, 75th, 95th, and 99th percentile values were 23, 29, 38, 46, 56, 75, and 94, while in women these values were 29, 36, 48, 59, 72, 95, and 119. Therefore in our population, about 30% of men have HDL-C values < 40 mg/dL and about 30% of women have HDL-C values < 50 mg/dL. These values have been classified as being in the high risk category. Moreover patients with HDL-C values < 20 mg/dL, classified as marked HDL deficiency, are well below the 1st percentile for both men and women.

Levels of nonHDL-C calculated by subtracting HDL-C from total cholesterol levels have been shown in a number of studies including the Framingham Offspring Study to represent a stronger predictor of CVD risk than calculated LDL-C levels (66). The Third Adult Treatment Panel of the National Cholesterol Education Program classified a nonHDL-C value > 190 mg/dL as a high risk value, a value between 130 - 160 mg/dL as a borderline value, and a value < 130 mg/dL as optimal in high risk patients, while a value of < 100 mg/dL was considered optimal in patients with established CVD (1,4). The benefit of this calculation is that it can be used in the fasting and non-fasting state regardless of triglyceride values. Moreover this calculation maybe especially useful for CVD risk assessment in patients with diabetes mellitus (1,4). In our own population studies in 222,738 subjects for nonHDL-C in mg/dL the 1st, 5th, 25th, 50th, 75th, 95th, and 99th percentile values in our population were 57,74, 84, 104, 161, 212, and 259. Therefore our 75th percentile value was 161 mg/dL, indicating that values > 160 mg/dL are associated with increased CVD risk. In addition significant reductions in this parameter can be achieved with statin therapy or the combination of a statin and ezetimibe.

Lipoprotein Subfraction Assessment by Precipitation Methods

Gidez, Miller, Burstein, Eder, and colleagues in New York developed a combined heparin precipitation method and dextran precipitation to measure HDL cholesterol and HDL₃ cholesterol, allowing for the calculation of HDL₂ cholesterol (67). Using this methodology it was documented that CHD patients had significantly lower HDL cholesterol levels, especially lower large HDL₂ cholesterol levels, than control subjects (67). More recently Hirano and colleagues in Japan using the same principles have developed an even more simplified procedure for measuring the cholesterol concentration in HDL cholesterol and HDL₃ cholesterol, and then

calculating HDL₂ cholesterol by difference (68). This latter test will be available in the future for automated analyzers without pretreatment from the Denka-Seiken Company of Niigata, Japan. Hirano and Ito of Japan have developed an assay for small dense LDL cholesterol, and a value of > 40 mg/dl has been associated with increased CHD risk (69-71). An optimal value for small dense LDL cholesterol is < 20 mg/dl, and these particles can be effectively lowered with statin therapy (72). A newer assay has been developed for sdLDL-C that does not require pretreatment of the plasma or serum sample (73). This assay has been applied to samples from both the ARIC and Multi-Ethnic Study of Atherosclerosis (MESA) studies and has been found to be significantly better than LDL-C in prospective CVD risk prediction (74,75). In MESA it was also found to be superior to total and small LDL-P assessment in CVD risk prediction (75). In both ARIC and MESA, the 75th percentile value for sdLDL-C was about 50 mg/dL, while in the Framingham Offspring Study it was about 40 mg/dL, reflecting population differences (71,74,75). In our own population studies in 222,738 subjects for sdLDL-C in mg/dL the 1st, 5th, 25th, 50th, 75th, 95th, and 99th percentile values in our population were 9, 12, 19, 25, 36, 62 and 86. Therefore our 75th percentile value was 36 mg/dL, and in our view a value > 40 mg/dL is associated with increased CVD risk.

In our view sdLDL-C will become part of standard CVD risk assessment, and efforts to optimize this parameter with lifestyle modification and if necessary statin therapy are indicated. About 30% of our population had optimal values < 20 mg/dL. One of the reason sdLDL-C levels may superior to total cholesterol and LDL-C is that sdLDL apoB-100 is catabolized with a residence time of about 3.0 days versus 1.5 days for large buoyant LDL. This longer residence time may allow more sdLDL to be become modified and taken by macrophage scavenger receptors in the artery wall, promoting atherosclerosis. Moreover while LDL-C is very highly correlated with total cholesterol (r=0.92), in the same population sdLDL-C was not as strongly correlated (r=0.71). Remnant lipoprotein particles have long been considered to also be atherogenic. An antibody based precipitation assay for measuring cholesterol in remnant lipoproteins was developed by Nakajima and associates of Japan (76). Using this assay an elevated level of remnant lipoprotein cholesterol > 30 mg/dl was associated with dysbetalipoproteinemia, and a value > 10 mg/dL was associated with an increased risk of CVD, especially in women (76-78). Remnant lipoprotein cholesterol are increased in diabetic subjects, and their values increase significantly in the post-prandial state (79.80). Their values can be readily reduced by statin therapy (81). Newer direct on-line homogeneous assay have recently become available from the Denka-Seiken Company of Niigata, Japan and are now being tested in propspective epidemiologic studies.

Seman and Schaefer developed an assay for assessing Lp(a) cholesterol using lectin affinity in collaboration with the Genzyme Corporation, and an elevated value of > 10 mg/dl was associated with coronary heart disease (CHD) (82,83). Guadagno and colleagues developed an Lp(a) particle assay using electrophoresis followed by immunoblotting, but this assay has never been clinically validated (84). However, in our view neither of these assays has clinical utility because of lack of sensitivity. We recommend measuring Lp(a) by immunoassay (see below).

Lipoprotein(a) Measurements

Immunoassays for Lp(a) are now widely available and have been reasonably well standardized, mainly by the laboratory of Dr. Santica Marcovina of the Northwest Research Clinics Laboratory of the University of Washington in Seattle, WA. The first immunoassay for Lp(a) was developed by Albers and colleagues (85). Subsequently it was learned that lipoprotein(a) consists of an LDL-like particle to which apolipoprotein(a) is covalently linked to apoB-100 (85). The LDL-like moiety is composed of a central core of cholesteryl esters and triglycerides surrounded by phospholipids, free cholesterol, and a single molecule of apolipoprotein B (apoB). Apo(a) contains 10 different types of plasminogen kringle 4-like repeats as well as regions homologous to the kringle 5 and protease (P) regions of plasminogen. The kringle 4 type 2 domain (4_2) is present in multiply repeated copies from 2 to >40 that differ in number between apolipoprotein(a) isoforms. Apo(a) is linked to apolipoprotein B100 by a single disulfide bond involving an unpaired cysteine residue in kringle 4 type 9 (86).

Many studies have shown that elevated Lp(a) levels (> 50 mg/dL) as measured by various immunoassays are an independent CVD risk factor (87-89). In our own studies in the Framingham Offspring Study the measurement of Lp(a) cholesterol using the lectin method previously mentioned or apo(a) isoforms measured in Dr. Marcovina's laboratory did not add information about CVD risk above and beyond that obtained from the measurement of Lp(a) by immunoassay (90). Dr. Marcovina has recommended that Lp(a) be expressed in nmoles/L and has reported 75th percentile values ranging from 73 nmoles/L in a Caucasian population, to 130 nmoles/L in an African American population to 40 nmoles/L in a Japanese population (90). We understand her reasoning for recommending the use of nmoles/L, but we prefer using mg/dL, since in clinical practice apo(a) isoforms are not measured.

