ARIC

ATHEROSCLEROSIS RISK IN COMMUNITIES STUDY

MANUAL 8

Lipid and Lipoprotein Determinations

The National Heart, Lung and Blood Institute of the National Institutes of Health

MANUAL 8: LIPID AND LIPOPROTEIN DETERMINATIONS

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I. OBJECTIVE AND SPECIFIC AIMS

The objectives of ARIC are to identify factors associated with atherosclerosis and to study the incidence of clinical manifestations of atherosclerotic disease among men and women, both black and white, in four U.S. communities.

The function of the Central Lipid Laboratory in this multicenter study is to provide lipid measurements in the entire cohort and to conduct special studies related to lipid transport and atherogenesis in selected samples of the cohort. The results of these measurements shall serve to increase our understanding of the etiology and pathophysiology of atherosclerotic disease.

The specific aims for the Central Lipid Laboratory are:

1. To provide measurements of plasma cholesterol, plasma triglycerides, HDL-chol, and glucose in the entire cohort for visits 3 and 4. These measurements shall be fully comparable with those of visits 1 and 2 and shall be reported to the respective agencies within a week of receipt of samples.

2. To provide, in defined samples of the cohort, measurements of genetic and phenotypic markers of plasma lipid transport and endothelial cells that are known or suspected to play a role in plasma lipid transport and atherogenesis.

3. To provide storage space for aliquots of study specimens at conditions preserving the integrity of factors thought to be of pathophysiologic relevance in atherogenesis.

4. To interact with the Steering Committee in the design and implementation of special lipid studies to gain the greatest knowledge at the lowest cost.

5. To participate actively in the analysis and publication of study results within the guidelines set forth by the Steering Committee.

II. RATIONALE AND APPROACH FOR SELECTION OF MEASUREMENTS

II.A. ANALYTE SELECTION FOR ENTIRE COHORT

II.A.1. Plasma-Cholesterol

Elevated plasma cholesterol levels are generally accepted as a risk factor in the development of accelerated atherosclerosis and may lead to an increased incidence of CHD (1-3). The contribution of plasma cholesterol to the risk of CHD is determined by its partition among various lipoprotein fractions. A relatively large proportion of cholesterol carried in VLDL, chylomicron remnants, IDL, and LDL are "atherogenic," whereas a large proportion carried in HDL appears to be "protective" against the development of atherosclerotic manifestations (4-11). Clearly, levels of plasma cholesterol and its subfractions are influenced by a wide variety of environmental and genetic factors. The ARIC study design provides three unique opportunities for identification of those factors underlying CHD risk factor variability. The design permits investigations of the complex interactions between multiple environmental and genetic factors as they influence serum analyte concentrations. The strata to be considered include,

but are not limited to, racial groups, gender, and hormone use in females. The genetic factors to be considered include, but are not limited to, the apoE polymorphism, apoB polymorphism, and apo(a) length variation. Such studies of genotype by environmental interaction begin to shed light on the causes underlying CHD risk factor heterogeneity among races, populations, genders, and other important groups.

The second opportunity, afforded by the large sample size of the ARIC project, is the comparison of subsamples from the extreme tails of the analyte distribution. For example within a single racial group by gender strata, the ARIC study will permit detailed comparisons between, and investigations of, the upper and lower deciles of the total cholesterol distribution. Even with such informative restrictions, the comparison will still have sufficient sample size for detecting effects of small or moderate size. Of course, all investigations will ultimately include each racial group and both genders.

Finally, the ARIC sample encompasses individuals extending beyond the normal range of serum analyte variation. The work proposed here include, but is not limited to, the rare dyslipoproteinemias, since such studies have proved invaluable for understanding the mechanisms by which gene alterations can give rise to increased CHD risk. Hence, the measures and studies mentioned shall lead to a better understanding of both the differences between the tails of the analyte distributions and the factors that give rise to normal variation.

Among the factors that elevate plasma cholesterol levels and influence its transport by atherogenic lipoproteins are genetic abnormalities such as familial hypercholesterolemia (FH), dysbetalipoproteinemia, and familial combined hyperlipoproteinemia (FCH) (12). These dyslipoproteinemias result from changes in the rate of synthesis, post-secretory processing in plasma, and/or removal of lipoproteins from the circulation. Indeed, several mutations of genes regulating lipid metabolism have been described, which explain the biochemical abnormalities found in the plasma of various disorders of lipid transport.

While major advances have been made in the understanding of the pathophysiology of dyslipoproteinemias, the mechanisms by which arterial wall damage occurs in these disorders are still not fully understood, though several working hypotheses have been presented (13- 18). In FH, for instance, mutations in the LDL receptor gene lead to malfunctioning of the LDL-receptor pathway and accumulation of LDL in plasma (19). Increased residence time of LDL may lead to modification of these lipoproteins, thus rendering them susceptible to endocytosis by macrophages and endothelial cells (220-22). Such a scavenger pathway may induce the development of lipid depositions and foam cells, typical for the atherosclerotic process (23). Exposure of LDL to endothelial cells can lead to peroxidation of LDL components (24). This results in the alteration of the structural and biological properties of LDL particles. These modified LDL become ligands for the scavenger receptor, may attract monocytes, and are cytotoxic (24-27). Thus, cholesteryl ester may accumulate in macrophages and induce foam cells and plaque formation.

Although the dyslipoproteinemias mentioned confer greatly increased risk for CHD to affected individuals, only a minority of patients with clinical manifestations of atherosclerosis have these well-defined abnormalities of the lipid transport system (28). Thus, the impact of the classical dyslipoproteinemias on the overall incidence of CHD is relatively small. Other factors may be more important for the prevalence of atherosclerosis in the general population. Such factors may include more subtle genetic dispositions to abnormalities in lipid transport, and lipid metabolism which may only

become apparent under certain environmental conditions.

II.A.2. Triglycerides

Many studies have shown an association between fasting plasma triglycerides (TG) and CHD (28-32). This association is often not maintained in prospective studies upon correction for other lipid parameters, mainly because of a strong inverse association of TG with HDL-chol (33,34). Even though HDL-chol is a more powerful indicator of risk of CHD, evidence suggests that it is the metabolism of TG-rich lipoproteins (TGRL) that influences plasma levels of HDL-chol and not vice-versa (35,36). The connection between the metabolism of TGRL and HDL-chol has been clearly established in the postprandial state, and the mechanisms by which high TG reduce HDL-chol are reasonably well understood (37-39). Recognizing the potential importance of the metabolism of TGRL for atherogenesis, ARIC participates in a collaborative study to determine the relationship of TGRL-metabolism ascertained in the postprandial state with subclinical atherosclerosis. TG analysis is also necessary for calculation of LDL-chol (40).

II.A.3. HDL-Cholesterol

Levels of HDL-cholesterol have been shown to be inversely correlated with the risk of CHD (7-11). More importantly, the predictive value of HDL- cholesterol levels was stronger than any of the other lipid measurements, and a wide range of level were found in "healthy" subjects not affected by the classical dyslipoproteinemias (41). HDL, one of the four families of plasma lipoproteins in humans, are heterogeneous with respect to physical, chemical, and functional properties (42-48).

The mechanism(s) by which HDL "protect" against atherosclerosis may be related to the following observations: (1) HDL seems to be a major factor in the proliferation of vascular endothelial cells (49); (2) HDL reduces the uptake of LDL by several cell types (50-52); and (3) HDL enhances the net removal of cellular cholesterol from a variety of cells such as aortic smooth muscle cells and fibroblasts (54-56). Importantly, HDL increases the egress of cholesterol from macrophages preloaded with cholesterol with chemically modified LDL (57). These observations support the hypothesis that HDL functions in reverse cholesterol transport (58); thus, HDL would serve as a scavenger of tissue cholesterol, including that of the arterial wall. A second hypothesis which is not exclusive of the former relates to the metabolic interrelationship of HDL and TGRL. According to this hypothesis, high HDL-chol levels reflect effective catabolism of TGRL (37).

Irrespective of the mechanism(s) involved, HDL-chol is an essential part of the lipid profile. It is required for minimal characterization of changes in lipid transport in study participants and is also necessary for the calculation of LDL-chol.

II.A.4. Plasma Glucose

Plasma glucose levels have been measured in the first two visits by the Central Chemistry Laboratory. Glucose measurements represent a minimal assessment of carbohydrate metabolism, which is interrelated to lipid transport and has been implicated in atherogenesis. Since the Central Chemistry Laboratory will no longer provide services, we propose to include glucose measurements in our panel of measurements for visit 3 and 4 for the entire cohort.

II. B. PREVIOUSLY PERFORMED CASE-CONTROL STUDIES

Case-control studies during visits 1 and 2 as well as some measurements performed in the entire cohort will be available on a case-control basis for visits 3 and 4. The rationale for these studies has been given in our previous manual. The studies include:

II.B.1. Plasma ApoA-I
II.B.2. Plasma ApoB
II.B.3. Plasma Lp(a)
II.B.4. Lp(a) Phenotype
II.B.5. LDL-Chol/LDL-ApoB
II.B.6. LDL-Size
II.B.7. ApoE Genotype
II.B.8. RFLP in the A-I/C-III/A-IV Gene Cluster

II.C. NEW CASE-CONTROL STUDIES

II.C.1. Studies on HDL and TG-Metabolism

1.1. LpA-I and LpA-I/A-II
1.2. Studies on ApoA-I, ApoC-III, and ApoA-IV Gene Expression
  i. ApoA-IV and ApoC-III plasma levels
  ii. ApoA-IV Gene Sequence Polymorphism
  iii. ApoA-I promoter variability

1.3 Cholesteryl Ester Transfer
  i. Cholesteryl Ester Transfer Protein (CETP) activity and/or mas

1.4 ApoB Signal Length Polymorphism

II.C.2. Studies on Lp(a)

2.1 Lp(a) levels and phenotypes in hypercholesterolemic subjects
2.2 Lp(a) levels and phenotypes in blacks and whites
2.3 Age-dependent increases of Lp(a) in women and their relationship to phenotype
2.4 Apo(a) Genotyping

II.C.3. Plasma Endothelin Levels

Four types of lipoprotein abnormalities are commonly associated with atherosclerosis in populations. These include: i) decreased HDL-chol levels, often accompanied by elevated fasting TG levels or exaggerated postprandial lipemia; ii) elevated levels of LDL; iii) increased plasma concentrations of chylomicron remnants and IDL; and iv) increased concentrations of Lp(a). One or more of these lipoprotein phenotypes is often present in survivors of myocardial infarction (59). These four lipoprotein phenotypes are of multifactorial origin and may mediate, at least in part, the risk of CHD that is conferred by other risk factors such as insulin resistance and obesity. Furthermore, risk factors such as cigarette smoking and hypertension are exaggerated by disorders of lipid transport. Nevertheless, other etiologies including disturbances in homocysteine metabolism (60) or clotting disorders may cause atherogenesis as well, even though lipid disorders may accelerate disease progression in these conditions.

Finally, the susceptibility of the arterial wall to atherogenic lipoproteins or other noxious stimuli may play a significant role in precipitation of atherosclerotic disease. This hypothesis is based on the well known occurrence of CHD in the absence of any of the classical risk factors and observations, that there exists wide variability in the severity and extent of atherosclerosis despite similar disorders of lipid transport. For example, patients with the heterozygous form of FH may die in their thirties from atherosclerotic complications, while others may live into their seventies and die from other causes (12). Clinical and epidemiological studies in man are limited to studies of blood factors, even though many of the genes that cause disease are expressed in tissues that are inaccessible for sampling such as liver and intestine. Hence, the researcher is confronted with a complex phenotype that results from the interaction of a large number of genes. However, by choosing well defined phenotypes, we believe that the complexity of genes involved may, at least in part, be disentangled. We propose a research program that should allow the definition of subsets within the phenotype entities described above. In addition, we propose studies that should provide some information as to the contribution of arterial wall factors. The role of increased concentrations of chylomicron- and/or VLDL remnants in atherogenesis is not discussed here since the respective pathomechanisms are investigated in the "Postprandial Lipoprotein Study."

II.C.1. Studies on HDL and TG Metabolism

II.C.1.1. Plasma Concentrations of LpA-I and LpA-I/A-II

Human HDL are traditionally divided into two major subclasses termed HDL_2 and HDL_3. These HDL-subclasses are heterogeneous with respect to physical, chemical and functional properties (42-47), and can be further divided into HDL containing apoA-I without apoA-II (LpA-I) and HDL containing both apoA-I and apoA-II (LpA-I/A-II), the two major HDL apolipoproteins (48). Clinical studies showed that drug treatment and alcohol consumption alter the proportions of LpA-I to LpA-I/A-II particles (61,62). The well established male-female difference in plasma levels of HDL_2 was shown to result from increased concentrations of LpA-I in HDL_2 of females (63). We have data to show that a high cholesterol diet affects the amount and proportions of LpA-I/A-II in HDL_2 and HDL_3. Furthermore, the molar ratio of apoA-I to apoA-II in HDL_2, most likely reflecting different proportions of LpA-I to LpA-I/A- II, was correlated with lipoprotein lipase activity and HDL_2 levels, but inversely correlated with TG abundance in HDL_2, hepatic lipase activity, and the magnitude of post-alimentary lipemia (39). *In vitro* incubations of TG-rich lipoproteins (TGRL) and postprandial HDL_2 with hepatic lipase suggested that apoA-II containing particles are preferentially converted to HDL_3-like particles (38). This suggestion has now been confirmed in our laboratory by incubating hepatic lipase with LpA-I or

LpA-I/A-II (64). According to experiments by Fielding et al., particles containing apoA-I may be the physiologic acceptor of cellular cholesterol, while HDL containing both apoA-I and apoA-II may be involved in the esterification of cell-derived cholesterol (65). All of these studies indicate that LpA-I and LpA-I/A-II are metabolically distinct and fulfill different functions. That measurement of LpA-I and LpA-I/A-II could be of clinical relevance, is suggested by preliminary studies showing lower LpA-I levels in patients with angiographically- verified CHD when compared to controls (66). Clearly, a more comprehensive evaluation of the risk associated with differences in plasma levels of LpA-I and LpA-I/A-II is needed as well as comparison of the relative predictive power of these levels with other established risk factors. Such information may be obtained by using cases and controls of the cohort. Furthermore, the significance of differences in plasma levels of LpA-I may be greater in patients with low HDL-chol.

II.C.1.2. Apolipoprotein A-I, C-III and A-IV Gene Expression

II.C.1.2.(i). Measurement of ApoA-IV and ApoC-III Plasma Levels

Apolipoproteins comprise a multigene family dispersed into the genome. Seven of the eight major apolipoproteins arose from a primordial gene via multiple partial and complete gene duplication, translocation, unequal crossover, and perhaps gene conversion (67-69). As a result, apolipoprotein genes acquired distinct sequences that confer functional specificity and permit differential expression in response to metabolic stimuli. In addition to structural similarities, some of the apolipoprotein genes are closely linked and tandemly organized (70-73). The AI-CIII-AIV gene cluster is located on chromosome 11 within a 20-kb DNA segment (70). ApoA-I and apoA-IV are closely related in structure and share several functions. Both are activators of LCAT (74,75), and both have been suggested to mediate the uptake of HDL by hepatocytes (76-78). ApoA-IV may facilitate the transfer of apoC-II to TGRL and thereby enhance their catabolism (79). We have additional evidence in the rat that apoA-IV gene expression is associated with hepatic TG-secretion. Increased hepatic TG-production as induced by a sucrose-rich diet is associated with nearly 2-fold increases in the abundance of apoA-IV mRNA, which is primarily due to enhanced transcription from the apoA-IV gene. Conversely, reduced hepatic TG-production resulting from diets rich in menhaden oil is associated with a 80-90% decrease in the transcription from the apoA-IV gene (80). An association between TG-transport and apoA-IV has been suggested in man because of a positive correlation of apoA-IV plasma levels and plasma triglycerides (81). In contrast, apoC-III may reduce the catabolic rate of TGRL since it inhibits the activity of lipoprotein lipase (82,83) and apoE-mediated remnant removal (84,85). Hence, the relationship of apoA-IV and apoC-III gene expression may be critical for the catabolism of TGRL. In fact, overexpression of apoC- III in transgenic mice is associated with hypertriglyceridemia (86), and a defect in or close to the AI-CIII-AIV locus may be present in as many as 50% of patients with FCH (87). We are in the process of completing a study on the XmnI restriction fragment polymorphism in the AI-CIII-AIV gene cluster in ARIC study participants with phenotypes of combined hyperlipemia, isolated hypertriglyceridemia, and isolated hypercholesterolemia. Our preliminary data fully support the results of Scott's group showing an increased frequency of the allele in subjects with elevations of TG and cholesterol.

Since the lod-score in the initial studies was 6.86 and since this RFLP is probably associated with FCH in the ARIC population, it is highly likely that a mutation in the AI-CIII-AIV gene cluster is present in patients with FCH. Such mutations may be located in the structural portion of the gene resulting in functionally-defective gene products or in regulatory regions affecting either basal transcription or

transcriptional adaptation to metabolic stimuli. To approach this problem, we propose to measure both apoA-IV and apoC-III levels in patients with FCH with or without the respective polymorphism. In addition, apoA-IV and apoC-III levels will also be measured in subjects with isolated hypertriglyceridemia, isolated hypercholesterolemia and normal lipid values for control purposes.

II.C.1.2.(ii). Measurement of ApoA-IV Gene Sequence Polymorphism

The gene for human apoA-IV has been cloned and is well characterized (88). Considerable polymorphism exists as a result of allelic differences in the apoA-IV gene (89,90). The well known apoA-IV polymorphism (AIV-1 and AIV-2) is due to Gln to His substitution at codon 360. The frequency of the Gln allele is 0.91, and the frequency of the His allele is 0.09. Average plasma triglyceride levels have been reported to be lower in His-containing heterozygotes than in the common homozygote. In contrast, HDL-chol showed the opposite pattern. Codon 347 of apoA-IV is also polymorphic; the frequency of the Thr allele is 0.78, and the frequency of the Ser allele is 0.22. In this study, we will type both of these relatively common apoA-IV amino acid polymorphisms.

For each of the proposed studies relating gene variation in apoA-IV to lipid, lipoprotein and apolipoprotein levels, or directly to CHD, plasma apoA-IV levels will be measured by immunoassay. The close biological link between plasma apoA-IV levels and the apoA-IV gene indicate that small effects of the gene will be more readily detectable as differences in apoA-IV levels. Indeed, an influence of the apoA-IV polymorphism on plasma apoA-IV levels and metabolism is known. In addition, information on the effects, or lack thereof, of the apoA-IV gene on plasma apoA-IV levels may lend information concerning the mechanism by which this gene influences plasma triglycerides and HDL-chol levels.

II.C.1.2.(iii). Determination of Sequence Variation in the ApoA-I Promoter

Even though there is overwhelming evidence for an inverse relationship between HDL-chol and elevated risk of atherosclerosis, there is a paucity of data concerning the mechanism(s) of reduced plasma HDL-C concentrations. Several studies have shown that the major determinant of reduced HDL-chol is an elevated fractional catabolic rate of the HDL-apolipoproteins (91), which may, at least in part, be due to enhanced transfer of TG to HDL (92). In addition, the rate of apoA-I synthesis can influence plasma apoA-I and HDL-chol levels (93), and overexpression of the apoA-I gene can lead to elevated plasma HDL- chol concentrations (94). In the 5' regulatory region of the apoA-I gene there is a common G to A substitution disrupting the normal apoA-I promoter (95). We propose to type this polymorphism in a sample of ca 100 individuals with elevated apoA-I and HDL-chol and a sample of ca 100 subjects with low apoA-I and HDL-chol to test if the above G to A promoter polymorphism is associated with altered apoA-I and HDL-chol levels. Equal numbers of both genders will be tested to determine an interaction of gender- or hormone-specific effects with the altered promoter sequence. Because of the possible interaction with plasma triglycerides, this study will be repeated in hyper- and normo-triglyceridemic groups. The -76 G to A substitution will be typed by routine methods of PCR followed by SSCP (96).

II.C.1.3. Cholesteryl Ester Transfer Studies

II.C.1.3.(i). Cholesteryl Ester Transfer Protein (CETP, Lipid Transfer Protein I, LTP-I)

CETP circulates in the blood in association with HDL_3 and very high density lipoproteins. It has a molecular weight of 74 KD (97). The primary structure of CETP has been deduced from the cDNA sequence (98) and the organization of the CETP gene has been elucidated (99). The function of CETP is to transport neutral lipids among the cores of plasma lipoproteins (100). Since CETP has the ability to transport both cholesteryl ester and TG, the cores of lipoproteins approach equilibrium as a result of CETP action. CETP participates in reverse cholesterol transport since it transfers cholesteryl ester generated by the LCAT reaction from HDL to apoB-containing lipoproteins in exchange for nearly equimolar amounts of TG. Thus, CETP provides a mechanism for returning cholesteryl ester formed in HDL to the liver using the LDL receptor pathway or the chylomicron remnant pathway. The latter pathway can function independently of LDL-receptor mediated clearance, is not saturable (101), and may become of major quantitative importance when the LDL receptor pathway is either defective or saturated.

Current concepts suggest that the CETP-mediated transfer of cholesteryl ester from HDL to apoB-containing lipoproteins may be atherogenic. Animal species such as the pig, rat, and mouse, which are resistant to atherosclerosis, have little, if any CETP activity. Conversely, animal species such as monkeys and rabbits, susceptible to dietary induced atherosclerosis, have CETP activity in their plasma (102). In the rabbit, such diets lead to increased abundance of hepatic CETP mRNA (103). Furthermore, a splicing defect in the CETP gene that results in complete absence of CETP appears to be a frequent cause of hyperalphalipoproteinemia in the Japanese population (104,105). In this inborn error of metabolism, HDL_2 do not acquire TG from TGRL in exchange for cholesteryl ester. Indeed, the CETP-deficient patients exhibit high HDL levels with a preponderance of HDL_2 and even larger HDL particles. Atherosclerotic manifestations are conspicuously absent in these patients supporting the hypothesis that transfer of cholesteryl ester to the atherogenic apoB-containing TGRL is a fundamental event in atherogenesis. This concept is also consistent with the pathogenic mechanisms that have been proposed by us to explain the relationship of increased postalimentary lipemia with CHD. When clearance of TGRL in the postprandial state is delayed, transfer of cholesteryl ester from HDL to TGRL is extensive. Hence, cholesteryl ester-rich remnants which remain in the circulation for prolonged time are formed and may lead to deposition of cholesterol in the vessel wall. Thus, defective triglyceride metabolism may determine the partitioning of cholesteryl ester between anti-atherogenic HDL and the potentially atherogenic apoB-containing lipoproteins (39).

The rate of neutral lipid transfer depends on the enzyme concentration of substrate and acceptor lipoproteins and enzyme mass. Enzyme mass as determined by radioimmunoassay varied nearly 4-fold in 50 normolipemic subjects (106). With functional assays of CETP activity, which showed a good correlation with enzyme mass (107) and are independent of endogenous lipoproteins present (108), decreased cholesteryl ester transfer was found in chronic alcoholics, providing an explanation for elevated HDL in these patients (109). In addition, cholesteryl ester transfer appears to be enhanced in diabetic patients (110), and we have data showing increased enzyme activity in 12 patients upon eating a cholesterol-rich diet. Thus, measurement of CETP activity and/or mass may allow us to estimate the magnitude of cholesteryl ester transfer to the potentially atherogenic lipoproteins and may be complementary to the analyses performed in the postprandial state in a subset of ARIC participants. We propose to measure CETP activity in cases and controls of the cohort, in particular in those subjects

who had postprandial studies. We would also suggest studies in participants with combined hyperlipemia, isolated hypertriglyceridemia, isolated hypercholesterolemia and diabetic patients. For measurement of CETP activity, a functional assay is already established. A specific radioimmunoassay will be developed, if this is deemed necessary. Antibodies for such an assay are already available.

II.C.1.4. ApoB Signal Length Polymorphism

Boerwinkle et al have described polymorphic length variation in the signal peptide of the human apoB gene (111,112). Eukaryotic signal peptides are required for the posttranslational processing of most secretory proteins, and information specifying cellular or subcellular localization may also reside in the signal peptide sequence. The signal peptide alleles consist of the following: the longest allele (designated 5' SP-29) encodes 29 amino acids in the signal peptide and contains two copies of the sequence (CTG GCG CTG) encoding Leu-Ala-Leu, and a consecutive run of 9 (CTG) codons encoding 8 leucine residues; the medium sized allele (5' SP-27) encodes 27 amino acids and contains 2 copies of (CTG GCG CTG) but a run of only 6 (CTG) codons; the shortest allele (5' SP-24) encodes 24 amino acids and contains a single copy of (CTG GCG CTG) and a run of 6 (CTG) codons. To our knowledge this polymorphism is the only naturally occurring signal peptide variation in humans. In three separate studies, the apoB signal peptide has been associated with altered levels of plasma glucose, TG and HDL- chol (112-114). We propose to ascertain the frequency of respective alleles in study participants who underwent postprandial lipid testing, since such an analysis would further define potential determinants of TGRL metabolism.

II.C.2. Studies on Lp(a)

II.C.2.1. Lp(a) Levels and Phenotypes in Hypercholesterolemic Subjects

Subjects with etiologically defined elevations of LDL-chol provide a unique model to analyze the relationship of Lp(a) plasma levels with apoB metabolism. Utermann and colleagues found 2-to 4-fold higher levels of plasma Lp(a) in patients heterozygous for a LDL receptor mutation. These investigators considered effects of apo(a) size on plasma levels of Lp(a) in their analyses and concluded that there exists a multiplicative interaction between the LDL receptor locus and plasma levels of Lp(a) (115). The results of this study are somewhat puzzling for a number of reasons. First, in a rhesus monkey model no such association was found (116). Second, drugs which enhance the expression of the LDL receptor and lead to an increased clearance of LDL do not lower plasma levels of Lp(a) (117,118). By contrast, some antihyperlipemic drugs which lower plasma levels of VLDL reduce the plasma concentration of Lp(a) (119). Third, diets that increase or decrease LDL-chol have only minimal effects on plasma levels of Lp(a) (120). Fourth, turnover studies suggest that plasma levels of Lp(a) are primarily determined by its synthetic rate (121). Patients with the heterozygous form of FH also accumulate IDL in their plasma. This is in contrast to subjects with familial defective apoB-100, who can clear IDL from the circulation due to the interaction of apoE with a normal LDL receptor. It would thus be interesting to compare plasma levels of Lp(a) in subjects with FH, polygenic hypercholesterolemia and defective apoB-100. Since differences in apo(a) isoform size account for 40% of Lp(a) plasma level variability(122), apo(a) phenotypes must be determined in these subjects to correct for influences of apo(a) size.

There are several well-known syndromes including familial abetalipoproteinemia and familial hypobetalipoproteinemia, which are characterized by low or absent plasma apoB levels. Recent studies showed that some forms of familial hypobetalipoproteinemia are caused by mutations in the apoB gene

that result in truncations of the apoB molecule. In the homozygous form characterized by 2 defective apoB alleles, LDL-chol and apoB are undetectable. In heterozygous subjects possessing 1 defective apoB allele, plasma LDL-chol and apoB levels are greatly reduced. These persons, because of their low LDL-chol, may be protected from CAD and may have increased longevity. The prevalence of the heterozygous form has been estimated to be between 0.1 and 0.8% in the general population (123,124). Besides familial hypobetalipoproteinemia, other genes may lower plasma LDL-chol. In a family with FH, Hobbs et al recently showed a trait that lowers LDL- and plasma cholesterol. This trait did not co-segregate with the apoB or the LDL receptor gene locus (125).

Patients with familial hypobetalipoproteinemia represent another model to study Lp(a) metabolism as the secretion of Lp(a) may depend on the intracellular formation of a disulfide bond with apoB. Since the apoB species that result from mutant alleles are often truncated, the disulfide bond with apoB thought to be in the carboxy-terminal region of apoB can not be formed (126,127). If this hypothesis is correct, plasma levels of Lp(a) should be reduced in these subjects, commensurate to the reduction of apoB levels. In our opinion, there is no other population as well characterized and of the size required to address this question. In year 1, approximately 15 subjects with LDL-chol levels of less than 35 mg/dl were identified. Assuming a similar prevalence during years 02 and 03 and increasing the cutoff to 45 mg/dl, we would estimate that 100 study participants could be included in this study. This study would require Lp(a) phenotyping to correct for phenotype- specific effects on Lp(a) plasma concentrations and analyses of apoB species by immunoblotting subsequent SDS gel electrophoresis to ascertain apoB truncations.

II.C.2.2. Lp(a) Levels and Phenotypes in Blacks and Whites

Plasma levels of Lp(a) are associated with risk of CHD (128-133). While the risk of CHD conferred by Lp(a) appears to be independent of other conventional risk factors, the atherogenicity of high plasma Lp(a) levels may be potentiated by high plasma LDL-chol levels (128). Apo(a), the unique protein component of Lp(a), exists in several isoforms distinguished by their molecular weight (133,134). The molecular basis for the variation in apo(a) size lies primarily in multiple apo(a) alleles which code for 9 to 35 repeats of kringle 4 (135,127). In addition, minor variability in size might be due to differences in the glycosylation of apo(a). The molecular weight of apo(a) shows an inverse association with plasma concentrations of Lp(a). However, only 40% of the variability of Lp(a) plasma levels can be explained by effects of apo(a) size in populations. Environmental factors seem to play only a minimal role in Lp(a) plasma concentrations. In patients, who underwent heart transplantation, Lp(a) levels fell, perhaps as a result of cyclosporin treatment while all other plasma lipid parameters rose (136).

There seems to be a consensus that plasma levels of Lp(a) are primarily inherited. The quantitative trait for Lp(a) plasma concentrations may be determined by a locus distinct from the apo(a) locus or by the apo(a) locus itself. A logical mechanism for the latter possibility would be variability in promoter strength or differences in enhancer or silencer elements determining the transcription rate of apo(a), while differences in trans-acting factors could be involved to explain the former possibility. Additional mechanisms controlling posttranslational processing of apo(a) could be involved as well. All these scenarios would be consistent with turnover data pointing towards synthesis as the primary determinant of Lp(a) plasma levels.

We have recently shown that plasma levels of Lp(a) are higher in blacks than in whites. This has been confirmed in the ARIC population as well as in other studies. This race-specific difference could be due

to a prevalence of smaller apo(a) isoforms in blacks, should the inverse association between apo(a) size and Lp(a) concentrations also exist in blacks. Hence, we propose Lp(a) phenotyping in representative samples of blacks and whites. A large number of white subjects of ARIC have been phenotyped previously. One could thus enrich this sample with a representative sample of black study participants to study this problem.

Should apo(a) size not differ between blacks and whites, the above suggested mechanisms, i.e., race-specific effects in promoter-strength or trans-factors would become the primary hypothesis. There is now active work going on in several laboratories to characterize the positive and negative regulatory elements controlling the expression of the apo(a) gene. Our laboratory is actively engaged in research on apolipoprotein gene expression, and techniques have been developed to address specific problems, especially when the regulatory elements in apo(a) gene expression have been clarified.

II.C.2.3. Age-dependent Increases of Lp(a) in Women and Their Relationship to Phenotype

Data from the Procam-Study showed that Lp(a) concentrations in women are influenced by age, while no such age-dependence was observed in males. Furthermore, postmenopausal increases were 3-fold higher when compared to premenopausal increases (139). This finding is of particular interest as the risk of CHD increases in postmenopausal women. Elevated levels of Lp(a) compounded by postmenopausal increases in LDL-chol may contribute to the increased risk of CHD in these subjects. ARIC has visit 1 data of Lp(a) levels in all female study participants. Furthermore, in female cases and controls of the cohort, Lp(a) was measured again and the Lp(a) phenotype was determined. We therefore propose in a subset of female participants to measure Lp(a) blood concentrations during visit 04, which would provide a 9 year difference to ascertain age-dependent changes. Furthermore, we propose to determine Lp(a) phenotypes in these women, to determine whether Lp(a) related changes depend on the phenotype and are related to the incidence or progression of disease. The design of this study will require careful consideration in the selection of study participants, i.e., women with high, medium and low Lp(a) levels, use of hormones, incidence of new disease, and optimal use of measurements already available to keep the cost/benefit ratio low.

II.C.2.4. Lp(a) Genotype

Recent studies by Lackner et al. have identified the molecular basis of apo(a) size polymorphism. These researchers identified a highly polymorphic apo(a) gene fragment using restriction with Kpn I, pulsed-field gel electrophoresis, and genomic blotting. This segment varied in length from 48 to 190 kb and was suggested to contain 9 to 35 kringle 4-encoding repeats. In 102 unrelated Caucasian Americans, 94% had 2 different alleles as defined by the length of kringle-encoding DNA (140). These results contrast with expression data, i.e., phenotyping, showing that 60% of subjects studied expressed only one apo(a) polymorph. A small part of this discrepancy may be due to the fact that some isoforms may not be resolved in gels because of their similarities in molecular weight. A much more likely explanation is however, that some of the apo(a) alleles are not expressed in detectable quantities. Our studies in blacks and whites should partially address this question.

The second important finding of the studies of Lackner et al. was that the relationship of apo(a) genotype with Lp(a) plasma concentration was similar to that in unrelated subjects. For example, genotypes differing in the number of kringle 4-coding sequences explained part of the variability of Lp(a) plasma levels. Importantly, a much higher association of apo(a) genotype with Lp(a) plasma

levels was found within families. Hence, the quantitative trait of Lp(a) plasma concentrations was largely determined by the apo(a) locus in these Caucasian families. This then implies that those regions of the apo(a) gene distinct from the regions encoding the number of kringle 4-units, play a major additional role. Thus, the Lp(a) locus contains not only the 19 or more alleles identified on the basis of kringle 4-encoding sequences, but additional sequence heterogeneity, perhaps in the promoter region, would occur on top of each of the other 19 or more alleles. Given this complexity, an extremely large sample number would be required to study the precise role of the apo(a) genotype in atherogenesis. As already mentioned, knowledge about the apo(a) gene is rapidly emerging and we would expect that information critical for population studies, i.e. promoter heterogeneity, will be available within the next 2-3 years. We than could genotype a sample of the ARIC population, i.e., blacks and whites, subjects with low or high Lp(a) plasma levels, with respect to sequences encoding kringle numbers and regulatory sequences controlling the expression of the apo(a) gene product. Developmental work is ongoing in our laboratories to measure kringle 4-encoding sequences.

II.C.3. Plasma Endothelin Levels

Endothelial cells release substances that affect vascular tone and platelet function (141). Endothelin induces long-lasting potent vasoconstrictions in systemic, renal and coronary arteries (142). A recent study described that circulating endothelin concentrations were higher in patients with symptomatic atherosclerosis than in control subjects, and there was a highly significant correlation between plasma endothelin levels and the number of sites of disease involvement (143). While this study clearly showed that endothelin is associated with disease, the design of the study did not allow conclusions as to whether endothelin was solely a marker for atherosclerotic disease or whether it participates in the atherogenic process. Endothelin may be released by damaged endothelial cells thus signaling the extent of the disease process. However, endothelin can also be released from the intimal layer of intact blood vessels (144). Evidence suggests that endothelin may function as a mitogen (145) that could promote proliferation of vascular smooth muscle cells. We believe that ARIC is in the unique position to i) verify the finding that endothelin is a marker of atherosclerosis and ii) determine whether endothelin is involved in atherogenesis. Thus, endothelin levels could be measured in stored aliquots and specimens collected during visits 3 and 4 to determine whether elevated plasma concentrations precede disease, or whether increased levels signal a more rapid progression of disease. Because of carefully performed characterization of the lipid transport system and the coagulation system, interactions of endothelin plasma levels with other risk factors of CHD can be ascertained as well.

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III. FEASIBILITY, ACCURACY AND PRECISION OF MEASUREMENTS

III.A. Feasibility in Frozen Plasma Specimens

The validity of cholesterol and triglyceride determinations in frozen plasma is generally accepted (1). In our pilot study, we showed only minor effects of freezing on HDL-chol measurements (-5%) (2). Glucose determinations have been performed in frozen plasmas during visits 1 and 2. The measurement of glucose proposed in our laboratory for visits 3 and 4 employs similar methodology used at the Chemistry Central Laboratory. This procedure requires only 2-4 µl of plasma, thus not depleting the plasma aliquots for other test measurements.

The validity of measuring apolipoproteins A-I (3) and B (4) levels by radioimmunoassay and Lp(a) concentrations by enzyme-linked immunoabsorbant assay in frozen plasma has been established in visits 1 and 2 (5). There is no effect of freezing on the quantitation of apoA-I (3) and Lp(a), while freezing and thawing decreases apoB levels by 6.8% compared to apoB levels in fresh samples (4). We have shown through quality control pools that no further decrease in apoB levels occurrs upon prolonged storage time.

In case - control studies of the cohort, the determination of Lp(a) phenotypes has been established and is currently performed in frozen plasma samples by Dr. Morrisett (5). Analysis of LDL subclass patterns by nondenaturing gradient gel electrophoresis is performed on 40 ul of frozen plasma specimens. Our pilot studies established that within analytical precision, LDL subclass patterns observed in fresh plasma samples are not altered by freezing and thawing.

The isolation of genomic DNA from frozen buffy coats is well established. Examination of the restriction length fragment polymorphism at the AI-CIII-AIV gene locus in selected cases and controls is currently being performed. With the introduction of the polymerase chain reaction technology, sufficient amounts of specificifed DNA sequences are generated which allows for examination of a number of gene loci responsible for the expression of various proteins regulating lipid metabolism. Proposed studies include apoE genotyping which is currently ongoing, apoA-IV polymorphism, apoA-I promotor variability, apo(a) genotyping and apoB signal length peptide polymorphism. Some of these tests are already in use in our laboratory or Dr. Boerwinkle's laboratory.

Measuring abundance levels of mRNA of the LDL receptor and HMG CoA- reductase from frozen buffy coats will require preliminary testing to establish accurate quantification. The long term storage conditions of the buffy coats are probably not optimal, but perhaps acceptable, for the preservation of mononuclear mRNA. Pilot tests will ascertain the degree, if any, of mRNA degradation under these conditions.