We recently assessed Lp(a) levels using the Denka-Seiken assay in 216,331 mainly Caucasian subjects (53% women, median age 58 years) at Boston Heart Diagnostics. Subjects with known CVD, non-fasting status, or those on lipid lowering medications were excluded from this analysis. Correlation analysis was carried using the Spearman method. In this analysis Lp(a) levels were significantly (p<0.001) higher in women than in men. Lp(a) values at the 50th, 75th, 90th, 95th, and 99th percentile values in women in mg/dL were 17, 51, 99, 121, and 189, respectively. Corresponding values in men were 11, 39, 83, 107, and 158, respectively. An elevated Lp(a) > 50 mg/dL has been designated as a high risk value by the European Atherosclerosis Society (88). About 25% of the women and about 20% of the men in our population had Lp(a) values > 50 mg/dL. The Europeans have recommended the use of statins to optimize LDL-C levels in those at high CVD risk, especially those with elevated Lp(a) levels. In addition this group recommended the use of niacin at 2 grams/day to lower Lp(a) levels. It should be noted that post-menopausal women with CVD that got significant benefit from oral hormonal replacement were those with elevated Lp(a) values (92). No such benefit was associated with the 21% Lp(a) lowering with niacin 2 grams/day observed in the Atherothrombosis Intervention in Metabolic Syndrome with Low HDL/High Triglyceride and Impact on Global Health Outcomes (AIM-HIGH) study (93). However these investigators did report on subjects with elevated Lp(a) values in this study. Recently it has been shown that inhibitors of proprotein convertase subtilisin kexin type 9 (PCSK9), which prevent breakdown of

the LDL receptor, can not only lower LDL-C by more than 50%, but also lower Lp(a) by about 35%, and reduce CVD risk on top of statin therapy by about 50% (94-96). Therefore PCSK9 inhibitors are a therapeutic option in CVD patients with elevated Lp(a) values.

Measurement of Apolipoprotein A-I and B

Most studies indicate that HDL-C serves as a better predictor of risk than does total serum apoA-I, as we have shown in the Framingham Offspring Study (66). Low values (25th percentile) for apoA-I for men are < 120 mg/dL, while for women such values are < 140 mg/dL. In many studies including those of Dr. Allan Sniderman of Montreal elevated levels of total apoB are a better predictor of CVD risk than is total cholesterol or calculated LDL-C (96-100). A high risk total serum apoB value has been classified as > 120 mg/dL, while an optimal value is < 80 mg/dL, representing about the 80th and 33rd percentiles in our population analysis of 251,522 subjects. In this population sampled in the fasting state and without CVD and not on lipid lowering medication the 1st, 5th, 25th, 50th, 75th, 95th, and 99th percentile values for apoB in mg/dL were 43, 55, 75, 91, 110, 143, and 172, respectively. In our view apoB values > 120 mg/dL associated increased CVD risk and values < 40 mg/dL are consistent with some form of hypobetalipoproteinemia, while undetectable values indicate the presence of abetalipoproteinemia.

ApoB is the integral protein found in chylomicron and their remnants, VLDL, LDL, and Lp(a). However apoB-48, the form of apoB made in the intestine, only contributes a very small amount to total apoB (approximately 0.5 to 2.0 mg/dl), and therefore can be ignored in terms of total plasma measurements which are usually about 100 fold higher (101,102) In our analysis from Framingham the total cholesterol/HDL cholesterol ratio provided identical information to that provided by the apoB/apoA-I ratio (96). A more recent study indicated that total serum apoB did increase the C statistic significantly for CVD risk prediction in the Framingham Offspring Study from 0.72 to 0.73 as compared to a model using standard risk factors (100). However this increase in our view is clinically not very significant.

PLASMA FATTY ACID MEASUREMENTS

Fatty acids are essential to heart health, and are chains of carbons (usually 14 - 22) with hydrogens attached. There are three fatty acids attached to glycerol to form triglycerides. Triglycerides are stored in fat cells, and are the major form in which energy is stored in the body. There are two fatty acids attached to phospholipids, which form bilayers that comprise all the cell membranes in the body. Embedded in these membranes are protein receptors, transporters, and ion channels critical for maintaining cell function. There is one fatty acid attached to cholesterol to form cholesteryl esters. About 70% of plasma or serum cholesterol is in the esterified form, and this form of cholesterol is found in the core of lipoprotein particles along with triglycerides and fat soluble vitamins. Phospholipids and apolipoproteins form the surface of lipoprotein particles. The type of fatty acids attached to triglycerides, phospholipids, and cholesteryl esters are mainly determined by dietary intake (103).

Plasma fatty acids obtained from subjects in the fasting state are a better measure of overall dietary fatty acid changes than are red blood cell fatty acids (103). Using red blood cell fatty acids has the disadvantage of not reflecting recent dietary changes, but only those changes in omega-3 fatty acids over a 3 month period, rather than the past 3-4 weeks. Moreover red blood cell fatty acids are not good measures of the intake of saturated fat, monounsaturated fat, polyunsaturated fat, or trans fats. It should be noted that only the essential fatty acids linoleic acid (18:2n6) and α -linolenic acid (18:3n3) cannot be made in the body and must be obtained from the diet. All other fatty acids, they can be formed from their precursor fatty acid. Linoleic acid is converted to arachidonic acid, while α -linolenic acid is converted to eicosapentaenoic acid and further to docosahexaenoic acid; however the efficiency of these latter conversions appears to be limited and quite variable among individuals.

Fatty acids have been classified as saturated if they have no double bonds, and the major saturated fatty acids in the diet and the bloodstream are palmitic acid (16:0) and stearic acid (18:0), along with smaller amounts of myristic acid (14:0). The sum of these three fatty acids in fasting plasma divided by the percentages of all measured fatty acids can serve as a guide to saturated fat intake (103). Foods rich in saturated fatty acids are beef, pork, and lamb meat, dairy products including butter, cheese, and whole milk, as well as lard, tallow, coconut oil, and palm oil (103). Saturated fatty acids can also be produced in the body. They are solid at room temperature, and generally comprise about 30-33% of plasma fatty acids. In membranes these fatty acids decrease membrane fluidity, resulting in less LDL receptor recycling and activity, and higher levels of low density lipoprotein cholesterol (LDL-C) in the bloodstream (104). Therefore these fatty acids in the diet and the bloodstream are associated with increased CVD risk. In our population based on 110,010 subjects sampled in the fasting state, the 25th, 50th, and 75th percentile values for the sum of myristic, palmitic, and stearic plasma fatty acids in % were 29.9, 31.4, and 33.0, respectively. We have therefore classified a saturated fatty acid index value > 33% as elevated and associated with increased CVD risk, while an optimal value is < 30%, and is associated with decreased CVD risk. Having a high saturated fat index in our view indicates that the patient needs to choose poultry without skin, fish, low fat dairy products, and very lean cuts of meats, and replace butter with vegetable oil or tub margarine, and whole milk with low fat or skimmed milk. When foods rich in saturated fatty acids are replaced in part by vegetable oils there is lowering of total cholesterol and low density lipoprotein cholesterol (LDL-C) due to enhance LDL clearance, decreased plasma saturated fatty acids, and a reduction in CVD risk (105-111). Moreover when dietary saturated fatty acids were reduced to < 6% of calories in the Women's Health Initiative there was significant CVD risk reduction (112).