The validity of measuring concentrations of HDL particles containing only apoA-I in fresh or frozen plasma samples is established (6). The principle of quantifying particles containing only apoA-I using electroimmunodiffusion relies on that apolipoprotein epitope expression is not altered by freezing and thawing. We showed no effect of freezing and thawing on the total

immunoreactivity of apoA-I in plasma samples using RIA (4).

Cholesteryl ester transfer protein (CETP) activity is currently being measured in frozen plasma samples in this laboratory. Using published procedures, the purification of CETP and the development of an immunoassay for CETP measurement appears feasible because of our track record in protein purification and development of immunoassays (3,4,7-9). The applicability of frozen samples for CETP quantification by an immunoassay has been reported (10). Other biologic tests to monitor cholesteryl ester transfer, include the quantification of plasma phosphatidylcholine and free cholesterol. No change in concentration is expected, provided that the sample processing is followed as lecithin cholesterol acyltransferase (LCAT) activity is not active under these conditions. The concentrations of these analytes will be determined by commercially-prepared enzymatic kits. Small amounts of frozen plasma will be required for each of these determinations.

The direct measurement of LDL-cholesterol requires refrigerated plasma as used currently. Use of frozen plasma would require a pilot test. The conventional ultracentrifuge technique is used for isolating VLDL-free plasma (11) followed by determinations of HDL-chol in the infranate. LDL-apoB concentration is determined by RIA in the VLDL- free plasma which is assayed at the same dilution as whole plasma samples.

Concentrations of plasma endothelin-1 levels will be determined by a commercially available radioimmunoassay kit. The validity of the RIA for frozen plasma specimens has been established (12). This will be confirmed in a pilot study. It is reasonable to assume that freezing and thawing should not disrupt the relevant epitope expression because the endothelin structure which is relatively short sequence that is stabilized by two disulfide bonds.

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III.B. Accuracy and Precision of Measurements

Accuracy of cholesterol is traceable to reference values established by the CDC. Unfortunately, the CDC no longer provides control materials to monitor laboratory performance internally. However, the external surveillance program is maintained by CDC, and our laboratory will continue to participate in this program that certifies measurements of cholesterol, triglycerides and HDL-cholesterol. Furthermore, our laboratory is one of the network laboratories which serve as reference laboratories for these analytes. For our clinical determinations, we use pooled human serum from Pacific Biometrics as a standard approved by the CDC. Through our quality control program, internal limits for accuracy and precision of measurements in these pools have been established by overlapping analysis with CDC quality control materials that were available. Thus, all measurements performed in visits 3 and 4 will be directly comparable to measurements in visits 1 and 2.

Accuracy of apoA-I measurements is traceable to a well characterized apoA-I standard and apoA-I determinations in CDC pools for which consensus values have been developed. Locally prepared pools of plasma containing low, intermediate, and high analyte concentrations serve to maintain precision and to support accuracy. Accuracy of apoB measurements is traceable to zonally isolated LDL, which contains apoB as the sole apoprotein (1) and whose protein content is quantified by the Lowry procedure (2). In addition, analyses of apoB plasma pools produced by our laboratory will be used as in visits 1 and 2. Accuracy of Lp(a) determinations are determined by direct measurement of protein content in the standard. Plasma obtained from three individuals with low, intermediate, and high Lp(a) levels serve as control material. Accuracy of plasma glucose determinations is monitored using commercially available control materials from Sigma, and the quality control principles stated above are followed. Accuracy of measurements utilizing commercial kits (LpA-I, ApoC-III, Free Cholesterol and Endothelin) will be established through control materials provided by the manufacturer. Accuracy of apoA-IV and CETP measurements will be traceable to immunoreactivities of a standard with known mass as determined by amino acid analysis and/or protein measurements. Accuracy of phosphatidylcholine will be established by using standards of known phosphatidylcholine concentration. Comparability of mRNA measurements in unknowns will be assured by using a control RNA pool. In this pool, we may be able to determine the abundance of the respective mRNA by solution hybridization. The concept of accuracy is less important in those tests of genetic analysis which provide yes or no answers as long the assays are working and error-rate due to non-analytical factors such as transcription can be estimated. The functioning of assays will be demonstrated in control samples with known outcome and error-rates will be estimated by blinded duplicates. Precision of test measurements is monitored internally and externally through analysis of blinded duplicate samples. Table 1 shows the coefficient of variations (CV) determined through internal and external quality control components.

Table 1. Quality Control Performance for Tests in Cohort for Visits 1 and 2

INTERNAL CV			EXTERNAL CV[1]
TEST	ACTUAL	PREDICTED	PRECISION
Cholesterol	2.5	2-3	<3
Triglyceride	2.7	2-3	<3
HDL-Chol	3.7	<6	<4

[1] From QC of 1986 - 1989.

Hence, the laboratory stayed within performance limits predicted for these tests. The precision performance was decreased for a two month period in 1990 due to defective sample cups. We anticipate our laboratory CV of glucose determinations to be 3-4%.

We had anticipated an overall assay variability of less than 10% for apoA-I and apoB measurements. In quality control data from 1986 to mid 1990, the overall laboratory CV as determined by internal pools was less than 8% for apoA-I and less than 8% for apoB, respectively. Overall CD's based upon external quality control by blinded duplicates between 1987 and 1989 were 10-11% and 10-12% for apoA-I and apoB, respectively. In the Intraindividual Variability Study, an analytical CV of 11% was reported (3), while apoB analysis was not performed. CV's based upon external blinded duplicates may include errors that could have occurred prior to arriving to the laboratory. Previous experience on cholesterol measurements in other studies indicate that about 2% points of the analytical CV may be attributed to preanalytical and transcriptional errors. ApoA-I and apoB measurements will be performed only in case-control studies or selected populations during Visits 3 and 4. We anticipate improved precision because of the smaller sample size in the case-control studies. The CV for Lp(a) measurements was less than 15% which can be easily tolerated because of the wide range of Lp(a) plasma levels found in individuals.

Assessment of precision of tests employing kits or immunoassays to be developed will be conducted using the principles stated above. Table 2 shows performance of proposed tests as reported by manufacturers or research laboratories.

Table 2. Precision Performance of Proposed Tests

TEST	REPORTED CV	REFERENCE
LpA-I	<10% (inter-assay)	4
ApoC-III	<10% (intra-assay)	5
Endothelin	<10% (inter-assay)	called
CETP (RIA)	< 7% (inter-assay)	6
ApoA-IV	< 8% (inter-assay)	7

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IV. METHODS

IV.A. TEST MEASUREMENTS IN THE ENTIRE COHORT

The standard procedures for cholesterol and triglyceride measurements are the Monotest Cholesterol procedure (1) and the GPO Triglyceride procedure (2) of Boehringer Mannheim. Both of these tests are adapted to automatic analysis using a Roche Cobas-Fara II Analyzer By using these reagents and instrumentation we have demonstrated that measurements meet the criteria of precision and accuracy set forth by CDC. A number of factors are involved in the interference in the enzymatic determination of plasma lipids (3,4). Among common drugs, only ascorbic acid and `L-methyldopa produced borderline negative interference at therapeutic plasma concentrations. In Trinder systems (5), bilirubin has been reported to interact in the peroxidase-catalyzed reaction by competing for hydrogen peroxide, which results in lower lipid levels (6). The inclusion of hexacyanoferrate (II) into the reagent mixture minimizes this

chemical interference, and bilirubin concentrations of up to 15 mg/dl did not produce significant changes in results. Positive interference may be caused by hemoglobin, which acts as a pseudoperoxidase. However, no distortion of results was observed by concentrations of up to 2 g/dl.

Since the proper functioning of the Cobas-Fara II Analyzer is essential for the timely analysis of samples, a detailed maintenance program is in operation, which includes a service contract with 24 hour response time of a field engineer. Should the machine be out of operation, a back-up procedure will be implemented. In this procedure, the same reagents are used, automated pipettes are used for reagent and specimen delivery, and absorbance readings are done manually on a calibrated spectrophotometer. Results obtained are identical to the automated method.

In the unlikely event that the commercial reagent kits would deteriorate or would become unavailable, back-up procedures (7,8) will be set up which are, in essence, identical to the primary method, but differ in the source of reagents. The equivalence of a CDC-method to measure cholesterol (7), to the Monotest cholesterol method of Boehringer Mannheim (1), has been established (9).

IV.A.1. Automated Determination of Cholesterol

Cholesteryl esters are hydrolyzed to free cholesterol and fatty acids by cholesteryl esterase. The free cholesterol produced and that already present in the sample is oxidized by cholesterol oxidase to form cholest-4-en-3-one and hydrogen peroxide. Peroxidase catalyzes the reaction of hydrogen peroxide, 4-aminoantipyrine, and phenol (or a substituted phenol) to produce a quinoneimine dye, with maximum absorbance at 500 nm. The absorbance readings at this wavelength are proportional to the concentration of total cholesterol in the samples (1).

1. MATERIALS

1.1. Equipment and Supplies

- Cobas-Fara II Analyzer(Roche)
- Gilson pipetman
- Sample cups
- Disposable rotors
- Reagent boats
- Reagent-tips
- Sample needles

The Cobas-Fara II is a compact self-contained centrifugal analyzer which utilizes the horizontal light path principle. It is equipped with the following modules:

a. An automatic pipetting station. This consists of a robotic sample arm, a sample needle, sample tubing, wash-tower, reagent arm, reagent head, reagent tips, a reagent drip cup, and individual reagent racks, sample racks and calibration racks. An optical rack reader identifies racks for specimen identification. The Fara is equipped with optical sample selection and is capable of loading one or two reagents with sample and diluent into a

twenty-five-place disposable plastic cuvette-rotor. Specimens are withdrawn from thirty-place specimen trays. Each cuvette in the rotor has two compartments, one for reagent and one for sample, and a second reagent if desired. Reagents and samples are thus kept separate until the rotor accelerates and mixes by means of vibration and breaking.

b. Optical system. The Fara employs a high intensity xenon flashlight source in combination with a holographically inscribed grating monochromator to give linear measurements of absorbance up to 3.0 at 340 nm. The monochromator is capable of selecting any wavelength from 285 nm to 750 nm with a half bend pass of 5.5 nm at 500 nm. Absorbance measurements are made using a horizontal placement of the cuvettes relative to the light path.

c. Rotor including photometer arm, photometer lenses, and cuvette rotors.

d. Microprocessor. The Cobas-Fara II is equipped with a 64 K 8080A microprocessor into which the user may program 40 parameters for each of 130 tests via a keyboard and a display panel on the system. The programmed tests are retained in memory until changed by the operator. In the event of power failure, programs are protected by a battery backup within the system for a period of two weeks. Hard copy print-outs are made by an alphanumeric printer. Each printout is identified with test name, number, abbreviated parameter list, and both disk and cup number (10).

1.2. Reagents

Two reagents are used: deionized water and a standard reagent. Tap water is deionized by a Barnstead apparatus. The water is pre-purified by passing it through an organic removal cartridge, a submicron filter assembly, and a reverse osmosis membrane. This pre-purified water is then passed through four research grade ion exchange cartridges and a 0.2 micron filter cartridge. Conductivity is then measured by an on-line ohmmeter and is consistently 17 megaohms or greater.

1.3. Standard

Standard cholesterol reagent is purchased from Boehringer (Cat No. 236691). The shelf life of this product is 2-3 years. Prepare the reagent by transferring the premeasured powder from the plastic shipping bottle to a 500 ml volumetric flask using a plastic funnel. Add approximately 400 ml of deionized water and dissolve the powder using a magnetic stir bar. Remove the stir bar after 10 min. and add water up to the 500 ml mark. Transfer the final solution, which is light pink in color, to a glass bottle and store at 4°C. The reagent is stable for up to four weeks when stored at this temperature.

The following backup procedure (7) is used if the sale of the commercially available reagent is stopped or if the quality of the reagent deteriorates:

a. For PIPES buffer, 50 mmol/L, pH 6.9: Add 8.84 g PIPES to 450 ml deionized water in

a glass beaker. Stir for approximately 10 min. until the solution clears. Equilibrate the solution to 37°C, and adjust, if necessary, to pH 6.9. Transfer the solution to a 500 ml volumetric flask and after cooling to ambient temperature, adjust volume to 500 ml with deionized water.

b. For stock phenol reagent: Prepare from phenol crystals stored in a desiccator at room temperature. Carefully but quickly, weigh 1.43 g of phenol crystals into a 150 ml beaker, add 50 ml PIPES buffer and swirl to dissolve; transfer solution to a 250 ml volumetric flask, rinse beaker into flask several times with PIPES buffer, and adjust volume to 250 ml with buffer. This reagent may be stored for up to one month at 4°C in a tightly-closed glass container.

c. For stock mixed reagent: Transfer to a 250 ml volumetric flask: i) 0.101 g 4-amino-antipyrine, ii) 0.65 g sodium cholate, and iii) 3.73 g KCL. Add approximately 200 ml PIPES buffer to the flask and swirl to dissolve; add 0.50 ml Triton X-100; adjust volume to 250 ml with buffer to avoid bubbles, and mix. This reagent is stable for at least one month at 4°C.

d. For working reagent: Mix 50 ml of the stock mixed reagent and 50 ml of the stock phenol reagent. Add 25U of cholesterol oxidase, 25U of cholesterol esterase, and 1250U of peroxidase (based on the respective specific enzyme activities) either from solutions of the concentrated enzymes or by weighing the dry enzyme preparations.

2. ANALYTICAL PROCEDURE

2.1. Cobas-Fara II

After performing the daily maintenance on the Cobas-Fara II analyzer, transfer 25 ml of the standard cholesterol reagent into a 35 ml disposable container with a snap closure lid to prevent incorrect positioning in the rack. Pipette 1.0 ml of the Cholesterol standard, Level I, Level II and Level III (Pacific Biometrics) into sample cups and place them in the black metal calibration rack. Pipette 1.0 ml of each plasma specimen into a specimen cup (without top) and then fill each sample tray with thirty plasma specimens. Each sample rack has thirty unique sample positions which are not duplicated with any other sample rack; i.e. rack A has specimen number 1- 30, while sample rack C contains specimen numbers 61-90. Following the loading of the specimens, the number of specimens and quality control standards are programmed into the cholesterol program. Sample volume, diluent volume, and reagent volumes are 4, 10, and 330 fL, respectively. Incubation temperature is 37°C, and running time is 6 min. Cholesterol content of samples is calculated by the microprocessor of the instrument using the Autoblank Endpoint Mode.
A factor is derived by:

$$F = \frac{C_{standard}}{(An - Ao)_{stand} - (An - Ao)_{blank}}$$

where An and Ao are absorbance readings at 0 and 6 min. at 500 nm. At the end of the run a data printout is generated which contains the factor derived, and results of unknowns expressed in the units of the standards. Absorbance readings may also be printed out when desired. The printout of the conversion factor provides a convenient check for the quality of the reagents. Identification numbers of samples are then transferred from the work sheet to the printout which is kept as a permanent record. The method is linear for up to 500 mg/dl. Measurements exceeding this value are flagged by the instrument. Analysis is repeated subsequent to diluting the plasma with an equal volume of saline.

2.2. Manual Back-up Procedures in Case of Instrument Failure

In case of machine problems exceeding two working days, the manual backup procedures will be in effect. These procedures are as follows: Add 20 µl of water (blank), standard, plasma, and control material to 10x75 mm glass tubes using an automatic dispenser. Add 2.0 ml of standard cholesterol reagent or backup reagent also using the automatic dispenser. Vortex tubes and incubate for 12 min. at room temperature. Read absorbance of blanks, standards, pools, and unknowns at 500 nm on a Gilford 250 spectrophotometer equipped with an automatic sampling device. Calculate cholesterol content of unknowns by the formula:

$$Conc_X = \frac{(Ax - Abl) \times Conc.st}{(Ast - Abl)}$$

where Ax, Abl, and Ast are the absorbances of the unknown, reagent blank, and standard, respectively.

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IV.A.2. Automated Determination of Plasma Triglycerides

Triglycerides are hydrolyzed to glycerol and fatty acids by lipase. The glycerol formed is then phosphorylated in the presence of ATP. Oxidation of the resulting glycerol-3-phosphate to dihydroxyacetone phosphate and hydrogen peroxide is catalyzed by glycerol phosphate oxidase. An intense red chromogen is produced by the peroxidase catalyzed coupling of 4-aminoantipyrine and sodium 2-hydroxy-3,5-dichlorobenzene-sulfonate with hydrogen peroxide. The absorbance readings at 510 nm are proportional to the concentration of total glycerol content (free and esterified) in the samples (1).

1. MATERIALS

1.1. Equipment and Supplies

- Gilson pipetman -Reagent tips
- Sample cups -Sample needles
- Disposable rotors -Reagent boats
- Cobas-Fara II analyzer.

1.2. Reagents

Deionized water and standard triglyceride reagent are used. Triglyceride reagent is purchased from Boehringer Mannheim Biochemicals-Indianapolis (Cat. No. GPO 701912). Prepare the reagent in the same plastic bottle in which the pre-measured solution is shipped. Using a pasteur pipette, transfer the solution to the vial containing a premeasured amount of reagent powder; dissolve powder to obtain a clear yellow solution. Transfer the clear yellow solution back to the plastic bottle and mix. Rinse the vial which contained the premeasured powder three times with the working solution for quantitative transfer. This reagent may then be stored in the original plastic bottle for up to two weeks at 4°C.

The following backup procedure is used if the sale of the commercially available reagent is stopped or if the quality of the reagent deteriorates:

a. Stock-reagent buffer: Add 6.055 g Tris-HCl to 900 ml distilled water in a 1000 ml beaker and stir to dissolve. Add 1.016 g magnesium chloride and 203 mg 4-aminoantipyrine and dissolve. Adjust pH to 7.6 with dilute hydrochloric acid and add 100 mg Triton X-100, rinse the weighing boat which contained Triton X-100 several times with buffer; stir for 10 min. and transfer to 1000 ml volumetric flask; adjust to volume with distilled water. This reagent is stable for at least 1 month at 4°C.

b. HBDS-reagent: Weigh out 2.4 g 2-hydroxy-3,5-dichlorobenzenesulfonate, transfer to a 50 ml glass beaker, add 100 mg Triton X-100, dissolve in 40 ml Tris-HCl buffer (pH 7.8, 0.05 M). Transfer to a 50 ml volumetric flask, make volume up to 50 ml with Tris-

HCl buffer. This reagent is stable for at least one month at 4°C.

c. Working reagent: Weigh out the following enzymes and place in an iced beaker containing 50 ml stock reagent buffer: 10KU lipase, 25U glycerokinase, 400U glycerol-3-phosphate: 0_2-oxidoreductase, 1KU peroxidase, 25.4 mg adenosine 5'-triphosphate. Mix well and quickly transfer to a 100 ml volumetric flask. Add 1.0 ml of HBDS reagent and make volume up to 100 ml with stock reagent buffer.

1.3. Standard Solutions

Glycerol (0.210 g) dissolved in one liter of 0.9% NaCl containing 0.5 g sodium azide is used as primary standard. Alternatively, glycerol standards are also available through commercial sources such as Boehringer Mannheim (Precimat Glycerol, Cat. No. 166 588, Boehringer Mannheim, Indianapolis, Indiana).

2. ANALYTICAL PROCEDURE

2.1. Cobas-Fara II

After performing the daily maintenance on the Cobas-Fara II analyzer, transfer 25 ml of the standard triglyceride reagent into a 35 ml disposable container with a snap closure lid to prevent incorrect positioning in the rack (2). Pipette 1.0 ml of the Triglyceride glycerol standard and Level 2 and Level 3 (Solomon Park) into sample cups and place in the black metal calibration rack. Pipette 1.0 ml of each plasma specimen in specimen cup (without top) and then fill each sample tray with thirty plasma specimens. Each sample rack has thirty unique sample positions which are not duplicated with any other sample rack; i.e. rack A has specimen number 1-30, while sample rack C contains specimen numbers 61-90. Following the loading of the specimens, the number of specimens and quality control standards are programmed into the triglyceride program. Sample, diluent, and reagent volumes are 5, 40, and 350µl. Incubation temperature is 30°C and running time is 5 min. Data are calculated by the Autoblank Endpoint Method as described under section A.2.2.1. At the end of the run, transfer sample identification numbers to the printout which is kept as a permanent record. The measurements are linear up to 800 mg/dl. Measurements exceeding this limit are repeated after dilution of samples with an equal volume of 0.9% saline.

2.2. Manual Backup Procedure in Case of Instrument Failure

In case of machine problems exceeding two working days, a manual backup procedure will be in effect. Add 20 µl of water (blank), standard, plasma, and control material to 10x75 mm glass tubes by using an automatic pipettor. Add 2 ml of standard triglyceride reagent or backup reagent by using the automatic dispenser. Vortex tubes and incubate for 10 min. at 37°C. Read absorbance at 510 nm within one hour. TG content of samples is calculated by the formula given in section A.3.

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IV.A.3. Measurement of HDL-Cholesterol

Methods to quantify plasma levels of HDL include various kinds of ultracentrifugation (1-3), electrophoretic techniques (4), and procedures which rely on precipitation of lipoproteins other than HDL (5). While the classical ultracentrifugation techniques are impractical for analyzing such large numbers of samples as required in this program, precipitation techniques have been proven useful in previous epidemiological studies (6-9). In the majority of these studies, VLDL and LDL were precipitated by heparin and Mn2+, and the cholesterol of HDL remaining in solution was measured by a modified Lieberman Burchard method. The use of heparin and Mn2+ is, however, inadequate when cholesterol is determined by enzymatic procedures, since Mn2+ interferes in the determination (10). To overcome this drawback, Mg2+ and dextran sulfate was used and evaluated to precipitate VLDL and LDL (5,11). The method was found highly reliable and reproducible, but revealed, on the average, 5% lower plasma levels of HDL-cholesterol, when compared with the LRC-procedure (11). The explanation for this negative bias may be that a more complete precipitation of apoB containing lipoproteins (11) and precipitation of apoE-containing lipoproteins (12) had occurred. The supernate contained less than 10% of total apo(a) immunoreactivity (13). These data are similar to a previous study in which about 10% of apo(a) immunoreactivity was found in the lipoprotein-free fraction subsequent to sequential ultracentrifugation (14). Thus about 10% of apo(a) is associated with little or no lipid.

Mixtures of dextran sulfate (molecular weight 50,000), an analog of heparin, and magnesium ions (Mg2+) cause precipitation of low density lipoproteins (LDL) and very low density lipoproteins (VLDL) while the high density lipoproteins (HDL) remain in solution. Precipitated lipoproteins are separated by centrifugation, and the supernate containing HDL is analyzed for cholesterol content.

1. MATERIALS

1.1. Equipment and Supplies

- Gilson pipetman, 1 ml
- Gilson pipetman, 100 or 200 µl
- Polystyrene conical centrifuge tubes with caps
- Refrigerated centrifuge
- Disposable transfer pipettes
- Swinnex filter holders equipped with 0.22 mm Millipore filters (25 mm diam.)
- 12 x 75 mm borosilicate glass test tubes
- Sample cups

- Reagent boats
- Reagent tips
- Sample needles
- Cobas-Fara II analyzer

1.2.. Reagents

Preservative solution: Dissolve 5 g NaN_3, 0.1 g chloramphenicol, and 50 mg gentamicine sulfate in 100 ml deionized water.

Magnesium Chloride, 0.5M: Dissolve 10.15 g $MgCl_2.6H_2O$ (stored in dessicator at room temperature) in 80 ml of deionized water in a 100 ml beaker; adjust to pH 7.0 with dilute NaOH solution and add 1.0 ml of preservative solution. Transfer quantitatively to 100 ml volumetric flask and adjust volume to mark with deionized water.

Magnesium Chloride, 1.5M: Dissolve 30.45 g $MgCl_2.6H_2O$ in 80 ml of deionized water in a beaker; adjust the pH to 7.0 with dilute NaOH solution and add 1.0 ml of preservative solution. Transfer quantitatively to 100 ml volumetric flask and adjust volume to mark with deionized water.

HDL-T Reagent: Place 0.5 g dextran sulfate in a 50 ml volumetric flask and dissolve in 0.5M $MgCl_2$ solution.

Standard cholesterol reagent or cholesterol backup reagent: See A.1.2.

2. ANALYTICAL PROCEDURE

2.1. Separation of HDL-Containing Fractions

a. Allow specimens, control materials, and precipitation reagents to equilibrate to room temperature.

b. Pipette 1.0 ml of plasma specimens, standards, and controls into conical plastic tubes; add 0.1 ml of HDLT reagent; immediately after the addition of this reagent, mix the contents of each tube in sequence for at least three seconds with a vortex type mixture.

c. Allow tubes to stand at room temperature for 10 min.; transfer tubes into refrigerated centrifuge and spin for 15 min. at 2000g.

d. Remove tubes from the centrifuge and inspect supernate for turbidity. Carefully remove the supernate and transfer into 10x75 mm glass tubes labeled HDLT.

Note: Any turbidity or cloudiness in the supernate indicates incomplete sedimentation of LDL/VLDL and to a consequent contamination and overestimation of HDL-cholesterol. Turbidity is usually observed in specimens with high triglyceride content. To clear supernates

from turbidity, use one of the following methods:

i. To the plastic separation tube, without separating the turbid supernate from the precipitate, add 1.0 ml of 0.9% NaCl solution (g/v) and another 0.1 ml of HDLT reagent. Vortex thoroughly, then centrifuge as previously described. Obtain the clear supernate for estimation of cholesterol content. Make note of the dilution on work sheet to correct final results by factor of 2.

ii. Alternatively, the turbidity can be removed by ultrafiltration. Assemble a 25 mm Swinnex filter holder with 0.22 fm Millipore filter, 25 mm diameter. Insert an AP15 and AP20 depth filter of 23 mm diameter within the O-ring over the filter; tighten the assembled filter and attach a 5 ml plastic syringe at the top fitting. Pour the turbid supernate solution in the syringe and force it through the filter with moderate pressure by using plunger of the syringe. The filtrate is usually clear and can be further processed (15).

2.2. Cholesterol Determination in HDL Fractions

a. Cobas-Fara II analyzer

After performing the daily maintenance on the Cobas-Fara II autoanalyzer, transfer 25 ml of the standard cholesterol reagent (Boehringer Cat. num. 236691) into a 35 ml disposable container with a snap closure lid to prevent incorrect positioning in the reagent rack. The Level 1 control (Pacific Biometrics) is used as a calibrator for low cholesterol and is placed in the calibration tray. To monitor the precision and accuracy of the dextran sulfate precipitation procedure, a precipitated serum HDL pool (Pacific Biometrics) is used and it also is placed in the calibration tray. Specimen dextran sulfate supernates are aliquotted into sample cups and are placed into the specimen trays. The HDL-cholesterol program is activated. The Cobas-Fara II system of specimen racks, reagent racks, and calibration racks has been described in section A.1.1.1. (16). Sample volume, diluent volume, and reagent volumes are 4, 10, and 200 ml, respectively. Data are calculated by the Autoblank Endpoint Method described in Section A.2.1. At the end of the run transfer sample identification number to the printout. Multiply cholesterol values by the factor of 1.1 to obtain HDLT- cholesterol concentration in plasma.

b. Manual Backup Procedure

Add 20 µl of water (blank), standard, supernates of specimens and control materials to 10x75 mm glass tubes by using an automatic dispenser. Add 1 ml of standard cholesterol reagent or backup cholesterol reagent; incubate tubes for 12 min. at room temperature. Read absorbance at 500 nm on Gilford 250 spectrophotometer. Calculate cholesterol content of fractions according to the formula given in Section A.3. Multiply values by 1.1.

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IV.A.4. Measurement of Plasma Glucose

Glucose will be determined using an enzymatic procedure in which hexokinase catalyzes the phosphorylation of glucose by ATP (1-3). Glucose-6-phosphate is then oxidized to 6-phosphogluconate in the presence of NADP by the enzyme glucose-6-phosphate dehydrogenase. No other carbohydrate is oxidized. Hence, the amount of NADPH formed during the reaction is equivalent to the amount of d-glucose in the specimen and can be measured by the change in absorbance at 340 nm.

1. MATERIALS

1.1. Equipment and Supplies

- Gilson pipetman, 1 ml
- Volumetric 25 ml
- Disposable transfer pipettes
- Sample cups
- Reagent boats
- Reagent tips

- Sample needles
- Cobas-Fara II analyzer
- Glucose-HK UV kit

1.2. Reagents

Two working reagents are supplied in the kit. Bottle 1 contains: 69.2 mM phosphate buffer at pH 7.7; 4.0 mM magnesium sulfate; 2.0 mM NADP; and 1.6 mM ATP. Dilute the contents of one vial by filling to the mark with deionized water to 50 ml. This is stable for three months when stored in an amber bottle at 2-8°C or one week at 19-25°C.

The second reagent, contains > 240U/ml glucose-6-phosphate dehydrogenase [E.C. 1.1.1.49.] and > 175U/ml hexose kinase [E.C. 2.7.1.1., yeast]. To prepare the daily working solution, transfer the contents of the buffer stock solution into bottle 2. This reagent is stable for three months when stored in an amber bottle at 2-8°C or for two weeks at 19-25°C.

1.3. Standards

A standard of 100 and 300 mg/dL provided by Sigma will be used. The expected population range of levels is 70-105 mg/dL. Precinorm (cat.# 125130) and Precipath (cat. # 125202), supplied by Boehringer, are quality control pools.

2. ANALYTICAL PROCEDURE

2.1. Cobas-Fara II

After performing the daily maintenance on the Cobas-Fara II analyzer (3), transfer 25 ml of the standard glucose reagent into a 35 ml disposable container with a snap closure lid to prevent incorrect positioning in the rack. Pipette 1.0 ml of the 100 mg/dL glucose standard (Boehringer Mannheim) into two separate sample cups and 1.0 ml of the 300 mg/dL glucose standard into a sample cup and place them in the black metal calibration rack. Pipette 0.5 ml of each plasma specimen into a specimen cup (without top) and then fill each sample tray with thirty plasma specimens. Each sample rack has 30 unique sample positions which are not duplicated with any other sample rack; i.e. rack A has specimen number 1- 30, while sample rack C contains specimen numbers 61-90. Following the loading of the specimens, the number of specimens and quality control standards are programmed into the glucose program. Sample volume, diluent volume, and reagent volumes are 2, 20, and 200 µl, respectively. Absorbance readings will be performed at 340 nm at 0.5 sec and at 140 sec. Incubation temperature will be 37°C. Glucose content of samples will be calculated by the microprocessor of the instrument using the Autoblank Endpoint Mode as described in section A.2.1. The method is linear for up to 600 mg/dL. Measurements exceeding this value are flagged by the instrument. Analysis is repeated subsequent to diluting the plasma with an equal volume of saline.

References:

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IV.B. ONGOING CASE-CONTROL MEASUREMENTS

IV.B.1. Plasma ApoA-I

Determination of plasma apoA-I was performed by radioimmunoassay in Visits 1 and 2. (See method in IV.B.1.2.). All new determinations of both plasma apoA-I and apoB will be performed by rate nephelometry (immunonephelometry), as described in IV.B.1.1. If instrument malfunction is encountered with this method, the manual radioimmunoassay method shall be used as a backup.

IV.B.1.1. Determination of Plasma ApoA-I (and ApoB) by Rate Nephelometry

An alternative method to radioimmunoassay, which measures plasma levels of apoA-1 and apoB with greater ease and precision, utilizes the principle of rate nephelometry (1). This method is based on the measurement of the rate of light-scattering resulting from an immunoprecipitin reaction between a specific antibody and antigen. The Array Protein System (Beckman) incorporates a rate nephelometer to automatically quantitate the concentration of specific proteins in plasma(2).

Patient plasma is applied to sample cups and diluted automatically. Diluted plasma samples and appropriate antibody are then delivered, via an automated diluter-dispenser, to a semi-disposable flow cell where the immunoprecipitin reaction takes place. The rate of light scattering as immuno-complexes are formed, is measured by a silicon detector. The rate signal is calculated and the concentration is derived automatically from a standard calibration curve of the specific protein being measured.

1. MATERIALS

1.1 Equipment and Supplies

- Array Protein System (Beckman)
- Dilution segments (Beckman # 757413)
- 120 Scatter Reference(Beckman # 662685)
- ICS Flow cell/stirrer kit (Beckman # 663495)

- Rainin pipetman
- Pipette tips

The Array Protein System is a compact, bench-top instrument equipped with the following modules :-

(a) Nephelometric optics. The Array employs a tungsten halogen lamp(400-620 nm) which provides a beam of light that is passed through a disposable flow cell. A rate nephelometer measures the intensity of light as it is scattered by particles in suspension in this flow cell.

(b) Fluid transport system. This consists of a buffer/diluent reservoir, an automated diluter dispenser, three peristaltic pumps and pinch valves, and robotic sample and antibody probes. The diluter dispenser controls the dilution of sample as well as delivery of the samples and the antibody to the flow cell. The pumps control the flow, and the sample and antibody probes maintain accurate pick-up and delivery of those fluids.

(c) Antibody wheel. Appropriate control antibodies are configured on the antibody wheel depending on the particular chemistry to be measured.

(d) Sample wheel. The 40-position sample wheel allows 40 samples to be placed on the system at one time. The wheel holds eight five-sample dilution segments which allow undiluted plasma samples to be placed directly on the outer segments. In addition, user-prepared dilutions of plasma specimens may be placed directly in middle wells.

(e) Card reader and disk drive. The card reader interprets coded program cards which contain all the parameters necessary to perform a particular assay. These parameters include test name, lot number, standard curve information and dilution requirements. A new program card is included with each antibody or calibrator kit.
The disk drive reads the instrument operation software provided on a 3.5 inch floppy disk.

(f) Electronic / Circuit board. This contains all the circuit boards necessary for system operation.

(g) CRT display and Alphanumeric keyboard. These are used to enter sample data into the system. All data is entered in response to prompts on the CRT screen.

(h) Printer. The printer provides a cumulative report of all assays performed.

1.2 Reagents

All of the following reagents are provided by Beckman:

- APO Control Serum (ApA and ApB-Part # 449350): Dissolve one bottle of APO control serum in 1 ml of deionized water. Use this diluted serum directly as an *Apo-B control* in the assay. Dilute the dissolved APO control with 2 parts deionized H_2O (1:3 dilution) and use as an *Apo-A control* in the assay.
- APO Calibrator (ApA and ApB-Part # 449370): Dissolve one bottle of APO Calibrator in 1 ml of deionized H_2O
- Apolipoprotein A-1, Reagent Pak Antisera (Part # 449300)--use as is, place directly on the left antibody wheel in position # 1.
- Apolipoprotein B, Reagent Pak Antisera (Part # 449310)--use as is, place directly on the left antibody wheel in position # 2.
- Buffer (Cat. # 663600)
- Apo Diluent (Cat. # 449380)

1.3 Quality Control Material

Internal quality control of apoA-I and apo-B determinations is maintained by overlapping plasma pools which are essentially the same to those described in IV.B.1.2.(b). These pools, prepared in-house and incorporating low, middle and high ranges, are included in each run.

External quality control of determinations is maintained by incorporating Beckman APO controls (both ApoA and ApoB) at the beginning and end of each run.

2. ANALYTICAL PROCEDURE

a. Calibration. The Array Protein System is designed for minimum calibration. Test calibrations are retained in system memory and are stable for a minimum of two weeks.

Calibration is performed as follows:

i) Insert and read the antibody cards for the apolipoproteins A and B.
ii) Insert and read the calibrator card. Follow the instructions on the System screen.
iii) Place the Apo-A and Apo-B Antibodies in positions 1 and 2 on the Antibody Wheel.
iv) Place 160 µl of Apo-A control in cup #1, and 160 µl of Apo-B control in cup #2 of the Sample Wheel.
v) Perform calibration, print results and place in maintenance log-book.
vi) After calibration is complete perform OPTICS SET since all previously stored values are erased by the calibration.

b. Optics Set. An OPTICS SET is performed after calibration of the system or when the OPTICS UPDATE is out of range. In addition, when a source lamp is replaced or if a new scatter reference is put in service, an OPTICS SET must be performed.
The procedure is accessed from the SYSTEM SET-UP Screen and is performed according to the instructions displayed on the screen.

c. Instrument Setup. Prime system before each run-choose *System Prime Mode 3* which will

prime all buffer and diluent lines.

d Optics Update. The optics update should be performed daily and also prior to any calibration. A 120 scatter reference is used to obtain scatter readings from the system. The range of the ADJUST % number is from +15% to -15%. Record the ADJUST % number in the maintenance log book each day the instrument is run. If the number is out of range perform an OPTICS SET to readjust the system.

c. Set-Up for Run. In the Antibody Tray, place the Apo-A antibody bottle in position #1. Place the Apo-B antibody in position #2.

In the Sample Wheel, place :

i) 160 µl of apo-A control into cups # 1 and # 39
ii) 160 µl of apo-B control into cups # 2 and # 40
iii) 160 µl of in-house plasma controls(low=L, middle = M, high=H) into cups #3,4,5, respectively.
iv) 160 µl of unknown plasma samples into cups #6- #38.

d. Sample Programming. Each sample cup may be defined by typing a sample I.D. for the appropriate cup #. After entering all sample I.D.s, the entire list may be re-checked from the Program Summary to avoid errors. There are three panels programmed into the Array which allow either ApoA-I or ApoB or both tests to be performed from the same plasma sample. In addition, a non-standard dilution (i.e. 1:32 or 1:72) may be programmed for a particular cup which is especially useful when plasma samples are highly concentrated. A user-prepared dilution is also possible. However, the resulting value must be multiplied by the dilution factor used in order to obtain a correct plasma apolipoprotein level.

e. Results. At the end of a run a printout is generated which contains the results of both controls and unknowns in units of mg/dL. In cases where the sample is too dilute or too turbid for analysis by the system, it will be flagged by the instrument and a message will appear beside this sample #. Thus appropriate measures may be taken to repeat the sample.
A hard copy of all results is kept as a permanent record.