Another class of fatty acids is the trans fatty acids. These fatty acids are formed when oils rich in monounsaturated and omega-6 polyunsaturated fatty acids are hydrogenated by repeated heating to high temperatures and then cooling. During this process the double bonds are either removed or converted to trans double bonds where the hydrogens are on opposite sides of the carbon chain instead of in the natural cis position where the double bonds are on the same side of the carbon chain. This results in a straightening of the carbon chain and a decrease in membrane fluidity (103). The major trans fatty acids in the blood stream are palmitelaidic

(16:1n9t) and elaidic (18:1n9t) acids, and these are the trans fatty acids that comprise the trans fatty index, divided by the total fatty acids. These fatty acids have one double bonds in the trans form at the 9th carbon position. They act like saturated fatty acids in that they are solid at room temperature. Dietary trans fatty acids raise LDL-C relative to monounsaturated or polyunsaturated fatty acids, by decreasing cellular LDL receptor activity and LDL clearance (103,113). They also lower HDL-C levels, by enhancing HDL apoA-I clearance. In our population of 110,010 subjects sampled in the fasting state the 25th, 50th, and 75th percentile values for the sum of palmitelaidic and elaidic acids in % were 0.36, 0.42, and 0.50. We have classified a trans fatty acid index of > 0.80% as elevated, and such values have only been observed in 2.6% of our population. In our view such values increases CVD risk, and patients with such values should restrict their dietary intake of fried foods, foods containing hydrogenated fats, shortening, stick margarine, and replace these foods with vegetable oils or trans fat free tub margarine. Elevated plasma trans fatty acids have been associated with an increased risk of developing CVD, and trans fatty acids are almost entirely obtained from the diet. Trans fatty acids are at low concentration in plasma because they are rapidly cleared from the circulation (113).

Fatty acids have been classified as monounsaturated if they have one double bond. The major monounsaturated fatty acids in the diet and in the bloodstream are oleic acid (18:1n9) and palmitoleic (16:1n7), which we measure. Oleic acid has a double bond at the 9th carbon from the omega or methyl end of the carbon chain, while palmitoleic acid has one at the 7th carbon position. Monounsaturated fatty acids are liquid at room temperature, and comprise about 20% of plasma fatty acids. These fatty acids can be produced in the body. They are found in foods of animal origin, as well as in vegetables like avocados, and in vegetable oils, especially olive oil and canola oil. Monounsaturated fatty acids, especially oleic acid, can also be made in the body. Double bonds in fatty acids improve membrane fluidity relative to saturated fatty acids because they cause a 37 degree kink in the carbon chain (103). Therefore their presence in cell membranes results in improved membrane fluidity and LDL receptor recycling and activity, lower LDL-C levels, and decreased CVD risk (103).

A European dietary trial documented that a Mediterranean diet enriched in either olive oil (1 liter/week) or 30 grams/day of nuts (walnuts, hazelnuts, and almonds) reduced CVD risk by about 30% relative to a low fat diet (110). These changes were associated with significant increases in plasma oleic acid in the olive oil group, and significant increases in alpha linolenic acid in the nut group (111). In our population of 110,010 subjects sampled in the fasting state the 25th, 50th, and 75th percentile values for the sum of oleic and palmitoleic acids in % were 18.7, 20.8, and 23.1, respectively. We have classified a monounsaturated fatty acid index of < 19% as being decreased, and associated with increased CVD risk, while a value > 22% has been classified as optimal and associated with decreased CVD risk. Having a low monounsaturated fat index indicates that the patient should increase their intake of olive oil and or canola oil in salads or use more of these oils in cooking.

Fatty acids have been classified as polyunsaturated if they have two or more double bonds. There are two types of polyunsaturated fatty acids, the omega-6 fatty acids, and the omega-3 fatty acids. The major omega-6 fatty acid in the diet and in the bloodstream is linoleic acid (LA, 18:2n6), which has two double bonds at the 6th and 9th carbon position. LA is the major fatty acid found in most vegetable oils including soybean, corn, safflower, and sunflower oils. LA is one of the essential fatty acids because the body cannot place a double bond at the n6 or omega-6 position of the carbon chain of fatty acids (1). LA must be obtained from the diet. In the body linoleic acid can be readily converted to arachidonic acid (AA, 20:4n6), which has four double bonds at the 6th, 9th, 12, and 15th carbon positions. Arachidonic acid is another major plasma fatty acid, and AA can also be obtained from the diet. AA has been reported to enhance the immune response, and in this regard may increase risk for auto-immune disease (103). Another omega-6 fatty acids that we measure is dihomogammalinolenic acid (20:3n6) which has three double bonds which have the same positions the first three for AA. This fatty acid has been reported to have anti-inflammatory and anti-thrombotic effects.

In large intervention trials where vegetable oils rich in LA such as corn oil and soybean oil were used to replace saturated fatty acids in the diet, there was lowering of lower total cholesterol levels and up to 50% reductions in CVD risk and mortality as documented in the Veterans Affairs Domiciliary Study, the Oslo Diet Heart Trial, and the Finnish Mental Hospital Study (104-109). Omega-6 fatty acids are liquid at room temperature, and confer increased membrane fluidity (103). In our population of 110,010 subjects sampled in the fasting state the 25th, 50th, and 75th percentile values for the sum of oleic, palmitoleic, and dihomogammalinolenic acids in % were 39.2, 42.6, and 45.5, respectively. We have classified an omega-6 fatty acid index as < 41% as being decreased, and a value > 46% as being increased, but because omega-6 fatty acid intake is already quite high in the United States, we have not made specific recommendations for therapy with regard to these values.

The unsaturated/saturated ratio is the sum of all beneficial monounsaturated and polyunsaturated fatty acids (oleic acid, palmitoleic acid, linoleic acid, arachidonic acid, dihomogammalinolenic acid, α -linolenic acid, eicosapentaenoic acid, docosapentaenoic acid, and docosahexaenoic acid) divided by the sum of all detrimental saturated and trans fatty acids (myristic acid, palmitic acid, stearic acid, palmitelaidic acid, and elaidic acid). In our population of 110,010 subjects sampled in the fasting state the 25th, 50th, and 75th percentile values for the unsaturated/saturated fat ratio were 1.99, 2.15, and 2.29, respectively. Having an unsaturated/saturated ratio index < 2.0 increases CVD risk, and indicates that the patient should increase their intake of vegetables, vegetable fats and oils (nuts, seeds, canola, soybean or other oils) and fish or fish oil and restrict their intake of animal and dairy fats (fatty meats. cheese, butter, dairy products, and ice cream), while an optimal value is > 2.25 (103). Omega-3 fatty acids are the other major type of polyunsaturated fatty acid. There are four major omega-3 fatty acids in the diet and in the bloodstream; alpha linolenic acid (ALA, 18:3n3). eicosapentaenoic acid (EPA, 20:5n3), docosapentaenoic acid (DPA, 22:5n3) and docosahexaenoic acid (DHA, 22:6n3). ALA has three double bonds at the 3rd, 6th, and 9th carbon positions, while EPA and DPA have five double bonds at the 3rd, 6th, 9th, 12th, and 15th carbon positions, and DHA has six double bonds at the 3rd, 6th, 9th, 12th, 15th, and 18th carbon positions. Flaxseeds, chia seeds, walnuts, as well as flaxseed oil, canola oil and soybean oil all contain considerable amounts of ALA. In addition to LA, ALA is an essential fatty

acid and must obtained from the diet since the body cannot place a double bond at either the 3rd or 6th carbon positions (103). ALA is liquid at room temperature and comprises about 1% of plasma fatty acids. In the Lyon Diet Heart Study when CVD patients were given a Mediterranean diet along with a margarine enriched in ALA (2 serving/day), there was a substantial reduction in CVD mortality as compared to patients who received the usual French diet (114). This reduction in risk was related to significant increases in plasma ALA levels in the intervention group.

Some ALA can be converted to EPA and DHA in the body, but most of the EPA and DHA must be obtained from the diet in the form of fish or fish oil. EPA and DHA are liquid at room temperature, and comprise about 3% of plasma fatty acids. EPA and DHA act in the liver to reduce triglyceride and very low density lipoprotein apoB-100 production (103). In addition both EPA and DHA have been reported to decrease inflammation in cells (103). Most over the counter fish oil capsules contain up to 50% EPA and DHA. In order to significantly lower triglyceride levels requires about 6 over the counter fish oil capsules/day or about four prescription fish oil capsules/day (115). These latter capsules are specially formulated to contain about 70-80% of their content as either EPA plus DHA, or DHA only, and have been approved for triglyceride lowering using 4 capsules/day in patients with fasting triglyceride values > 500 mg/dL (115).

Two over the counter fish oil capsules/day, one concentrated fish oil capsule/day, or two capsules containing almost solely EPA, have all been shown to decrease CVD morbidity, and in some cases mortality, as shown in the Diet and Reinfarction Trial, the GISSI study, and the Japan EPA Lipid Intervention Trial (JELIS) (116-122). In the latter trial getting plasma EPA levels to > 150 μ g/L was associated with very significant CVD risk reduction, especially in patients with dyslipidemia (triglycerides > 150 mg/dL and HDL-C < 40 mg/dL) (119-122). Capsules containing just EPA do not raise LDL-C levels, in contrast to capsules containing both EPA and DHA.

The Omega-3 Fatty Acid Index represents the sum of plasma EPA and DHA percentages divided by total fatty acids (103). In our population of 110,010 subjects sampled in the fasting state the 25th, 50th, and 75th percentile values for the sum of divided by total fatty acids in % were 2.23, 2.95, and 4.19, respectively. Having an omega 3 fat index of < 1.85% increases CVD risk, and indicates that the patient should increase their intake of oily fish such as herring, mackerel, salmon, sardines, sea bass, swordfish, or tuna, or take at least two fish oil or pure eicosapentaenoic acid (EPA) capsules daily. An optimal value is > 4.50%. It should be noted that both EPA and DHA significantly reduce inflammation in cells (123).

In our population of 110,010 subjects sampled in the fasting state the 25th, 50th, and 75th percentile values for concentration of ALA in μ g/L were 13.8, 18.2, and 24.5, respectively. Values of plasma ALA < 12.0 μ g/L have been associated with increased CVD risk, and having such levels indicates a need for the patient to increase their intake of ALA (flaxseed, canola, or soybean oils) (103). An optimal value is > 30.0 μ g/L. In our population the 25th, 50th, and 75th percentile values for concentration of EPA in μ g/L were 13.9, 21.3, and 34.9, respectively

Values of plasma EPA < 10.0 μ g/L have been associated with increased CVD risk and indicate a need for the patient to increase their intake of oily fish (herring, mackerel, salmon, sardines, sea bass, swordfish, or tuna) or fish oil or EPA capsules. An optimal value is > 50.0 μ g/L (20-22). Values > 150 μ g/L have been associated with significant heart disease risk reductions in a large clinical trial using EPA supplementation (122). Use of fish oil capsules has also been associated with heart disease risk reduction (116-122). In our population the 25th, 50th, and 75th percentile values for concentration of DHA in μ g/L were 51.3, 69.0, and 93.9, respectively Values of plasma DHA < 45.0 μ g/L are associated with increased CVD risk, indicating a need to increase the patient's intake of oily fish as for EPA or fish oil capsules. An optimal value is > 100.0 μ g/L (103).

Reducing and replacing dietary animal, dairy fats, and other foods rich in detrimental saturated and trans fatty acids with beneficial monounsaturated and polyunsaturated fatty acids in the form of vegetables oils (canola, soybean, and olive oils) and nuts (walnuts, hazelnuts, and almonds), and increasing dietary omega-3 fatty acids using fish or fish oil capsules have all been shown to reduce CVD risk (105-122). Knowing precise plasma fatty acid percentages and concentrations allows healthcare providers to make specific dietary recommendations based on current guidelines. While the focus for some doctors is just the omega-3 fatty acids, current guidelines also focus on restricting saturated fats and removing all trans fats from the diet (7,124). Therefore measuring plasma fatty acids serves as a useful guide for dietary assessment and recommendations (103).

MEASUREMENTS OF PLASMA STEROLS

Another useful measurement is the determination of plasma non cholesterol sterols by gas liquid chromatography/mass spectrometry after lipid extraction (125). One can then assess levels of lathosterol and desmosterol (markers of cholesterol production) and levels of β -sitosterol, campesterol, and cholestanol (markers of cholesterol absorption). The values of these sterols can be reported relative to total serum cholesterol as 100 x µmol per mmol of total cholesterol or in absolute concentration in mg/L. Plasma levels of lathosterol has been documented to be excellent marker of cholesterol synthesis, while levels of β -sitosterol and campesterol have been documented to be excellent marker of cholesterol absorption (126,127). In addition desmosterol is another marker of cholesterol and 20% via desmosterol (128). It should be noted that desmosterol levels are also determined by the rate of conversion of desmosterol to cholesterol via the enzyme 24-dehydrocholesterol reductase (DCHR24) (129). Therefore elevated desmosterol values relative to cholesterol are mainly a reflection of tissue desmosterol accumulation.

Using the methods outlined above we and others have documented that elevated plasma markers of cholesterol absorption, especially beta-sitosterol and campesterol, have been associated with cardiovascular disease (130-134). Moreover we and others have documented that elevated markers of cholesterol synthesis, especially lathosterol, are associated with insulin resistance, obesity, and familial combined hyperlipidemia, the most common genetic cause of

elevated low density lipoprotein (LDL) cholesterol and triglycerides (61,135-137). In addition we and others have observed that statins significantly lower markers of cholesterol synthesis, especially lathosterol, and increase markers of cholesterol absorption, while ezetimibe has the opposite effect (138-142). The statin/ezetimibe combination is the most effective way to lower LDL cholesterol levels by inhibiting both cholesterol synthesis and cholesterol absorption (141,142). Importantly while statin therapy has been shown to significantly lower CVD risk, it has also been reported that patients with heart disease on simvastatin got no benefit from therapy versus placebo if their baseline cholestanol levels were elevated (143). Markers of cholesterol synthesis and absorptions serve as a useful guide to optimize therapy to get patients to their LDL cholesterol goals. Getting patients to optimal LDL cholesterol levels has been associated with very significant benefit in terms of CVD risk reduction.