References:

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IV.B.1.2. Determination of Plasma ApoA-I by Radioimmunoassay

Determination of apoA-I by RIA is based on its immunological identity. Appropriate dilutions of specimens containing apoA-I are incubated with a fixed amount of iodinated apoA-I and limiting amounts of apoA-I antiserum. Antibody bound apoA-I is then separated from unbound apoA-I using formalin-fixed staphylococcus aureus cells which bind via protein A to the Fc region of IgG molecules. The amount of labeled apoA-I in the precipitate is compared with radioactivity in precipitates produced by known amounts of apoA-I, which allows the calculation of apoA-I in unknown specimens. In human plasma, most apoA-I is bound to lipids to form HDL, and the majority of antigenic sites of apoA-I is masked in intact HDL, when polyclonal antibodies raised against apoA-I are used to measure apoA-I in plasma (1). Exposure of relevant antigenic sites of apoA-I can be achieved by inclusion of Tween 20 into the incubation mixture (2,3).

1. MATERIALS

1.1. Equipment and Supplies

- Refrigerated centrifuge,
- Gilson pipetman, 200 µl, 1 ml, 5 ml.
- Dilution equipment: The Micromedic automatic pipette equipped with a 50 µl-sampling pump and 5.0 ml dispensing pump is used for the preparation of plasma dilutions. Calibration checks to maintain stability are performed at regular intervals. The two pumps are checked separately. Distilled water at 20°C is dispensed into a tap container and accurately weighed with an analytical balance. The volume dispensed is calculated from the weight of water at which the temperature measurements are made (the density of water at 20°C is 0.998 g per ml). To increase the accuracy of weighing, dispense in 10 pump cycles, 500 µl with the 50 µl sampling pump and 30 ml with the 5 ml dispensing pump set at a delivery of 60% of its total volume.
- Gamma counter: The Micromedic 4-600 is an automatic gamma counter with four detectors counting four tubes at the same time. The instrument is equipped with an MACC assay data reduction system, designed to handle data generated by radioimmunoassays. Data reduction may be achieved by the logit procedure or according to the four parameter method (4).
- Siliconized glass tubes: Soak glass tubes (borosilicate tubes, 10x75 mm,12x75 mm, 13x100 mm) in a 1% solution of Prosil R28 in water (v/v) for several minutes, assuring that all surfaces to be coated come in contact with the solution. Remove tubes from solution and air dry for at least 24 hours.
- Two Gilson fraction collectors.
- Econo columns,1 x 30 cm and 1.5 x 30 cm.

1.2. Reagents

- Sephadex G-50.
- Sephadex G-75
- Anti apoA-I antisera: Antisera are raised locally either in rabbits or goats. These antisera are filtered and stored in aliquots at -70°C.
- Barbital buffer, 0.05 M, containing 1mM EDTA, pH 8.6: Weigh out 103 g sodium barbiturate, 20 g barbital, and 3.72 g EDTA-Na_2, dissolve in 10 liters of deionized water.
- Bovine serum albumin, BSA, 10%: Weigh out 100 g of anhydrous BSA (Fraction V) and add slowly under constant stirring to a glass beaker containing 420 ml of deionized water. Stir until dissolved. Transfer solution into 500 ml volumetric flask and add deionized water up to the mark. Transfer solution into dialysis bags and dialyze at 4°C for two days against distilled water. Continue dialysis against 0.05 M barbital buffer, pH 8.6 for two days. Remove dialysis bags and adjust total volume to 1 liter in a volumetric flask. Transfer the 10% albumin solution into 50 ml plastic tubes and store at -20°C.
- Barbital buffer containing 2% BSA: Mix two volumes of 10% BSA with eight volumes of barbital buffer.
- ^{125}I-apoA-I

1.3 Procedure

a. Into a 10x75 mm glass tube add in this order:

   i) 0.5 mCi ^{125}I
   ii) 30 µl 0.05 M phosphate buffer, pH 7.6
   iii) 9 µl apoA-I (2.7 mg ApoA-I /ml in 3 M GuHCl)
   iv) 15 µl lactoperoxidase (0.10 mg/dL in 0.05 M phosphate buffer, pH 7.6), and
   v) 3 µl of 0.22 mM H2O2 (this is a 1:40,000 dilution of the 30% solution).

b. Incubate for 2 min. at room temperature.

c. Add 200 µl of 0.05 M phosphate buffer, pH 7.6 to quench the reaction.

d. Immediately load onto Sephadex G-50 column (1 x 30 cm) which has been equilibrated with 0.05 M barbital buffer, pH 8.6; elute with the same buffer and collect 50 fractions at 20 drops per fraction. Count each fraction for 0.1 min.

e. Graph counts (Y axis) versus fraction number (X axis). Pool peak fractions of the first peak.

f. Measure volume of pooled fractions and add half this volume as 10% BSA.

g. Rinse column with 200 ml of barbital buffer.

1.4 Standard Solution and Quality Control Material

a. Preparation of standard

Fresh pooled human plasma is adjusted to a solvent density of 1.063 g/ml by addition of potassium bromide and ultracentrifuged in a Beckman 50.2 rotor at 10°C for 24 hours at 44,000 rpm. The supernate is removed by aspiration and the infranate adjusted to a density of 1.21 g/ml by addition of solid potassium bromide. After 48 hours of ultracentrifugation in a 50.2 rotor at 44,000 rpm, the HDL is removed from the top of each tube and exhaustively dialyzed against 50 mM NH_4HCO_3. The HDL is then lyophilized, and the apo-HDL is isolated by exhaustive extraction of the lipid with absolute ethanol. After removal of ethanol by centrifugation and decantation, the sample is solubilized in 3 M guanidinium chloride (Gdm.Cl) and dialyzed against 50 mM NH_4HCO_3 to remove residual ethanol. The sample is made 3 M in Gdm.Cl and chromatographed on a 2.5 x 90 cm column of Sephadex G-75 superfine. The column is eluted with a buffer containing 3 M Gdm.Cl, 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.01% EDTA. The major peaks are collected and pooled. The apoA-I containing fractions are re-chromatographed on the same column. The apoA-I is identified by elution position during gel filtration, amino acid composition and electrophoretic mobility in SDS polyacrylamide gel electrophoresis . Aliquots of apoA-I are stored at -70°C at protein concentration of about 3 mg/ml. Protein is determined by the Lowry procedure (5) using bovine serum albumin as standard.

b. Quality control material

Internal quality control of apoA-I determinations is maintained by analysis of overlapping pools. Pools are either prepared from single subjects or from a group of donors. In each assay, at least three plasma pools are analyzed that contain high (more than 150 mg/dL), medium (100-150 mg/dL), and low (below 100 mg/dL) concentrations of apoA-I. Selection of donors is based either on their HDL-cholesterol levels or on previous apoA-I measurements. Blood is collected into EDTA-containing vacutainer tubes (1.5 mg/ml). Plasma is obtained by immediate centrifugation at 4°C. Plasma is then kept on ice, filtered, and divided into aliquots of 100 µl each, and stored at -70°C. In these pools apoA-I content is determined at several dilutions. The range of the apoA-I assay is 5 to 20 ng per dose. Appropriate dilutions of pools are prepared to cover this assay range. Temporary limits of pools are established in 15 assays at a period of stable performance; permanent limits require 30 assays. Our previous experience with such pools indicates stability for at least 12 months, when pools are stored at -70°C. Long term stability is maintained by overlapping analysis of fresh pools in assays controlled by previous pools. Additional quality control material will be derived from CDC. The frequency of analysis of CDC specimens depends on the availability of control material.

2. ANALYTICAL PROCEDURE

On the day before the assay, write assay protocol and label tubes. On the day of the assay, purify the apoA-I tracer, dilute plasma specimen, standard and the antiserum, prepare dilution of

Tween-20, and run the assay.

2.1. Purification of ^{125}I-apoA-I:

a. Equilibrate Sephadex G-75 column (Econo 1.5 x 30 cm) with BSA- barbital buffer.
b. Load 1.0 ml of ^{125}I-apoA-I, elute with equilibration buffer. Collect 60 fractions at 34 drops per fraction. Count all fractions for 0.1 min.
c. Graph counts per fraction and pool peak fractions of the first major peak. Do not include into the pool the shoulder on the leading edge of the ^{125}I-apoA-I peak if such a shoulder is present.
d. Dilute purified tracer for assay:
   i) Take 4 aliquots, 100 µl each, and count for 1 min.
   ii) Dilute with BSA barbital buffer to give 15,000-16,000 cpm/100 µl.
e. Rinse column with 200 µl of barbital buffer containing 0.1% sodium azide, but no albumin.

2.2. Dilution of Control Pools and Plasma Specimens

Place the frozen control pools and the plasma specimens in a rack (in the order of the assay protocol) in a water bath of ambient temperature for 20 min. and allow the specimens to equilibrate to room temperature. Dilute specimens 1:3,000 with 2% BSA-barbital in two steps as follows:

a. First dilution: Aspirate 30 µl of sample with the sampling syringe of the dilution equipment (pump setting 60) and dispense with 2.97 ml of 2% BSA-barbital (this corresponds to a setting of the dispensing pump of 59.4%) into siliconized 12 x 75 mm glass tubes; let stand for at least 5 min.

b. Second dilution: Aspirate 50 µl of the first dilution and dispense with 1.45 ml of BSA-barbital into siliconized tubes (this corresponds to a 100% setting of the sampling syringe and a setting of 29% of the dispensing syringe).

2.3. Dilution of standard :

Dilute standard with 2% BSA-barbital in siliconized tubes for a final concentration of 0.3 ug/ml. This is done by a two-step procedure similar to that described above for samples.

2.4. Dilution of anti-apoA-I antiserum:

Dilute the antiserum which has been previously diluted 1:20 with BSA- barbital according to the specifications given for the particular antiserum.

2.5. Preparation of Tween 20 dilution:

Weigh 0.75 g of Tween 20 and add 20 ml of BSA-barbital.

2.6. Preparation of Immunoprecipitin^R:

a. Centrifuge the commercial solution containing formalin-fixed Staphylococcus aureus cells at 3000 g for 10 min.
b. Aspirate supernate and suspend pellet in the original volume with phosphate buffered saline, pH 7.2, containing beta-mercaptoethanol (10%, w/v) and sodium dodecylsulfate (3%, w/v). Heat for 30 min. at 95°C.
c. Centrifuge cells at 3000g for 30 min. Aspirate supernate, and suspend cells in barbital buffer.
d. Centrifuge cells at 3000g for 30 min., aspirate supernate, resuspend cells in barbital buffer and store at 4°C.

2.7. Running the assay

a. Pipette in order of written protocol:

   i) Total tubes (tubes 1-6); these tubes receive only 100 µl of tracer and are not spun when harvesting the assay.
   ii) Background tubes (tubes 7-9); these tubes receive 350 µl of BSA-barbital, 50 µl of diluted Tween 20, and 200 µl of tracer.
   iii) Standard curve (tubes 10-42); volumes of 0, 5, 10, 15, 0, 30, 40, 50, 70, 100, and 150 µl of the standard containing 0.3 ng apoA-I / µl are pipetted in triplicates to give doses of 0, 1.5, 3, 4.5, 6, 9, 12, 15, 21, 30, and 45 ng/tube.
   iv) Controls (tubes 43-60); pipette 25 µl of the low, medium, and high control pool for two separate aliquots per pool in triplicates.
   v) Unknown plasma samples (tubes 100-end); pipette 25 µl of unknowns in triplicates.

b. Bring volume of all tubes except tubes 1-9 to 250 µl with 2% BSA-barbital.

c. Add 50 µl of diluted Tween 20 to all tubes except tubes 1-6.

d. Add 100 µl of diluted antibody to all tubes except tubes 1-9.

e. Add 100 µl diluted tracer to all tubes.

f. Incubate all tubes for 30-60 hours at 4°C.

g. Add to all tubes (except 1-6) 100 µl of Immunoprecipitin^R (which has been diluted five-fold with barbital buffer) and incubate assay for 15 min. at room temperature. Add 2 ml

of barbital buffer to tubes and centrifuge tubes for 30 min. at 3000 rpm.

h. Aspirate supernates and count tubes containing the pellets in the gamma counter for 1 min. after selection of the appropriate program. Data reduction is performed by the instrument (both logit and four parameter data reduction is available).

i. Subsequent to counting the assay, precipitate tubes 1-6 with trichloroacetic acid. Add 100 µl of 2% BSA-barbital to tubes, add 1.5 ml of ice cold 10% TCA, vortex, and incubate for 10 min. at 4°C. Spin at 1500 rpm at 4°C for 10 min. Siphon and count pellets.

j. Count the fraction of counts precipitated by TCA. Note: To convert results (ng/dose) into mg/dL, multiply results by 12.

k. The assay range is defined as 5 to 20 ng/dose. Unknowns reading outside this range are repeated in the next assay subsequent to two- fold higher or two-fold lower dilution of the specimen.

References:

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IV.B.2. Plasma ApoB

IV.B.2.1. Determination of Apolipoprotein B (apoB) in Plasma by Rate Nephelometry

ApoB measurements are performed in conjunction with those of apoA-I. See IV.B. 1.1.

IV.B.2.2. Determination of ApoB in Plasma by Radioimmunoassay

Determination of apoB by RIA is based on its immunological identity. The assay takes advantage of the fact that human LDL when isolated by zonal ultracentrifugation contains apoB as the sole apolipoprotein (1). Human LDL can therefore be used both as a tracer and as a standard. Approximate dilutions of apoB-containing specimens are incubated with a fixed amount of iodinated apoB (in form of iodinated LDL) and limiting amount of anti-apoB antisera. Antibody bound apoB is then separated from unbound apoB by using formalin-fixed-Staphylococcus aureus cells which bind via protein A to the Fc region of IgG molecules. The

amount of labeled apoB in the precipitate is then compared with the radioactivity in precipitates produced by known amounts of apoB (LDL) standard, which allows the calculation of apoB in unknown specimens (2,3).

1. MATERIALS

1.1. Equipment and Supplies

These are the same as described in Section 1.1 for the apoA-I assays.

1.2 Reagents

- Sephadex G-50
- Bio-Gel A-5M
- Anti-apoB antisera: Antisera are raised locally either in rabbits or goats. These antisera are filtered, divided into aliquots, and stored at -70°C
- Barbital buffer, 0.05M, containing 1mM EDTA, pH 8.6
- Bovine serum albumin, BSA, 10%
- BSA (2%)
- Phosphate buffer, 0.5 Mol, pH 7.6
- Phosphate buffer, 0.05 Mol, pH 7.6
- ^{125}I-ApoB (^{125}I-LDL).

a. Into a 10x75 glass tube in this order:

   i) 1.0 mC^{125}I,
   ii) 9 µl LDL (3 µg LDL protein/µl),
   iii) 16 µl of 0.5 M phosphate buffer, pH 7.6, and
   iv) 10 µl chloramine T (0.066 mg/ml in 0.05 M phosphate buffer, pH 7.6).

b. Incubate for 3 min. at room temperature.

c. Add 5 µl of sodium metabisulfite (0.2 mg/ml 0.05 M phosphate buffer, pH 7.6); add 0.5 ml barbital buffer, pH 8.6.

d. Immediately load onto Sephadex G50 column (Econo 1x30 cm) which has been equilibrated with barbital buffer; elute with the same buffer and collect 60 fractions at 20 drops per fraction. Count each fraction shielded for 0.1 min.

e. Graph counts versus fraction number and pool the 4 peak fraction of the first peak.

f. Measure volume of pool and add half of this volume as 10% BSA. Store at 4°C. Labeled LDL may be used as tracer for up to three weeks subsequent to iodination.

g. Rinse column with 200 ml barbital buffer.

1.3. Standard Solution and Quality Control Material

1.3.1. Preparation of standard

a. Fresh human plasma of 3-10 subjects is pooled to give a total volume of 30-50 ml. The density of the plasma is adjusted to 1.3g/ml by addition of sodium bromide as described previously (4) and subjected to ultracentrifugation in a Ti -14 Beckman zonal rotor using a density gradient of sodium bromide of 1.0-1.3g/ml, which is linear with the rotor volume. Ultracentrifugation is performed at 42,000 rpm, 140 min., and 14°C (1). During unloading, the effluent volume of the rotor is monitored continuously at 280 nm and 10 ml fractions are collected. The LDL peak typically eluting between 200 and 250 ml of the rotor effluent is pooled at half the peak height, and dialyzed against 0.9% sodium chloride containing 1 mM EDTA, pH 8.0 for one day. LDL is then removed from dialysis bags and ultracentrifuged in a 50.2 rotor for 16 hours at 40,000 rpm. The supernate of tubes is aspirated, and the yellow pellet is gently dissolved in 4 ml of EDTA saline at pH 8.0. LDL is filter sterilized by using 0.22 fm Millipore filters and stored in sterilized plastic tubes. The LDL standard is characterized by electrophoresis in 0.5% agarose and 3% polyacrylamide gels in the presence of 0.1% SDS (5). Protein content of LDL is determined by a modified Lowry procedure using bovine serum albumin as standard. Such LDL preparations maintain their immunological stability for at least four weeks.

b. If zonal ultracentrifugation is not available, LDL can also be prepared by conventional ultra-centrifugation in fixed angle head rotors. Plasma is ultracentrifuged in a 50.2 rotor at 45,000 rpm for 16 hours. The top layer containing VLDL is aspirated, and the bottom layers are mixed, pooled and adjusted to a density of 1.050 g/ml by addition of solid KBr. LDL is floated to the top of the tubes by ultracentrifugation in a 50.2 rotor at 45,000 rpm for 24 hours. The top layer containing LDL is re-centrifuged under the same conditions. The top layer is collected and 0.9% saline is added to bring the solution to a density of 1.025 gm/ml. The volume of saline added is calculated by the formula:

$$V_{saline} = \frac{(1.050 - 1.025)}{(1.025 - 1.006)} \times Volume_{top\ layer}$$

The resulting solution is ultracentrifuged for 16 hours at 40,000 rpm in a 50.2 rotor, the supernatant is aspirated, and the LDL containing pellet is processed as described above.

1.3.2 Quality Control Material

For internal quality control, 3 plasma pools containing low (below 65 mg/dL), medium (65-100 mg/dL), and high (above 100 mg/dL) pool levels of apoB are prepared. These pools are either

prepared from individuals or from several donors. Selection of donors is based on plasma levels of LDL-cholesterol as estimated by the Friedewald formula (6). Plasma specimens are kept on ice, filtered and divided into 100 µl aliquots, which are frozen at -70°C. As described in

paragraph *2.6.1.3.2,* limits of pools are determined in 30 assays during a period of stable performance. Limits of newly introduced pools are determined in assays controlled by previous pools. Quality control material provided by CDC is analyzed according to CDC specifications.

2. ANALYTICAL PROCEDURE

One day before assay write assay protocol, label tubes. On the day of the assay purify the apoB tracer, dilute plasma specimen, standard and antiserum, and run the assay.