Based on an analysis of 333,379 subjects sampled in the fasting state the 25th, 50th, and 75th percentile values for lathosterol were 73, 108, and 156 μ mol x 100/mmol of total cholesterol (TC). We have classified values in μ mol x 100/mmol of TC of < 90 as optimal, 90 - 160 as borderline, and > 160 as elevated, consistent with cholesterol overproduction. The 95th percentile value in our population studies was 263 μ mol x 100/mmol of TC. Moreover the 99th percentile for the absolute concentration of lathosterol was is 8.1 mg/L, and we have classified values > 15.0 mg/L as being markedly elevated.

In the same population the 25th, 50th, and 75th percentile values for desmosterol were 51, 60, and 73 µmol x 100/mmol of total cholesterol (TC). We have classified desmosterol levels of < 70 µmol x 100/mmol of TC as optimal, borderline values as being between 70 - 90 µmol x 100/mmol of TC, and elevated values as being > 90 µmol x 100/mmol of TC. The 95th percentile value in our population was 113 µmol x 100/mmol of TC). Moreover the 99th percentile for the absolute concentration of desmosterol is 3.5 mg/L, and we have classified values of > 10.0 mg/L as being markedly elevated and consistent with desmosterolosis. Such elevations are often caused by mutations in *DCHR24* gene, with a significant lack of conversion of desmosterol directly to cholesterol (129,144,145). Such markedly elevated desmosterolosis (129,144,145). More moderate elevations in desmosterol may be associated with a significant increased risk of cognitive decline with aging (146). Statin therapy to inhibit desmosterol production may be indicated in such cases.

In the same population the 25th, 50th, and 75th percentile values for β -sitosterol were 96, 133, and 185 µmol x 100/mmol of TC. We have classified β -sitosterol levels µmol x 100/mmol of TC of < 100 as optimal, borderline values as being between 100 - 180 µmol x 100/mmol of TC, and elevated values as being > 180 µmol x 100/mmol of TC, consistent with cholesterol over absorption. The 95th percentile value in our population was 261 µmol x 100/mmol of TC. Moreover the 99th percentile for the absolute concentration of β -sitosterol was 7.5 mg/L, and we have classified values of > 15.0 mg/L as being markedly elevated and consistent with phytosterolemia.

In the same population the 25th, 50th, and 75th percentile values for campesterol were 128, 180, and 252 µmol x 100/mmol of TC. We have classified campesterol levels of < 130 µmol x 100/mmol of TC as optimal, borderline values as being between 130 - 230 µmol x 100/mmol of TC, and elevated values as being > 230 µmol x 100/mmol of TC, consistent with cholesterol overabsorption. The 95th percentile value in our population was 381 µmol x 100/mmol of TC. Moreover the 99th percentile for the absolute concentration of campesterol was 10.3 mg/L, and we have classified values of > 20.0 mg/L as being markedly elevated and consistent with phytosterolemia.

Very high levels of β -sitosterol and campesterol in both relative and absolute terms are associated with phytosterolemia, a disease often characterized by the presence of tendinous xanthomas, elevated serum cholesterol levels, and premature CVD (147-149). The optimal therapy for this condition is ezetimibe 10 mg per day, and these patients have defects in the ATP binding cassette transporters G5 and G8, resulting in retention of phytosterols in the intestinal cell and increased absorption of phytosterols and cholesterol with enhanced delivery of these sterols into the bloodstream on lipoprotein particles (147-149). In our analyses since January of 2013 we have detected 233 individuals out of 325,543 subjects tested that had βsitosterol levels consistent with phytosterolemia; therefore the disease is rare, but may be more common than previously thought, with an estimated 200,000 cases in the United States. In the same population the 25th, 50th, and 75th percentile values for cholestanol were 79, 100, and 128 µmol x 100/mmol of TC. We have not classified cholestanol levels. The 95th percentile value in our population was 210 µmol x 100/mmol of TC. Moreover the 99th percentile for the absolute concentration of cholestanol was 5.4 mg/L, and we have classified values of > 10.0mg/L as being markedly elevated and consistent with cerebrotendinous xanthomatosis (CTX). Very high levels of cholestanol in both relative and absolute absolute terms are associated with CTX, a disease associated with tendinous xanthomas, moderately elevated cholesterol levels, and cholestanol deposits in tendons and in the brain, which can lead to seizures and severe neurologic impairment. The optimal therapy for this condition is the use of the bile acid chenodeoxycholic acid 250 mg orally three times daily. These patients have a defect in converting cholesterol to chenodeoxycholic acid, one of the major bile acids, due to defects in sterol 27 hydroxylase gene. This defect results in the buildup of the intermediate sterol cholestanol. By giving them chenodeoxycholate the production of cholestanol is suppressed. and the progression of their disease can be halted (150-153). In our analyses since January of 2013 we have detected 78 individuals out of 325,543 subjects tested that had cholestanol values consistent with CTX; therefore the disease is rare, but may be more common than previously thought, with an estimated 50,000 cases in the United States.

STATIN ASSOCIATED MUSCLE SYMPTOMS AND MYOPATHY

New onset muscle aches and pains in patients placed on statin therapy are quite common (about 15%) (154-159). The mechanisms for this phenomenon based on muscle biopsies are mitochondrial dysfunction, attenuated energy production, enhanced muscle degradation, and depletion of co-enzyme Q10. These findings are most apparent in patients receiving 40 - 80 mg/day of either simvastatin or atorvastatin, which are the most lipophilic statins. Most of these

patients have normal creatine kinase (CK) levels and they are known as having statin associated muscle pains (SAMS) (154-159). About 1% of patients placed on statins will develop significant muscle pain and marked elevations in CK levels, known as statin induced myopathy (160). Very rarely patients will develop marked muscle breakdown known as rhabdomyolysis, associated with very high serum CK levels, dark urine, and sometimes even kidney failure, and death.

Based on two large trials with simvastatin, the risk of statin induced myopathy (muscle pain and CK levels at least 3 fold higher than the upper limits of normal on treatment with normal levels at baseline) was about 1% in those with the normal solute carrier organic anion transporter (*SLCO1B1*) T/T genotype. This risk was 4.5 fold increased for those who carried the T/C genotype (about 24% of the population), and 17.0 fold increased for those with the C/C genotype (about 2% of the population) (160). The defect was shown to be due to an amino acid substitution (Val174Ala) in the organic anion transporter 1B1 that takes up statins in the liver. Individuals with this variant have less efficient liver uptake of statins, higher blood levels, and a greater chance of increased statin delivery to muscle cells, especially for the lipophilic statins. This relationship between *SLCO1B1* genotype and statin associated muscle symptoms with or without CK elevation was also observed for high dose simvastatin and atorvastatin, but not for the water soluble pravastatin (161,162). Moreoever patients with underlying muscle disorders may be particularly prone to statin-induced myopathy (163).