2.1. Purification of ^{125}I-LDL

a. Equilibrate Biogel A-5m column (Econo 1.5x30 cm) with BSA-barbital buffer.
b. Load 1.0 ml of ^{125}I-LDL, elute with equilibration buffer. Collect 50 fractions at 34 drops per fraction. Count all fractions for 0.1 min.
c. Graph counts per fraction and pool fractions of the first peak.
d. Dilute purified tracer for assay:
   i. Count 4 aliquots, 100 µl each, for 1 min.
   ii. Dilute with BSA-barbital to give 15,000-1,000 cpm/ µl.
e. Rinse column with 250 ml of barbital buffer containing no albumin, but 0.1% sodium azide.

2.2. Dilution of plasma specimens

Place quality control pools and plasma specimens in a rack in the order of the assay protocol. Dilute samples 1:1500 with 2% BSA barbital in two steps:

a. First dilution: Aspirate 30 µl of specimen with dispensing syringe and dispense with 2.970 µl of 2% BSA-barbital into siliconized tubes; let stand for 5 min.

b. Second dilution: Aspirate 50 µl of the first dilution with sampling syringe and dispense with 700 µl of BSA-barbital into siliconized tubes.

2.3. Dilution of standard

Dilute standard with BSA-barbital in siliconized tubes to give a final concentration of 0.5 ug/ml. This is done by a two-step procedure similar to that described above.

2.4. Dilution of anti-apoB antiserum

Dilute the antiserum, which has been diluted 1:20 previously, with BSA- barbital according to the specifications given for the antiserum in use.

2.5. Running the assay

a. Pipette in order of written protocol:

i) Total tubes (1-6); these receive only 100 µl of tracer and are not spun when the assays are harvested.
ii) Background tubes (7-9); these receive 400 µl of buffer and 100 µl tracer.
iii) Standard curve (tubes 10-45); volumes of 0, 5, 10, 20, 30, 40, 50, 60, 80, 100, 150, and 200 µl of the standard containing 0.5 ng/µl are pipetted in triplicate to give doses of 0, 2.5, 5, 10, 15, 20, 25, 30, 40, 50, 75, and 100 ng/tube.
iv) Control pools (tubes 46-63); pipette 50 µl of the low medium and high pool in triplicates. Use two aliquots per pool.
v) Samples; pipette 50 µl of samples in triplicates.

b. Bring volume of all tubes to 300 µl with BSA-barbital except tube 1-6.

c. Add 100 µl of diluted antibody to all tubes except tubes 1-9.

d. Add 100 µl of diluted tracer to all tubes.

e. Incubate the assay for 36-60 hours at 4°C.

f. Add to all tubes (except 1-6) 100 µl of Immunoprecipitin^{R} which has been prepared as described in 2.6.2.6 and diluted five-fold with barbital buffer, and incubate tubes for 15 min. at room temperature. Add 2 ml of barbital buffer to tubes and centrifuge for 30 min. at 3000 rpm at 4°C.

g. Aspirate supernatants and count tubes in gamma counter for 1 min. after selection of the appropriate program.

h. Precipitate tubes 1-6 with 10% trichloroacetic acid. Add 100 µl of 2% BSA-barbital to tubes, add 1.5 ml of 10% ice cold tricholoracetic acid, vortex, incubate for 20 min. at 4°C and spin at 2500 rpm. Siphon and count pellet. Calculate the fraction of counts precipitated by TCA. Note: To obtain results in mg/dL multiply results (ng/50 of 1:1500 dilution) by 3.

References:

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IV.B.3. Determination of Lipoprotein(a) by Double Antibody Elisa

Lp(a) is a lipoprotein that contains two protein antigens, apo(a) and apoB. Since LDL also contains apoB, this antigen cannot be used as a basis for measuring Lp(a). However, since apo(a) is found in no other lipoprotein than Lp(a), immunochemical measurements based on this antigen can be used to quantify the parent lipoprotein. Goat anti-human apo(a) (purified Ig-G) specific for the apo(a) antigen is bound to the surface of a plastic microtiter plate. When an Lp(a) sample is added to the plate, the lipoprotein binds to the solid-supported antibody. A second antibody, rabbit anti-human apo(a) (serum), is then added to the plate. This results in the formation of a sandwich: plate--goat Ab--Lp(a)--rabbit Ab. Finally, a peroxidase conjugated antibody, anti-rabbit Ig-G--peroxidase, is added. The peroxidase reduces hydrogen peroxide while oxidizing o-phenylenediamine to a colored compound which can be measured spectrophotometrically at 492 nm. The optical density at this wavelength is proportional to the amount of Lp(a) present in the triple antibody complex.

1. MATERIALS

1.1. Equipment and Supplies

- Eight (8) channel pipettor
- Immulon I U-bottom microtiter 96-well plates
- Pipette tips
- Rotary shaker
- Flow lab plate reader.

1.2. Reagents

- Deionized water.

- Phosphate buffered saline (PBS) 75 mM phosphate, 75 mM NaCl, pH 7.2: Accurately weigh out 2.485 g $NaH_2PO_4.H_2O$, 8.09 g Na_2HPO_4, and 4.38 g NaCl. Transfer these salts to a 1 L volumetric flask and add 900 ml distilled water. Stir magnetically until all salts are dissolved, then adjust pH to 7.2 with 1 M HCl. Dilute the mixture to 1L and store at 4°C until needed.

- Phosphate buffered saline and bovine serum albumin (PBS + 0.5% BSA): Weigh out 5g of anhydrous albumin and add portion-wise to one liter of vigorously stirred PBS. After complete dissolution, adjust pH to 7.2 with 1 M HCl if required. Store tightly closed at 4°C until needed.

- Phosphate buffered saline + bovine serum albumin + Tween 20 (PBS + 0.5% BSA + 0.10% Tween 20): Weigh out one-half gram Tween 20 and transfer 1 liter of PBS + BSA. Stir the mixture gently to avoid frothing. Store tightly sealed at 4°C until used.

- 0.1M citrate solution: Weigh out 21 g sodium citrate monohydrate and dissolve in 1 liter distilled water.

- 0.2 M phosphate solution: Weigh out 28.4 g Na_2HPO_4 and dissolve in 1 L distilled water.

- O-phenylenediamine substrate solution: Into a 25 ml volumetric flask pipette 6.0 ml of 0.1M citrate solution, and 6.5 ml of 0.2 M phosphate solution. Add 10 mg O-phenylenediamine and stir mixture magnetically until all solid is dissolved. Dilute the mixture with distilled water to the 25 ml mark. Add 10 µl of 48% hydrogen peroxide. This will provide sufficient substrate solution for 1 microtitre plate. Store in brown bottle and protect from light.

- Goat and rabbit anti-human apo(a): Anti-human apo(a) is purified by immunoaffinity chromatography of anti-human Lp(a) over LDL which has been covalently coupled to Sepharose (LDL-Seph) as described by Gaubatz et al (1). This purified Ig-G fraction is diluted 1:1000 in PBS before use.

- Peroxidase conjugate of goat anti-rabbit Ig-G: This commercial reagent is used at each dilution of 1:4000 in PBS-BSA-Tween 20.

1.3. Standard Solution and Quality Control Material

Purified Lp(a) prepared by the method of Gaubatz et al (2) is used as the primary standard. Criteria of purity include (1) absence of all apolipoproteins except apoB and apo(a) as judged by polyacrylamide electrophoresis and Western blotting with cognate antibodies, and (2) absence of LDL as judged by double-decker immunoelectrophoresis (no precipitin line in the upper anti-LDL deck). This primary standard is stored in the presence of proteolytic inhibitors (Trasylol and PMSF) and is prepared fresh on a monthly basis. It cannot be frozen without undergoing some precipitation and/or delipidation. However, in frozen plasma, Lp(a) does retain most of its immunoreactivity over an extended period. Accordingly, the plasma from six subjects with low, moderate, or high levels of Lp(a) have been aliquotted into separate vials and frozen for assaying at periodic intervals for retention or loss of immunoreactivity. Pooled plasmas will not be used since Lp(a) is known to occur as several different particle populations (3) (whose relative abundance varies with the donor) which may exhibit different stabilities when stored frozen over

extended times. The secondary standard will be prepared in a series of dilutions falling within the sensitivity range of the assay. Six wells are designated for standard curve samples. Nine wells will be designated for internal quality control. Results obtained from samples run in these wells will be used to determine the acceptability of the data obtained from the remaining sample wells on the same plate.

2. ANALYTICAL PROCEDURES

a. Coat microtiter plates with purified goat anti-human apo(a) (pure IgG fraction). Perform all incubation steps (e.g., 1, 2, 4, 7, 9, 11) using a rotating shaker. Dilute the IgG fraction 1:1000 in PBS buffer (10 µl of antibody in 10 ml of PBS). Pipet 100 µl of the above dilution into each well of the plate and let the plate sit in room temperature for two hours. After initial incubation at room temperature, place the plates in the cold at 4°C for overnight incubation.

b. Next day, remove plates and reagents from the cold and allow them to equilibrate at room temperature. Wash plates with PBS-BSA (100 µl) several times. Add 200 µl PBS-BSA and incubate for 2-4 hours at room temperature to block non-specific sites.

c. While plates are incubating in step 2, prepare dilutions of Lp(a) samples to be assayed using PBS-BSA Tween buffer as diluent. Wash plates with 2x100 µl of PBS-BSA Tween buffer just prior to dilution of samples.

d. Add 75 µl of sample per well and incubate at room temperature for two hours, then at 4°C overnight.

e. Next day, bring plates and reagents to room temperature and then wash each well with 100 µl of PBS-BSA-Tween 20 three times, followed by 200 µl/well one time.

f. Make 1:750 dilution of rabbit anti-human apo(a), in PBS-BSA-Tween 20 (20 µl of Ab /15 ml PBS-BSA-Tween).

g. Add 100 µl of this antibody to each well on the plate and incubate at room temperature for two hours.

h. Next, wash each well with PBS-BSA-Tween as in 2.8.2.5.

i. Prepare the third antibody (goat anti-rabbit IgG, peroxidase conjugate). Make a 1:2500 dilution of this antibody by adding 10 µl to 25 ml of PBS-BSA-Tween. Add 100 µl of the above antibody dilution to each well. Incubate at room temperature for two hours.

j. Next, wash plates with PBS-BSA-Tween 3x100 µl, 2 x 200 µl, followed by 2x100 µl with PBS alone.

k. Transfer 160 µl of the above substrate reagent into each well of the ELISA plate. Cover the plate with aluminum foil immediately and allow plates to incubate at room temperature for 30 min.

l. Remove foil and stop reaction by adding 40 µl of 5N sulfuric acid.

m. Read optical density of each well on the plate reader at 492 nm.

n. Calculate results from standard curve of concentration vs. optical density. If optical density > 1.6, sample is diluted appropriately and rerun.

References:

1. Gaubatz, J.W., Cushing, G.L., and Morrisett, J.D. 1986. Meth. of Enzymol. 129,
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IV.B.4. Lp(a) Phenotyping

In our laboratory, polyacrylamide gel electrophoresis and sodium dodecyl sulfate followed by immuno blotting has been used to determine the polymorphic pattern of apo[a] in the plasmas of 692 individuals (1). A total of 11 different bands ranging in apparent M_r from 419 kD to 838 kD could be resolved, but usually only 1 or 2 bands were present per individual. The polymorphic band pattern for an individual was assigned to 1 of the 66 different phenotypic designations representing the total number of possible single- and double-band combinations of the 11 detectable bands. All 11 of the possible single-band phenotypes but only 32 of the 55 possible double-band phenotypes were represented. There were 412 plasmas (59.5%) that contained a single band, 274 (39.6%) that contained two bands, and only 6 (0.9%) that had no detectable apo[a] band. A highly significant inverse correlation has been found between the M_r of the band(s) present and the plasma apo-Lp[a] concentration ($r = -0.461$; $p = 0.0001$). The correlation was better between apo-Lp[a] and single-band ($r = -0.495$; $p = 0.0001$) than double-band ($r = -0.382$; $p = 0.0001$) phenotypes. Of the 274 individuals exhibiting double band phenotypes, the lower M_r band was predominant in 141 individuals (51.4%), the two bands were equal in 85 persons (31.0%), while the higher M_r band was predominant in 48 cases (17.5%). Based upon the hypothesis that apo[a] polymorphism is controlled by different alleles at a single locus, the frequency of the 11 alleles determined from the observed phenotypes (low M_r to high M_r) was:

419 kD, 0.00875; 489 kD, 0.00510; 536 kD, 0.0555; 553 kD, 0.0758;
613 kD, 0.135; 680 kD, 0.0824; 705 kD, 0.104; 742 kD, 0.151;
760 kD, 0.246; 796 kD, 0.128; 838 kD, 0.00802.

1. Materials

1.1. Equipment and Supplies

- Lyophilizer
- Constant temperature water bath.
- Polyacrylamide gel electrophoresis apparatus
- Polyacrylamide gel electrophoresis transfer equipment
- Electrophoresis power supply
- X-omat XAR5 film (Eastman Kodak Co., Rochester, NY)
- Lightning Plus intensifying screen (Dupont, Wilmington, DE).
- Stainless steel cassettes

1.2. Reagents

- SDS-PAGE sample buffer: 10 mM TRIS, 1 mM EDTA, 1% SDS, 10% glycerol, 2% 2-mercaptoethanol, pH 8.8
- agarose - type C (Behring Diagnostics, La Jolla, CA).
- PAGE reagents
- Nitrocellulose filter paper
- ^{125}I-protein A (ICN Biochemicals, Inc., Irving, CA), specific activity >30)Ci/mg.
- phosphorylase B oligomers (Sigma, St. Louis, MO)

2. ANALYTICAL PROCEDURE

a. 50 µl aliquots of plasma specimens are lyophilized.

b. Add 500 µl of SDS-PAGE sample buffer to each specimen.

c. A 3.75% acrylamide - 0.10% bis-acrylamide - 0.75% agarose slab polyacrylamide gel is prepared.

d. A volume of the sample, normalized to 25 ng apo-Lp[a] (based on ELISA measurements), is loaded into the gel.

e. A reference plasma mixture containing four different M_r polymorphs of apo[a] from two individuals whose Lp[a] had previously been completely characterized (18), is run on each gel.

f. Electrophoresis is performed at 10°C for 18 h at 25 mA/gel constant current.

g. Slab gel is immediately removed from the glass plates and overlaid with a nitrocellulose filter paper followed by several layers of heavy gauge Whatman filter paper and fiber supports for Western transfer of protein from gel .

h. Transfer is carried out for 18 hours, 10°C at 50 volts on constant voltage.

i. The nitrocellulose paper is incubated for 30 minutes in 0.1 M Tris- HCl, 0.9% NaCl, pH 7.4 (Tris-saline wash buffer) containing 1 % bovine serum albumin (BSA) to block free binding sites.

j. The nitrocellulose is then incubated briefly (5-10) minutes in Tris- saline wash buffer containing 0.5% BSA and 0.25% Tween 20.

k. A highly purified rabbit Ig-G fraction is then added in the same buffer and incubated for 2 hours at room temperature.

l. The nitrocellulose is washed three times with Tris wash buffer containing BSA and Tween 20 , as above, followed by two further washes with deionized water.

m. ^{125}I-protein A in Tris saline wash buffer containing 0.5% BSA and 0.25% Tween 20 is incubated for 2 hours at room temperature with agitation. The nitrocellulose paper is then washed as previously described.

n. The nitrocellulose filter papers are exposed overnight at -70°C to X- omat XAR5 film using a Lightning Plus intensifying screen.

o. The film is developed using an automatic processor.

p. Molecular weight determinations are performed using apoB-100 (M_r 512 kD) and cross-linked phosphorylase B oligomers in the SDS-PAGE system in which the gels are stained with Coomassie Blue rather than blotted.

References:

1. Gaubatz, J.W., Ghanem, K.I., Guevara, J., Nava, M.I., Patsch, W., and Morrisett, J.D. 1990. J. Lipid Res. 31, 603-613.

IV.B.5. APO(A) GENOTYPING

Apo(a) genotypes as defined by the length of kringle 4-coding sequences are determined as described by Lackner et al (1). The current protocol requires isolation of DNA by pulsed-field gel electrophoresis from mononuclear blood cells. Isolation of mononuclear blood cells may be difficult to perform in Field Centers. We do, however, expect simplification of the procedure over the next 2-3 years. DNA is then restricted with KpnI and plugs are subjected to pulsed-field gel electrophoresis. The restricted genomic DNA is transferred to nylon membranes and hybridized with radioactively labeled probe.

References:

1. Lackner, C., Boerwinkle, E., Leffert, C.E., Rahmig, T., and Hobbs, H.H. 1991. J. Clin. Invest. 87,2077-2086.

IV.B.6. DIRECT MEASUREMENT OF LDL-APOB AND LDL-CHOLESTEROL

The ratio of LDL-cholesterol to LDL-apoB can be determined in plasma samples that have been subjected to ultracentrifugation to remove lipoproteins of density less than 1.006 g/ml. Following ultracentrifugation, the TGRL-free infranate is analyzed for contents of cholesterol and apoB. In an aliquot of the infranate, LDL is precipitated by dextran sulfate and Mg2+, to obtain a VLDL- and LDL- free supernate. The cholesterol content in this supernate represents HDL-cholesterol, which is subtracted from the cholesterol content in the total infranate, to give LDL-cholesterol. Since apoB is found only in VLDL and LDL, analysis of apoB by RIA in the VLDL-free plasma fraction provides a measure of the LDL-associated apoB.

1. MATERIALS

1.1. Equipment and Supplies

- Ultracentrifuge
- Ultracentrifuge rotor
- Ultracentrifuge tubes (Ultra-Clear tube, Beckman)
- Tube-slicer (Nuclear Supply and Service Co.)
- See IV.A.1. for total cholesterol measurements
- See IV.A.3. for HDL-cholesterol measurements

1.2. Reagents

- NaCl, 0.9 % (w/v)

2. ANALYTICAL PROCEDURE

2.1. Isolation of d < 1.006 g/ml Lipoproteins

a. 5 ml of plasma is transferred to an ultracentrifuge tube.

b. The plasma is overlaid with 1.0 ml of normal saline.

c. The centrifuge tubes are capped and placed in a Beckman 40.3 ultracentrifuge rotor and spun for 26 h at 35,000 rpm at 4°C. No brake is applied for stopping.

d. Tubes are carefully removed and individually inserted into a tube slicer until the bottom

edge of the cap is 0.8 cm from the top of the tube slicer and the small dent in the tube faces away from the point of the slicer blade.

e. The tube is sliced 1.5 cm from the top of the tube.

f. The top fraction is carefully aliquotted into a 5.0 ml glass stoppered volumetric flask using a 2.5 ml disposable syringe.

g. The cap and tube slicer are rinsed with a small volume of 0.15 M saline and the washings are added to the volumetric flask.

h. The flask for each tube is brought up to 5.0 ml with normal saline.

i. The contents of the tubes are inverted several times.

j. The remaining bottom fraction is removed and aliquotted into a 5.0 ml glass-stoppered volumetric flask.

k. The centrifuge tube is washed with normal saline and the washings are added to the flask.

l. The flask was brought up to 5.0 ml volume with normal saline.

IV.B.7. LDL-Size

Electrophoresis of human plasma in polyacrylamide gradient gels separates plasma proteins, including lipoproteins, according to their apparent Stoke's radii when electrophoresis is performed until equilibrium is reached. Electrophoresis in 2-16% polyacrylamide gradient gels has been shown to separate several subspecies of LDL (1).

1. MATERIALS

- Polyacrylamide gradient gels (PPA 2/16, Pharmacia, Sweden)
- Tris-borate buffer: 0.09 M Tris (M.W. 121.12) 174.4 g, 0.08 M boric acid (MW 61.83), 79.2 g, Na-EDTA 14.88 g, deionized water to 16.0 L, pH 8.4.
- Sample buffer: Dissolve 40.0 g sucrose (40%) and 25 mg bromophenyl blue (0.025%) in 100 ml of Tris-borate buffer, pH 8.4.
- Sudan Black B stain: Dissolve 8.0 g zinc acetate in 400 ml deionized water by heating at 100°C for 30 min. under slow stirring. Add 200 ml ethylene glycol monoethyl ether (Cellosolve, Sigma Chem.) and heat with moderate stirring for 30 min. Add another 200 ml of Cellosolve and heat with moderate stirring until volume is reduced to ca 700 ml. Add 5.0 g Sudan Black B and continue heating and stirring until volume is reduced to 450 ml, which takes about 3 h. Filter the hot mixture using medium porosity filter paper.
- Power supply

- Pharmacia GE 2/4 apparatus
- Circulating refrigerated water bath

2. PROCEDURE

a. Fill gel apparatus with 3.5 L fresh Tris-borate buffer, cool to 2°C for 20 min., insert gels and pre-run at 125 V for 15 min.

b. Sample preparation: to 40 µl aliquots of fresh plasma add 20 µl of sample buffer. Run 2 control specimens stored at -70°C on each gel.

c. Run gel at 70 V for 20 min., then increase voltage to 125 V and run for 18 h.

d. Stain gel in filtered Sudan Black stain for 20 h, destain for 48-72 h in 50% Cellosolve, store in 25% Cellosolve for several days to restore gel size and shape.

e. Scan gels from top to bottom using the LKB Ultrascan XL laser densitometer. Compare R_f values of unknown samples to the known R_f values of controls to assign LDL size-type.

Reference:

1. Krauss, R.M. and Burke, D.J. 1982. J. Lipid Res. 23,97-104.

IV.B.8. Apolipoprotein E Genotyping

The structural gene of apoE is polymorphic, coding for three common isoforms of apoE (E2, E3, and E4). The three alleles produce three homozygous (E2/2, E3/3, E4/4) and three heterozygous (E2/3, E2/4, E3/4) states. Amino acid substitutions at either residue 122 or 158 result in the common isoforms. The E2 isoform contains two cysteine residues at positions 112 and 158, while the E4 isoform contains two arginine residues in these positions. The E3 contains a cysteine at position 112 and an arginine at position 158. Because of charge differences, isoforms E2, E3, and E4 can be separated by isoelectric focusing. The physiologic significance of the E-isoforms has been reviewed (1).

The different E-alleles can be typed by restriction enzyme isoform genotyping (2). This procedure is based on the amplification of a discrete gene region spanning the coding sequences from amino acid 91 to amino acid 165 of the mature apoE protein. DNA amplified by the polymerase chain reaction is restricted with HhaI and separated in polyacrylamide gels under non-denaturing conditions. The recognition sequence of HhaI is GCG'C. In the -4 allele, a total of 6 cleavage sites are present. The T for C change at the codons for amino acids at positions 112 and 158 alters the cleavage sites in the -2 and -3 alleles, which have 4 and 5 cleavage sites, respectively. The expected lengths of fragments are 16, 18, 33, 38, 48 and 72 bp for the -4 allele, 16, 18, 33, 38, 48, and 91 for the -3 allele, and 16, 18, 38, 81, and 91 for the -2 allele.

1. MATERIALS

1.1. Equipment and supplies

- Oligonucleotide primers; 5'-TAAGCTTGGCACGGCTGTCCAAGGA-3'
  5'-ACAGAATTCGCCCCGGCCTGGTACAC-3'

- Enzymes: Taq polymerase (Perkin Elmer Cetus)
  HhaI (New England Biolabs)
- DNA Thermal Cycler (Perkin Elmer Cetus)
- Cyclone Nucleic Acid Synthesizer (MilliGen/Biosearch)

2. PROCEDURE

2.1. Oligonucleotide Synthesis

Oligonucleotide primers are synthesized on a Cyclone nucleic acid synthesizer and further purified using a Surepure kit (U.S.B.)

2.2. DNA preparation

Genomic DNA for polymerase chain reaction is extracted from frozen buffy coat by using the salting out procedure as described by Miller et al. (3):

a. Resuspend thawed buffy coats of nucleated cells in 10 ml of STE buffer consisting of 10 mM Tris, pH 8, 99 mM NaCl and 1 mM Na_2EDTA) and spin at 2700 rpm at 4°C for 14 min.

b. Aspirate the supernate to within 0.5 inches of the pellet and repeat STE wash again.

c. Add 3 ml of lysis buffer (10 mM Tris, 400 mM NaCl and 2 mM Na_2EDTA, pH 8.2), 0.2 ml of 10% SDS and 0.5 ml of proteinase K (1 mg/ml) and digest the cell lysates overnight at 37°C.

d. After digestion, add 3 ml of lysis buffer and 2 ml saturated NaCl (6 M), and shake vigorously.

e. Centrifuge the mixture at 2500 rpm for 15 min.

f. Transfer the supernate containing the DNA to a new tube and add 2 volumes of absolute ethanol.

g. After mixing, remove precipitated DNA strands, wash with 70% ethanol and dry under vacuum.

h. Resuspend the DNA pellet in 500 µl of TE buffer, determine DNA concentration by reading absorbance at 260 nm, use for polymerase chain reaction.

2.3. DNA amplification

a. Add to PCR tube 1 ug of genomic DNA, 0.2 µM of each primer, 0.2 mM dNTP, 10 µl PCR Buffer II (Perkin Elmer Cetus), 2.5 mM $MgCl_2$, 7 µl of DMSO and RNase-free water to 100 µl.

b. Overlay with 80 µl of mineral oil and incubate at 95° C for 5 minutes.

c. Add 2.5 units of Amplitaq^{TM} DNA polymerase and spin the mixture for 30 seconds in a microcentrifuge.

d. Start cycling using the following conditions: 94°C for 1 min., 60°C for 1 min. and 72°C for 1 min. for 30 cycles. The last cycle is 94°C for 1 min., 60°C for 1 min. and 72°C for 5 min.

e. Remove mineral oil and save aqueous PCR-product.

2.4. Restriction enzyme digestion and polyacrylamide gel electrophoresis

a. To 90 µl of PCR-mixture, add 15 units of HhaI and incubate for 4 h at 37°C.

b. Each digested reaction mixture is ethanol precipitated, the precipitate is dissolved in TE-buffer, heated to 70° C for 10 min. and subjected to electrophoresis in 14% polyacrylamide gels (14x18 cm). Running conditions are 2 hours at 250 V.

c. After the run, the gel is stained with ethidium bromide (0.2 mg/l) for 10 minutes and destained in distilled water. DNA fragments are visualized by UV illumination and fragment size is compared with marker DNA.

References:

1. Mahley, R.W. and Rall, S.C. Jr. In: The Metabolic Basis of Inherited Disease. 1989. pages 1195-1214.
2. Hixson, J.E. and Vernier D.T. 1990. J. Lipid Res. 31,545-548.
3. Miller, S.A., Dykes, D.D., and Polesky, H.F. 1988. Nucleic Acid Res. 16,1215.

IV.C. NEW CASE-CONTROL MEASUREMENTS

IV.C.1. Measurement of LpA-I Plasma Concentrations

Anti apoA-II antibody incorporated in excess quantities into agarose gels inhibits almost completely the electrophoretic migration of apoA- II containing particles (LpA-I/LpA-II), while the electrophoretic migration of particles containing no apoA-II (LpA-I) is inhibited by apoA-I antisera. However, apoA-I antiserum is present in the agarose gel at concentrations to produce immunoprecipitates (rockets) which have a height that is proportional to the concentration of LpA-I in the specimen. A sample gives two rockets: a very small one that corresponds to the apoA-II containing particles and a higher, lighter stained one that corresponds to particles containing no apoA-II, i.e., LpA-I. Comparison with standards allows the calculation of LpA-I. ApoA- I present in particles which also contain apoA-II can be calculated by subtracting LpA-I from total plasma apoA-I (1,2).

1. MATERIALS

1.1. Equipment and supplies

- LpA-I kit (Hydragel, France)
- Power supply
- Electrophoresis apparatus (LKB Multiphor II)
- Gilson pipetteman, 20 µl
- Drying oven
- Water bath
- Hydragel accessories

1.2. Reagents

- Tris/glycine/MES buffer containing 7.05 g/L Tris-HCL, 5.65 g/L glycine, 16.2 g/L MES, and 0.2 g/L of sodium azide, pH 7.0.
- Coomassie Blue stain, 0.13% (w/v)
- Destaining solution (methanol 27%, v/v, acetic acid 7% v/v in distilled water.
- Normal saline

1.3. Standards

Reconstitute the lyophilized standard serum with 0.5 ml of distilled, deionized water. Let stand at room temperature for 30 min. and vortex gently. Dilute the dissolved standard as follows:

Saline	Standard	Conc.
1010 µl	10 µl	STD x 0.5
1000 µl	20 µl	STD x 1.0
990 µl	30 µl	STD x 1.5
980 µl	40 µl	STD x 2.0

In addition, quality control pools produced in house will be analyzed within each assay.

2. ANALYTICAL PROCEDURE

a. Warm the unknown specimens and controls in a 37°C water bath for 20-30 min. Vortex gently.

b. Dilute the standard, see above.

c. Dilute 20 µl of each unknown speciman with 1000 µl of normal saline.

d. Apply 5 µl of the diluted standards and unknown specimens into the wells. To avoid any edge effect, one well at the end of each gel is not used and is filled with saline. Allow specimens to diffuse for 20 min. before applying current to obtain a homogeneous diffusion in all wells.

e. Cool the LKB Multiphor II system to 4°C. Place the Hydragel on cooling plates with the wells on the cathodic side.

f. Place wicks soaked in Tris-buffer on either side of the gel (1 cm).

g. Fill each of the two compartments with 300 ml of buffer.

h. Set the voltage at 300 V and run for 60 min.

i. Take the gel off and place it on a flat surface.

j. Absorb the proteins not precipitated by applying one thin filter paper previously soaked with saline, and two thick filter papers under a pressure of approximately 1 kg for 20 min.

k. Wash the gel vertically in saline for 60 min.

l. Press again with one thin filter paper, previously soaked with saline, and two thick filter paper under a pressure of approximately 1 kg for 20 min.

m. Dry the gel in an oven at 55°C.

n. Immerse the gel in staining solution for 5 min.

o. Immerse the gel in the destaining solution until the background is clear.

p. Calculation: The smaller rocket corresponds to particles containing apoA-II and is not used for quantitation. The higher lighter stained rocket corresponds to particles containing apoA-I and is measured in mm. The slope, intercept, and regression coefficient of the standard curve are determined, and concentrations of LpA-I in unknowns are determined according to the formula:

$$\text{LpA-I} = \frac{\text{P.H.} - b}{m}$$

where P.H. = peak height, b = intercept and m = slope

q. Repeat any sample with a peak height outside the range of the standard curve after appropriate dilution.

References:

1. Koren E, Puchols P, Alaupovic, Fesmire J, Kandoussi A, Fruchart J-C. Clin Chem 1987;43:38-43.
2. Luc, G., Parsa, H.J., Zylberberg, G., and Fruchart, J.C. 1991. Eur. J. Clin. Invest. 21,118-122.

IV.C.2. Quantification of Plasma ApoC-III Levels

ApoC-III is determined by single radial immunodiffusion (1).

1. MATERIALS

1.1. Equipment and Supplies

- Gilson pipetteman, 20 µl
- Glass test tubes, 12 x 75 mm
- Gel Scanner, LKB Laser Gel Scanner
- ApoC-III Plates (Daiichi, Tokyo, Japan)

1.2. Reagents

Physiological saline is required for specimen and standard dilution. An apoC-III pre-treatment reagent is provided by the vendor.

1.3. Standards

An apoC-III standard serum is provided by the vendor. One vial of standard is reconstituted with 0.5 ml of saline, which is stable for two weeks at 2-10°C.

2. ANALYTICAL PROCEDURE

i) The frozen plasma specimens are brought to room temperature in a water bath set at 27°C and gently vortexed.

ii) Specimens are then diluted with the apoC-III pre-treatment reagent as described by the manufacturer.

iii) 10 µl of either standards or specimens are pipetted into the test wells of the apoC-III plate

iv) The plates are closed and developed for 48 h at 25°C.

v) For reading of precipitation rings, plates are placed upside down in the LKB Laser Scanner. The apoC-III levels of the unknown samples measured by extrapolating ring diameters against the ring diameters of standards with known apoC-III concentration.

References:

1. Goto Y et al. J Clin Biochem Nutr 1986;1:73-88.

IV.C.3. Apolipoprotein A-I Promoter Heterogeneity

The -76 G to A substitution will be typed after DNA extraction, PCR, and SSCP as described above (1,2).
References:

1. Pagani, F., Sidoli, A., Giudici, G.A., Berenghi, L., Vergani, C., and Baralle, F.E. 1990. J. Lipid Res. 31,1371-1377.
2. Orita, M., Suzuki, Y., Sekyia, T., and Hayashi, K. 1989. Genomics 5,874-879.

IV.C.4. ApoB Signal Peptide Length Polymorphism

Genomic DNA is extracted and amplified using [5'- CAGCTGGCGATGGACCCGCCCGA-3' AND 5'-ACGGGCCCTGGCGGCCCGCCAGCA-3'] as primers (1,2). Amplification products are analyzed by electrophoresis in 8% polyacrylamide gels.

References:

1. Boerwinkle, E. and Chan, L. 1989. Nucleic Acids Res 17,4003.
2. Boerwinkle, E., Brown, S.A., Rohrbach, K., Gotto, A.M., Jr., and Patsch, W. 1991. Am. J.
. 49, 1145-1154.

IV.C.5. Quantification of Plasma Cholesteryl Ester Transfer Protein Mass and Activity

The measurement of plasma cholesteryl ester transfer protein (CETP) mass, i.e., immunoreactivity, requires:

a) the purification of CETP from human plasma (1);
b) the production of antibodies to CETP (2);
c) the development of an assay for CETP levels (3).

A functional assay which is independent of endogeneous substrate and product lipoproteins (4) is already established in our laboratory. This assay allows determination of activity in frozen plasma samples, independent of substrate lipoproteins.

IV.C.5.1. Functional Assay for CETP Activity in Plasma Samples

VLDL- and LDL-free plasma is obtained by treatment of plasma, that has been stored at -70°C, with polyethyleneglycol. Treated plasma is incubated with ^{14}C-cholesteryl oleate-labeled LDL and HDL_2 in the presence of 5,5-dithiobis(2-nitrobenzoic acid), a LCAT inhibitor. LDL is precipitated by addition of dextran sulfate and Mg^{2+}. Radioactivity in the supernate is measured and CETP activity is calculated (4).

1. MATERIALS

1.1. Equipment and Supplies

- Sonicator (equipped with microtip)
- Ultracentrifuge equipped for zonal operation

1.2. Reagents

- 9.5% polyethyleneglycol (w/v) in 0.1 M phosphate buffer, pH 6.5 containing 0.1 M NaCl
- LDL, HDL_2, and lipoprotein deficient plasma, typically prepared by zonal ultracentrifugation
- 140 mM 5,5-dithiobis(2-nitrobenzoic acid) in 0.1 M NaCl, titrated to light yellow color
- 8% fatty acid free bovine serum albumin in deionized water
- 2% dextran sulfate in deionized water
- 2 M $MgCl_2$ in deionized water

Preparation of ^{14}C-cholesteryl oleate-labeled LDL :

i. Mix 5 μCi cholesteryl[1-^{14}C]oleate (50-60 mCi/mmol), 800 μg of egg phosphatidylcholine dissolved in chloroform and 20 nmol of hydroxytoluene (20 μl of a 1 mM solution in chloroform). Evaporate under stream of nitrogen.

ii. Add 2.0 ml of 50 mM Tris-buffer, pH 7.4 containing 0.01% Na_2EDTA and flush with N_2.

iii. Sonicate the suspension twice for 5 min. each at 25°C under a stream of nitrogen.

iv. Add the sonicated lipids to a mixture of 0.5 ml of LDL (6 mg protein/ml), 5 ml of lipoprotein deficient plasma, 600 µl of 10 mM 5,5-dithiobis(2-nitrobenzoic acid) and 80 µl of 10% EDTA and incubate for 24 h at 37°C.

v. Re-isolate the LDL by zonal ultracentrifugation, dialyze against 10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.4, and determine specific activity of esterified cholesterol in LDL.

2. ANALYTICAL PROCEDURE

2.1. Radiolabel *In Vitro* Assay

a. Add 400 µl of 9.5% polyethylene solution to 200 µl plasma in a 1.5 ml disposable cup for 15 min. at room temperature.

b. Centrifuge at 3500 x g for 10 min. at 4°C and collect the supernatant.

c. Aliquot the following into a 1.5 ml disposable tube:

i) 60 µl of unknown supernate;
ii) 500 nmol of total cholesterol of [1-^{14}C]-Cholesteryl oleate-labeled LDL;
iii) 200 nmol of total cholesterol of HDL_2 in 50 µl of 10 mM Tris-HCl buffer, pH 7.4 containing 0.15 M NaCl, 1 mM EDTA, and 0.01% NaN_3;
iv) 10 µl of the 140 mM 5,5-dithiobis(2-nitrobenzoic acid) solution;
v) 350 µl of 0.1 M phosphate buffer, pH 7.4
vi) Bring to total volume of 700 µl with 0.9% NaCl solution.

d. As a sample blank, substitute normal saline for the supernate in the reaction mixture above.

e. Incubate the reaction mixtures for 16 h at 37°C.

f. Terminate the reaction by placing assay tubes on crushed ice for 15 min.

g. Add 300 µl of 8% bovine serum albumin solution and 100 µl of unlabeled LDL (equivalent to 20 mM total cholesterol) to the assay tubes and vortex.

h. Add 110 µl of an equivolume mixture of 2% dextran sulfate and 2 M $MgCl_2$, and vortex.

i. Place the assay tubes on crushed ice for 15 min. followed by centrifugation at 3500 x g for 10 min. at 4°C.

j. Aliquot 1.0 ml of the supernate into a scintillation vial together with 10 ml of scintillation fluid and count.

k. Calculate cholesteryl ester exchange activity in unknown plasma samples according to the formula:

$$F = \frac{-\ln\left[1 - \frac{C_H}{M_V(S_{V(0)} - S_{Eq})}\right]}{\frac{M_V + M_H}{M_V \cdot M_H}\, t}$$

where F : The rate of cholesteryl ester exchange between VLDL and HDL(nmol/incubation/h)

M_V : Pool size of triglycerides (nmol/incubation) in VLDL

M_H : Pool size of cholesteryl ester and triglycerides (nmol/incubation) in HDL

$S_{V(0)}$: Counts/M_V (cpm/nmol) at time 0

S_{Eq} : Counts / (M_V+ M_H) (cpm/nmol) at equilibrium

C_H : Radioactivity in HDL (counts/min) at time t (h)

Cholesteryl ester exchange activity in plasma equals :

$$F \times \frac{1000\ (ul)}{60\ (ul)} \times 3 \quad (nmol/ml/h)$$

IV.C.5.2. Immunoassay of CETP in Human Plasma

CETP is purified from fresh human plasma by a series of steps including ultracentrifugation and 3 sequential chromatographic procedures using butyl-Toyopearl 650, CM-Toyopearl 650, and Toyopearl HW-55 (1). Comparable matrices may be used instead. Monoclonal and/or polyclonal antibodies will be generated by standard procedures. Alternatively, synthetic peptides of CETP may be used for generation of antibodies against surface-specific epitopes. A radioimmunoassay will be developed as reported (4).