Other risk factors for statin induced myopathy documented in the large trials were: age ≥ 65 years of age (2.2 fold increased risk), being female (1.8 fold increased risk), having an estimated glomerular filtration rate of < 60 mL/min/1.73 m² (2.5 fold increased risk), the use of a calcium channel blocker (1.7 fold increased risk) and the use of amiodarone (6.4 fold increased risk) (160). Therefore a 68 year old woman with a SLCO1B1 T/C genotype, and an estimated glomerular filtration rate (eGFR) of 50 mL/min/1.73 m² would have an estimated risk of statin induced myopathy of about 45%. Other known risk factors for SAM and statin induced myopathy include hypothyroidism and deficiencies of vitamin D and co-enzyme Q10.

Patients who have the *SLCO1B1* T/C or C/C genotype are at high risk for statin induced myopathy. In such patients it is particularly important to also rule out hypothyroidism, vitamin D deficiency, and co-enzyme Q deficiency, since such conditions have also been associated with statin induced myopathy. If these are present, one should treat accordingly. Doing this may be particularly important in women, those over 65 years of age, those with an eGFR < 60 mL/min/1.73 m², as well as those on calcium channel blockers and amiodorone. Since patients with CVD, LDL-C \geq 190 mg/dL, and and/or a 10 year CVD risk of \geq 7.5% are candidates for statin therapy, it is important to assess the risk in such subjects of statin-induced myopathy or muscle symptoms. Therefore in addition to SLCO1B1 genotyping it is also important to assess for the other risk factors mentioned above. Moreover the use of water soluble statins such as pravastatin or rosuvastatin at lower doses is generally well tolerated in high risk patients.

NEXT GENERATION DNA SEQUENCING FOR THE MOLECULAR DIAGNOSIS OF LIPID DISORDERS

The first two steps in the process of making the diagnosis of lipid disorders is to get complete measurements of lipid, lipoproteins, and plasma sterols as reviewed previously. The next step is to rule out secondary causes of lipid abnormalities as previously reviewed. It should be noted that common familial lipid disorders associated with premature CVD do not require DNA sequencing. These disorders include: Lp(a) excess, familial combined hyperlipidemia, familial dyslipidemia, and familial hypoalphalipoproteinemia (61). The third step is to determine if any of the following are present: fasting LDL-C values > 190 mg/dL, fasting triglyceride values > 1,000 mg/dL, HDL-C values < 20 mg/dL, VLDL-C or remnant cholesterol levels > 50 mg/dL and a VLDL-C/TG ratio > 0.5, desmosterol values > 10 mg/L, β -sitosterol values > 15 mg/L, campesterol levels > 20 mg/L, cholestanol levels > 10 mg/L, or evidence of cholesterol ester storage disease with moderate lipid abnormalities and evidence of liver disease (CESD). Thereafter next generation DNA sequencing is critically important for the definitive diagnosis of various lipid disorders of including familial hypercholesterolemia (FH), dysbetalipoproteinemia (DBL), phytosterolemia, CTX, desmosterolosis, and as well as diagnosis of the causes of significant statin induced muscle problems or myopathy (164). This testing allows for the formulation of the best personalized, targeted and effective treatments of: 1) FH using lifestyle modification, statins, ezetimibe, colesevelam, and if necessary proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors for the prevention of cardiovascular disease (CVD), 2) DBL with lifestyle modification, statins, fibrates, and niacin for CVD prevention, 3) phytosterolemia with lifestyle modification and ezetimibe for CVD prevention, 4) CTX with chenodeoxycholate for prevention of neurologic disease, 5) desmosterolosis with statin therapy for prevention of neurologic disease, and 6) CESD with newly available enzyme replacement for the prevention of liver failure and CVD. In addition one can diagnose the genetic causes of causes of marked hypertriglyceridemia and high density lipoprotein (HDL) deficiency, as well as provide genetic risk scores for elevated LDL-C and triglycerides, as well as decreased HDL (164).

The genes that need to be analyzed on an Illumina MiSeq platform for elevated LDL-C > 190 mg/dL and FH include the LDLR (LDL receptor), APOB (apolipoprotein or apoB), PCSK9 (proprotein convertase subtilisin/kexin type 9), LDLRAP1 (low density lipoprotein receptor adaptor protein 1), and STAP1 (signal-transducing adaptor protein). The gene sequenced for the diagnosis of DBL is APOE (apolipoprotein E). The genes sequenced for the diagnosis of phytosterolemia are ABCG5 and ABCG8 (ATP binding cassette transporters G5 and G8), for the diagnosis of CTX is CYP27A1 (sterol 27 hydroxylase), for the diagnosis of desmosterolosis is DHCR24 (24-dehydrocholesterol reductase), and for the diagnosis of CESD is LIPA (lysosomal acid lipase A) (165). An analysis of 10 specific single nucleotide polymorphisms at the LDLR (rs6511720), APOB (rs515135), PCSK9 (rs11206510), ABCG8 (rs6544713), CELSR2 (cadherin, rs12740374), HMGCR, (rs3846663, 3-hvdroxy-3-methyl-CoA reductase), TIMD4 (rs1501908,T-cell immunoglobulin and mucin domain containing 4, HNF1A (rs2650000, homeobox A), NCAN (rs10401969, neurocan), and MAFB (rs6102059, V-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B) gene loci allows one to calculate a genetic score for risk of elevated LDL-C levels since many such patients do not have a monogenic cause for this abnormality (164).

Because lifestyle modification and statin therapy remain the cornerstones of treatment for elevated LDL-C levels, after standard *SLCO1B1* genotyping, it is important to carry out gene sequencing at the following 8 gene loci to assess for true risk of statin induced myopathy: *SLCO1B1*, *AMPD1* (adenosine monophosphate deaminase 1), *CPT2* (carnitine palmitoyltransferase 2), *CoQ2* (coenzyme Q2 4-hydroxybenzoate polyprenyltransferase), *CYP2D6* (cytochrome P450, family 2, subfamily D, polypeptide 6), *PPARA* (peroxisome proliferator-activated receptor alpha, *PYGM* (phosphorylase, glycogen, muscle) and *SLC22A8* (solute carrier family 22 organic anion transporter, member 8). This latter testing will allow for the selection of the optimal statin type and dose for LDL-C lowering and compliance, and will also serve to diagnose underlying muscle disorders that be greatly aggravated by statin treatment (163,164).

For patients with marked hypertriglyceridemia the following 5 genes need to be sequenced: *LPL* (lipoprotein lipase), *GPIHBP1* (glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1), *APOA5* (apoA-V), *APOC3* (apoC-III), and *APOC2* (apoC-II) (164,165). One can also provide a genetic risk score for elevated TG levels based on 11 SNPs at the following 9 gene loci: *ANGPTL3* (rs10889353, angiopoietin-like3), *APOB* (rs7557067), *GCKR* (rs1260326, glucokinase or hexokinase 4 regulator), *MLXIPL* (rs714052, MLX interacting protein-like), *XKR6* (rs7819412 XK Kell blood group complex subunit related family, member 6), *LPL* (rs328, rs12678919), *TRIB1* (rs2954029, tribbles pseudokinase 1), *FAD Cluster* (rs174547, fatty acid desaturase cluster) and *APOCluster* (rs964184 and rs3135506, apolipoprotein AI/CIII/AIV/AV gene cluster) (164).

In order to provide a molecular diagnosis of specific disorders associated with marked HDL deficiency the following genes need to be sequenced: *ABCA1* (ATP binding cassette transfer protein AI), *APOA1 (apoA-I), and LCAT* (lecithin cholesteryl acyltransferase). There are also 4 genes associated with elevated HDL and lack of normal HDL function, namely *CETP* (cholesteryl ester transfer protein), *SCARB1* (scavenger receptor B1, *LIPC* (hepatic lipase), and *LIPG* (endothelial lipase). This analysis allows for the definitive diagnosis of apoA-I deficiency, apoA-I variants, Tangier disease, LCAT deficiency, and fish eye disease, as well as CETP, hepatic lipase, and SR-B1 deficiency (45-48,164,165). Based on a analysis of 14 SNPs at the following gene loci: *GALNT2* (polypeptide N-acetylgalactosaminyltransferase 2), *LPL, TTC39B* (tetratricopeptide repeat domain 39B), *ABCA1, FADCluster, APOCluster, MMAB* (methylmalonic aciduria cbIB type), *LIPC, CETP, LCAT, LIPG, ANGPTL4, HNF4A* (hepatocyte nuclear factor 4 alpha), and *PLTP* (phospholipid transfer protein) one can also generate a low HDL-C genetic score (164).

Familial Hypercholesterolemia (FH)

FH is occurs in about 1:250 individuals in the general population, and in about 1-2% of patients with premature CVD. Many experts and guidelines panels in the United States (National LIpid Association), Canada, Europe, and Australia) have recommended screening of families with FH and potentially genetic characterization of the molecular defect (166-170). FH is characterized by significantly elevated serum LDL-C levels > 95th percentile for age and gender adjusted

norms (usually > 190 mg/dL), while off cholesterol lowering medication. In FH families about 50% of the parents, siblings, and offspring of the affected patient will also have an elevated LDL-C value, although occasionally an autosomal recessive form is observed (171). FH has also been associated with human cholesterol 7alpha-hydroxylase deficiency in one kindred (172). FH heterozygotes in the untreated state generally develop CVD prior to age 60 years (average age of onset 45 years in men, and 55 years in women). Most patients with FH are heterozygous for mutations at the following five different gene loci in order of frequency: LDLR. APOB, PCSK9, LDLRAP1, and STAP1 (164). These patients can generally be well-managed with statins, ezetimibe, and if necessary colesevelam, and a PCSK9 inhibitor (93-95,173-175). Some rare patients (about 1:300,000 to 1:1,000,000 in the general population) are more severely affected and have LDL-C levels > 500 mg/dL off medications, and may have homozygous or compound heterozygous FH. In such patients it is especially important to identify the genetic defect in order to treat them properly. Most of these patients are unresponsive or minimally responsive to statin therapy. Such patients may also be unresponsive to PCSK9 inhibitors if they lack LDL receptors (null mutations) or their LDL receptors are totally dysfunctional. In the untreated state these patients often develop CHD prior to age 20 years, and disease detection can be optimized with the use of computed tomographic angiography (176,177). These patients may respond to statins, ezetimibe, PCSK9 inhibitors, mipomersan, lomitapide, but sometimes can only be treated with LDL apheresis (178-180). For patients with elevated LDL-C > 190 mg/dL without mutations in the LDLR, APOB, PCSK9, LDLRAP1, or STAP1, APOE, ABCG5, ABCG8, or LIPA genes, and a high polygenic score for elevated LDL-C, the standard therapy is identifying and treating any secondary cause of elevated LDL-C or statin induced myopathy, lifestyle modification as recommended by the ACC/AHA, and intensive statin therapy (2-4). The goals of therapy are LDL-C < 130 mg/dL in all patients, < 100 mg/dL in patients with a 10 year CVD risk > 7.5% based on the ACC/AHA risk calculator (3), and < 70 mg/dL in patients with established CVD as per the recent National Lipid Association guidelines (45). In some patients ezetimibe may have to be added to their regimen, and or colesevelam. Patients with CVD who are unable to tolerate statins or do not get to their LDL-C goal with the above therapies may be candidates for PCSK9 inhibitors.

Dysbetalipoproteinemia (DBL)

Sometimes patients will have markedly elevated fasting total cholesterol and triglyceride levels, and their direct LDL-C values may not be elevated. These patients often have very high levels of cholesterol in very low density lipoproteins (VLDL) with VLDL-C levels > 100 mg/dL, and a ratio of VLDL-C/total triglyceride of > 0.3 (180). Laboratories that measure direct LDL-C can help make this diagnosis, because by calculation one can obtain a direct measure of VLDL-C as well. Such patients may have tubo-eruptive xanthomas on their elbows and hands, as well as arcus and premature CVD. They have DBL due to defects in apoE or have apoE deficiency (181,183). Many of them are homozygous for the apoE2/2 variant, but also may have another familial lipoprotein disorder such as familial combined hyperlipidemia. DBL patients have a defect in removing both VLDL and chylomicron remnant particles from their bloodstream (182). These patients are very responsive to medical therapy. The standard therapy is identifying and treating any secondary cause of elevated LDL-C or statin induced myopathy, lifestyle

modification as recommended by the ACC/AHA, fenofibrate, and statin treatment. Niacin is also very effective in these patients. The goal of therapy is to get their VLDL-C levels to < 50 mg/dL.

Phytosterolemia

Some patients will present with significantly elevated LDL-C levels due to defects in *ABCG5* and *ABCG8* (147-149). Normally almost all the cholesterol and other sterols inside the intestines enter the intestinal cell via the transporter Niemann Pick C1-like protein 1, and then the intestinal cell regulates its sterol content by sending about 50% of the cholesterol and > 90% of β -sitosterol and campesterol via the ABCG5 and ABCG8 transporters back into the intestinal lumen for excretion from the body in the feces. Patients with phytosterolemia with defects in *ABCG5* and *ABCG8* overabsorb both cholesterol and the plant sterols β -sitosterol and campesterol in the intestine, and therefore have elevated levels of these constituents in the bloodstream, often leading to tendonous xanthomas and premature CVD. These patients have very high absolute plasma concentrations of β -sitosterol (> 15.0 mg/L) and campesterol (>20.0 mg/L). These patients are very responsive to ezetimibe therapy (149).

Cerebrotendinous Xanthomatosis

These patients have very high plasma levels of cholestanol (> 10 mg/L) and generally present with tendinous xanthomas and only moderately elevated LDL-C. They may suffer from neurologic problems, and if undiagnosed, they will develop severe ataxia by the third or fourth decade of life. They have defects in *CYP27A1* (sterol 27 hydroxylase) gene and have an inability to form the bile acid chenodeoxycholate. If these patients are diagnosed and treated, one can prevent the development of the severe neurologic disease. These patients with very high plasma levels of cholestanol (> 10 mg/L) present with tendinous xanthomas and only moderately elevated LDL-C. The standard therapy is identifying and treating any secondary cause of elevated LDL-C or statin induced myopathy, lifestyle modification as recommended by the ACC/AHA, and therapy with chenodeoxycholate 250 mg orally three times daily to prevent the development of neurologic problems (150-153).

Desmosterolosis

These patients have very high plasma levels of desmosterol because of defects in the *DHCR24* (24-dehydrocholesterol reductase) gene, causing a decreased ability to convert desmosterol to cholesterol. They may develop significant neurologic disease and cognitive dysfunction quite early in life if undiagnosed. These patients have plasma levels of desmosterol > 10 mg/L due to a decreased ability to convert desmosterol to cholesterol. The standard therapy is identifying and treating any secondary cause of elevated LDL-C or statin induced myopathy, lifestyle modification as recommended by the ACC/AHA, and therapy with a statin to decrease desmosterol production in order to prevent the development of neurologic problems (129,144,145).

Cholesterol Ester Storage Disease (CESD)

Patients with CESD or lysosomal acid lipase deficiency often present with more modest elevations in fasting serum LDL-C (> 160 mg/dL) as well as serum triglycerides (TG > 150 mg/dL). They often also have low levels of HDL-C (< 40 mg/dL in men and < 50 mg/dL in women). Based on their values they look like patients with familial combined hyperlipidemia, except that they have enlarged livers and elevated liver transaminases (> 120 U/L). They also have a deficiency of lysosomal acid lipase activity in their plasma, which can be measured (184-186). These patients have defects in lipase A (LIPA), the lysosomal acid lipase (also known as cholesterol ester hydrolase), the enzyme that breaks down cholesterol and triglycerides in the liver and other tissues. Enzyme replacement can prevent the liver failure that can develop in these patients. Patients with CESD or lysosomal acid lipase deficiency due to defects in the LIPA gene, have marked cholesteryl ester deposition in the livers, and will often develop liver failure if they do not receive appropriate enzyme replacement therapy (Sebelipase alfa, available from Synageva, Lexington, MA) (184-186).

Marked Hypertriglyceridemia

Patients with fasting triglycerides > 1,000 mg/dL are at increased for developing recurrent pancreatitus. Such patients generally have defects in one of the following genes: *LPL, GPIHBP1, APOA5, APOC3,* or *APOC2* (164). Occasionally they will have a very high genetic triglyceride risk score based on SNP analysis as previously mentioned (164). These patients usually respond very well to restriction of dietary fat and sugar, as well as treatment with fenofibrate and high dose omega-3 fatty acids. They may occasionally require statin therapy to optimize their LDL-C levels once their fasting triglyceride levels are < 400 mg/dL (187).

Marked HDL Deficiency

In the absence of marked hypertriglyceridemia, liver disease, uncontrolled diabetes, or increased inflammation, or patients receiving testosterone injections, most patients with HDL-C levels < 40 mg/dL have either familial combined hyperlipidemia, familial dyslipidemia, or familial hypoalphalipoproteinemia (61). It is well know that patients with dyslipidemia have high residual CVD risk, and these are the patients that have been shown to get significant benefit on top of statin therapy from either EPA in the JELIS Trial, with fenofibrate in the ACCORD trial in diabetic subjects, or with niacin in the AIM study in CVD patients (120,188,189). Such patients do not require a molecular diagnosis. However patients with HDL-C levels < 20 mg/dL, usually have familial apoA-I deficiency, an apoA-I variant, Tangier disease, or LCAT deficiency due to defects in the APOAI, ABCA1, or LCAT genes (45-48,164,165). Occasionally they may have a very high genetic score for low HDL-C (164). Patients with undetectable plasma apoA-I levels due to defects in the APOAI gene usually develop xanthomas and premature CVD, and are best treated by optimizing all other CVD risk factors (45,165). Patient with apoA-I variants have a very heterogeneous clinical picture, but their treatment is similar as for apoA-I deficiency. Patients with homozygous or compound heterozygous Tangier disease due to ABCA1 defects have cholesteryl ester deposition in many tissues in the body including their nerve sheaths, tonsils, liver, and spleen (165). Such patients often have peripheral neuropathy and

hepatosplenomegaly. Tangier patients with marked splenomegaly often have anemia and very low LDL-C levels, and generally do not develop premature CVD. In contrast such patients without marked splenomegaly and without anemia, usually have normal LDL-C levels, and they generally do develop premature CVD. These latter patients are best treated by optimizing their LDL-C levels with statin therapy and if necessary the addition of ezetimibe (46,165). Finally patients with LCAT deficiency generally present with marked corneal opacification, splenomegaly, and anemia (48,165). These patients usually have very low LDL-C levels, and do not develop premature CVD, but they do develop kidney failure usually in the 4th or 5th decades of life (165). In the future such patients will hopefully be treated with enzyme replacement therapy to prevent their kidney disease.

CONCLUSIONS

Standard lipid analysis often misses the excess CVD risk associated with elevated small dense LDL-C and Lp(a) levels, and decreases in large HDL particles. Therefore the measurement of these parameters is useful for assessing CVD risk. Common familial lipid disorders associated with premature CVD include Lp(a) excess, combined hyperlipidemia, and dyslipidemia. Plasma fatty acid analysis is important in order to assess for the adequacy of omega-3 fatty acid intake, and whether there are excess levels of saturated and trans fatty acids in order to provide personalized dietary recommendations. Ruling out the presence of diabetes, thyroid, liver, and kidney disease is also critical in the diagnosis and management of lipid disorders, as is a good history, especially with regard to the use of medications and supplements. Measuring plasma sterols is critical in evaluating causes of elevated very low density lipoprotein (VLDL-C > 30 mg/dL) and/or LDL-C (> 160 mg/dL) which includes familial combined hyperlipidemia (elevated lathosterol), familial hypercholesterolemia (normal sterols), dysbetalipoproteinemia (moderate increases in β -sitosterol), phytosterolemia (very high β -sitosterol), and cerebrotendinous xanthomatosis (very high cholestanol). The measurement of apolipoprotein (apo) A-I in HDL particles by gel electrophoresis is important assessing CVD risk, HDL functionality, and for the diagnosis of marked HDL deficiency states (HDL-C < 20 mg/dL due to apoA-I deficiency and variants, Tangier disease, and lecithin:cholesteryl acyltransferase (LCAT) deficiency. The measurement of apoB is important for the diagnosis of abetalipoproteinemia and hypobetalipoproteinemia. The definitive diagnosis of the above mentioned disorders along with causes of markedly elevated triglycerides (> 1,000 mg/dL) requires next generation DNA sequencing of the appropriate and relevant genes for these disorders in order to provide a definitive molecular diagnosis often necessary to formulate optimal therapy strategies.

Acknowledgements and Potential Conflicts of Interest

Drs. Schaefer, Diffenderfer, Polisecki, and Mehan are employees of Boston Heart Diagnostics, Dr. Asztalos is a consultant to Boston Heart Diagnostics. Drs. Asztalos, Schaefer, and Diffenderfer are also employees of Tufts University. None of the other co-authors have conflicts of interest. Boston Heart Diagnostics is a reference laboratory that offers many of the tests reviewed in this chapter.

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