References:

1. Ohnishi, T., Yokoyama, S., and Yamamoto, A. 1990. J. Lipid Res. 31,397- 406.
2. Hesler, C.B., Tall, A.R., Swenson, T.L., Weech, P.K., Marcel, Y.L., and Milne, R.W.

1988. J. Biol. Chem. 263,5020-5023.
4. Marcel, Y.L., McPherson, R., Hogue, M., Czanecka, H., Zawadzki Z, Weech PK, Whitlock ME, Tall AR, Milne RW. 1990. J. Clin. Invest. 85,10-17.

IV.C.6. Quantification of Plasma Endothelin-1

Plasma levels of endothelin-1 from frozen specimens will be determined by a commercially available competitive radioimmunoassay (1,2).

1. MATERIALS:

1.1. Equipment and Supplies

- Endothelin-1 RIA kit (Amersham)
- Low speed centrifuge [Beckman]
- Gamma counter [ICN Series 800 Gamma Counter]
- Centrifugal concentrator (Supervap)

1.2. Reagents

- ^{125}- iodine (Amersham)
- Endothelin-1
- rabbit Anti-human Endothelin-1 antiserum
- Triton X-100
- Goat anti-rabbit IgG serum
- Normal rabbit serum

1.3. Standards

- Human endothelin-1 [Peninsula Laboratories, Inc.]

2. ANALYTICAL PROCEDURE

A pre-purification step will be required for plasma specimens. A small amount of plasma will be acidified and endothelin-1 related compounds will be recovered. Recovery rates are reported to be approximately 90% for this method (1-3).

2.1. Specimen pre-treatment

Plasma specimens are thawed and 4.0 ml are required for this procedure.

a. Plasma specimens are acidified by adding 0.1% trifluoroacetic acid.
b. Samples are centrifuged at 3000 x g for 15 min at 4°C.

c. A SEP-column containing 200 mg of C18 is activated by washing with 60% acetonitrile in 0.1% trifluoroacetic acid.
d. Apply the sample to the column and wash in trifluoroacetic acid solution.
e. Elute the endothelin-like material in the acetonitrile solution.
f. Evaporate the eluate to dryness in a centrifugal concentrator.

2.2. Radioimmunoassay

The radioimmunoassay kit will measure endothelin-1 levels. The rabbit anti-human endothelin-1 has been shown to have the following cross-reactivities, in percent, compared to endothelin-1; 7% to endothelin-2; 7% to endothelin-3; and 17% to big endothelin-1. This kit is based upon the competition of standard or endothelin-1 in specimens with labeled endothelin 1 for limiting amounts of antibody.

2.2.1. Procedure

a. Dilute the Triton X-100 buffer to 200 ml with deionized water.

b. Reconstitute the RIA buffer with the Triton X-100 solution.

c. Reconstitute the endothelin-1 standard with 1.0 ml of the RIA buffer.

d. Reconstitute the rabbit anti-endothelin-1 serum with 13.0 ml of the RIA buffer.

e. Prepare dilutions of the standard to include points from 1.0 picogram to 126 picogram/tube.

f. In 12 x 75 mm polystyrene tubes, setup the RIA tubes.

g. To the standard tubes, pipette 200 µl into the non-specific blank tubes and 100 µl into the total blank tubes.

h. Pipette 100 µl of standards into duplicate tubes.

i. Pipette 100 µl of the diluted unknown specimens into duplicate tubes.

j. Pipette 100 µl of the primary antibody into all tubes except the non-specific blank tubes.

k. Vortex the contents of each tube.

l. Cover and incubate overnight at 4°C.

m. Reconstitute the radiolabeled endothelin-1 with 13 ml of the RIA buffer.

n. Add 100 µl of the diluted radiolabeled endothelin-1 to each tube.

o. Vortex the contents of each tube.

p. Cover and incubate overnight at 4°C.

q. Reconstitute the goat anti-rabbit IgG serum with 13 ml of RIA buffer.

r. Reconstitute the normal rabbit serum with 13 ml of RIA buffer.

s. Add 100 µl of the hydrated goat anti-rabbit IgG serum to each tube.

t. Add 100 µl of the hydrated normal rabbit serum to each tube.

u. Vortex all tubes and incubate at room temperature for 120 min.

v. Add 0.5 ml of RIA buffer to each tube and vortex.

w. Centrifuge all tubes at 1700 x g for 20 min.

x. Carefully aspirate the supernate.

y. Count the tubes in a gamma counter and calculate the amount of endothelin-1 concentrations against the corrected standard curve.

References:

1. Xuan, Y.T., Whorton, A.R., Shearer, P.E., Bovd, J., and Watkins, W.D. 1989. Biochem Biophy Res Commun 164,326-332.
2. Cernacek, P. and Stewart, D.J. 1989 Biochem. Biophy. Res. Commun. 161,562-567.

V. QUALITY CONTROL PROCEDURES

The success of the project depends on the accuracy and precision of measurements performed over the entire study period. Such a goal can only be achieved by the implementation of rigorous quality control procedures. For lipid measurements, guidelines have been established by the LRC Program and the CDC. Unfortunately CDC no longer provides control materials for cholesterol, triglycerides, or HDL-cholesterol.

The quality control standards and control pools are offered by a wide variety of vendors. Pooled serum samples are preferred to lyophilized quality control pools which require reconstitution which is a source of error. However, we will be using Pacific Biometrics control materials which are traceable to the CDC.

Key components of the quality control program are the use of internal quality control material

and the analysis of external surveillance samples. These two components assure that the accuracy of measurements is traceable to reference material. Precision of measurements is estimated from the analytical error obtained in analysis of quality control material, which is assumed to represent the error obtained in the analysis of participant samples.

V.A. QUALITY CONTROL OF PLASMA LIPID DETERMINATIONS

V.A.1. Internal Quality Control

V.A.1.1. Quality Control Pools

The internal quality control material for measurement of plasma cholesterol and triglyceride consists of Technicon standard and as well as the controls provided by Pacific Biometrics. Plasma cholesterol and triglyceride concentrations in these pools correspond to low-normal or high plasma levels of these two analytes when compared with their distribution in American populations (1).

In the Cobas-Fara, for every 30 samples a set of quality control pools will be analyzed. During normal operations, at least two specimen trays are run, which will provide a minimum of two analyses per pool per working day. Before a control pool is depleted, replacement pools are analyzed in overlapping fashion, to assure longitudinal stability of measurements. During periods of overlapping analyses, preliminary control limits for the new pools are derived from analyses in 20 working days, and final limits are calculated from 50 analyses performed on different days. A batch with a volume sufficient for 18 months to 2 years is prepared and stored at -70°C.

V.A.1.2. Calculation of Internal Quality Control Limits

The daily mean, $\bar{x}$, for each control pool, is calculated according to the formula:

$$\bar{x} = \frac{\Sigma x}{n}$$

where n is 4 or more for the QC-pools (pools for the low-normal and high cholesterol and triglyceride) and 2 or more for the serum calibrator to be used as primary standard of plasma cholesterol measurements. The mean of the daily means, the overall mean, x, is calculated by the formula:

$$\bar{x} = \frac{\Sigma x}{N}$$

where N is 20 days for preliminary limits and 50 days for permanent limits.

S_x is calculated by the formula:

$$S_x = \frac{\Sigma(x - x)^2}{N - 1}$$

where S_x is one standard deviation of the daily mean.
Control limits for the daily mean charts are:

Upper limit: $x + 3S_x$
Lower limit: $x - 3S_x$

Warning limits for the daily mean control charts are:

Upper warning limit: $x + 2S_x$
Lower warning limit: $x - 2S$

Apart from the $\bar{x}$ chart, a range chart is constructed and maintained, to monitor precision. The range, R, for control pools is the difference between the highest and the lowest value obtained in one day:

$$R = x_{high} - x_{low}$$

The average range, $\bar{R}$, is then:

$$\bar{R} = \frac{\Sigma R}{N}$$

Where N is 20 days and 50 days for preliminary and definite limits, respectively. Limits for the range are obtained by multiplication of R by the appropriate probability factor which, for four within-day values, is 1.855 for warning limits and 2.280 for control limits, respectively. In the determination of the definite limits, to be used in the daily charts, outliers will be excluded. An outlier is defined as any value of x or R outside the control limits. Limits are then recalculated.

Additional guidelines for the evaluation of internal quality control limits are based on the target value (TV) provided by CDC. The purpose of these additional rules is to place constraints on both, systematic and random errors. A large relative deviation of x from the TV must be accompanied by a small day-to-day variability to meet the criteria. Conversely, a smaller deviation of the x from the TV allows a larger between-day variability. These guidelines are:

QC-pools ------	Chol	$x \pm 2S_x$	within TV ± 5%
	TG	$x \pm 3S_x$	within TV ± 10%
	Chol, TG	x	within TV ± CDC limits

These limits are communicated to laboratories upon participation in this program.

V.A.1.3 Use of Internal Quality Control Limits

Warning signs that will alert the analyst and laboratory supervisor to prepare for corrective actions are:

i. x falls between $2S_x$ and $3S_x$.
ii. Shifts and trends on the x chart, including seven successive values above or below x and seven successive values in a pattern that indicates an abrupt or progressive change in one direction.
iii. Excessive variability, such as seven R values above R.
iv. R exceeds the warning limit.

Runs are repeated - subsequent to taking corrective actions - when:

i. x of any control pool falls outside any of the control limits defined in Section V.A.1.2.
ii. Two successive x values fall outside the warning limits, or
iii. R exceeds the control limit.

V.A.2. External Quality Control

V.A.2.1. Participation in Part III of CDC-NHLBI Lipid Standardization Program

The Central Lipid Laboratory has passed part I and II of this standardization program. Phase III of this program serves to maintain the performance of the laboratory by CDC. Surveillance samples sent by CDC usually cover the range of triglycerides and cholesterol, observed in more than 90% of the population to be studied. Surveillance samples are analyzed in duplicates at weekly intervals, and reported to CDC.

V.A.2.2 Analyses of Blinded Duplicates of Participant Samples

As part of the quality control of lipid measurements, 5% of samples consist of blinded duplicates prepared in the ARIC field centers to monitor intra- and interassay variability.

V.A.2.3 Participation in Proficiency Testing of the College of American Pathologists (CAP)

The Central Lipid Laboratory is accredited by CAP and will participate in its proficiency testing.

V.A.2.4. Network Reference Method Laboratory Network of the Centers for Disease Control (CDC)

Our laboratory participates in the cholesterol standardization program implemented by CDC.

V.A.3. Quality Control of HDL-Cholesterol Measurements

V.A.3.1. Internal Quality Control

Quality control of HDL-cholesterol measurements includes a low cholesterol calibrator pool and two pooled serum controls which are precipitated along with unknown samples. The first type of pool calibrates the instrument for cholesterol. Since at least two runs are performed in a typical working day, at least four values are obtained for each pool. The second type of pool is carried through the precipitation procedure like a regular plasma sample. Per batch of 39 samples, one sample is co-precipitated and is analyzed for cholesterol in duplicate. Target values for the quality control pool series are assigned limits in eight runs according to the CDC reference method. This method consists of ultracentrifugation at d < 1.006 g/ml, precipitation of LDL in the infranate by heparin and $MnCl^{2+}$, and analysis of cholesterol by the CDC reference method in the LDL-free supernate. Several reports indicate that the dextran sulfate Mg2+ method gives about 5% lower HDL-cholesterol values when compared with the heparin Mn2+ procedure (2,3), which is also our experience. For quality control purposes, target values from other laboratories using the Mn2+ procedure, these results will therefore be multiplied by a factor of 0.95 to overcome the systemic bias between the two procedures.

V.A.3.1.1. Calculation of Internal Quality Control Limits

The daily mean, $\bar{x}$, for the precipitated quality control pools are calculated according to the formula given in Section V.1.1.2. The number of analyses is 4 or more for the cholesterol calibrator pool and 2 or more for each precipitated quality control pool. Calculation of $\bar{x}$, $S_{\bar{x}}$, temporary and permanent warning and control limits is described in V.1.1.2 as well. Precision is monitored by using a range chart. Limits for the range chart are obtained by multiplication of R by the appropriate probability factors. For the quality control pools (two within-day values), the range warning limit is 2.46 R, and the range control limit is 3.27 R. For the cholesterol calibrator quality control pool (four within-day values), the range warning limit is 1.855 R, while the range control limit is 2.280 R.

Additional guidelines for HDL-cholesterol determinations are:

Chol = $\bar{x}$ within 5% of TV or within 3 mg/ml of TV

Chol = $x \pm 3S_x$ limits within TV $\pm$ 25%

Chol = $x \pm 3S_x$ limits within TV $\pm$ 15%

The TV of AQ is defined as 95% of the TV provided by CDC, when assignment of TV was based on precipitation by heparin and Mn2+. Warning signs which will trigger a review of procedures are the same as described in Section V.1.1.3.

Cholesterol analyses in HDL-containing supernates are repeated when:

i. x falls outside any of the control limits,
ii. Two successive x values of calibrator fall outside the warning limits, or
iii. R of calibrator exceeds the control limit.

The HDL precipitation procedure is repeated when:

i. x of local pool falls outside any of the control limits,
ii. Two successive x values for the local pool fall outside the warning limits, or
iii. R of the local pool exceeds the control limit.

IV.A.4. Quality Control of Instrumentation

A.4.1. Maintenance Program for the Cobas-Fara II Analyzer

Each day laboratory personnel will check the sample needle and sample syringes, clean the optical lenses with methanol, and check the printer paper, the seating of sample tubing, the action of the pipettor arm, reagent delivery, and the uniformity of the diluent stream of the sample needle. Each week the staff will empty and refill the pipette reservoir, empty the waste reservoir, check the sample loop tubing, clean the reagent drip cup, perform precision checks for reagent and sample delivery, fill reagent boat and all sample cups with bichromate solution, activate the appropriate program on the keyboard and record coefficients of variation for sample delivery and reagent delivery, which must be below 2%. Each month the teflon syringe tips and sample needles will be replaced and the photometer check performed.

The field service engineer of Cobas instruments will be responsible for performing preventive maintenance every three months. In addition, an emergency service (24-hour response time) is part of the service contract.

V.B. Quality Control of Plasma Glucose Measurements

Internal quality control for the proposed glucose determinations will be established and maintained through the use of control standards and pools offered by a number of vendors. The

glucose standards and control materials offered by Boehringer Mannheim will be utilized for establishing the accuracy and precision performance. The analysis of blinded duplicates will provide the necessary external surveillance required for our quality control program. The statistical principles of quality control outlined in section A.1.1.2. for cholesterol quality control

will apply to the quality control program for glucose measurements. The glucose determinations will be performed on the Cobas instrument. The maintenance program outlined for cholesterol will apply for this test. Typically, individual glucose measurements will be performed on the same day from the same sample cup dedicated to cholesterol and triglyceride measurements.

Comparability of our glucose determinations (performed either in plasma or serum) to glucose determinations (performed in serum) by the Central Chemistry Laboratory during visits 1 and 2 will be ascertained.

V.C. QUALITY CONTROL OF APOA-I AND APOB MEASUREMENTS

Despite the progress made in the characterization of apolipoproteins and in the understanding of apolipoprotein function, maintaining accuracy and precision in their quantitation is still a major challenge for a laboratory. Major variables in the quantitation of apolipoproteins by RIA are the standards, tracer, and antisera. It is therefore important to keep these sources of variation as constant as possible. Thus, one of the principles in maintaining long term stability is to avoid changing more than one of these variables within a reasonable period of time. There is no need to change the method of labeling apolipoproteins. ApoA-I standard is prepared in a quantity to last for the entire study period provided that this standard retains immunologic stability. ApoB standard (human LDL) may need to be prepared in monthly intervals because of instability of LDL. To avoid possible effects of inter-individual differences in LDL on apoB quantitation, LDL is prepared from fresh plasma obtained from several subjects. With regard to antisera, we intend to use one batch of antiserum per analyte for the duration of this study. However, it has been our experience that identical results can be obtained with different antisera. Should a change in antiserum become necessary, equivalence of results with the previous assays will be established by using internal quality control pools.

V.C.1. Internal Quality Control

V.C.1.1 . Quality Control Pools

Three plasma pools are prepared that contain high, medium, and low levels of the apolipoprotein to be measured. As a guideline for the preparation of pools, low, medium, and high plasma levels of apoA-I are defined to be less than 100 mg/dL, 100-150 mg/dL, and above 150 mg/dL. Low, medium, or high levels of plasma apoB are defined to be less than 65 mg/dL, 65-100 mg/dL, and above 100 mg/dL. Aliquots of pools are stored at -70°C. The range of the apoA-I assay is defined as 5-20 ng/dose, which corresponds to plasma apoA-I values of 60 to 240 mg/dL when the dilution of samples is taken into account. More than 95% of specimens

analyzed should fall in this range. The range of the apoB assay is defined as 10-70 ng/dose. corresponding to plasma apoB values of 30 to 210 mg/dL. Again, more than 95% of unknowns should fall within this range. Analyses of unknowns falling outside the range specified will be repeated. Twice the usual amount of plasma will be analyzed in samples falling below the cutoff point, and half the usual volume of plasma will be analyzed in samples whose apparent apolipoprotein content is above the upper limit of the assay range.

Control pools are treated like regular samples. In each assay, two aliquots of each of the three control pools are analyzed in triplicate. One aliquot of unknown samples is analyzed in triplicate.

Calculation of control limits for accuracy:
The daily mean, x, for each control pool is defined as the mean of the two aliquots, analyzed in triplicate. The overall daily mean, x, is calculated by the formula:

$$x = \frac{dx}{N}$$

where N is 15 or 30 days for preliminary or definite limits. S_x is calculated by the formula:

$$S_x = \frac{d(x - x)^2}{N - 1}$$

Warning limits for the daily mean chart are:

Upper limit: $x + 2S_x$
Lower limit: $x - 2S_x$

Control limits for the daily mean chart are:

Upper limit: $x + 3S_x$
Lower limit: $x - 3S_x$

Precision of analyses is monitored in two ways. Each sample is analyzed in triplicate, which provides a mean and CV for each sample. In addition two aliquots of each pool are analyzed in triplicate, which provides additional information on errors produced in preparing the dilutions of plasma lipids.

A range chart is constructed to monitor intra-assay variability in a strict sense, i.e., taking into account all dilutions. The range, R, for control pools is the difference between the two means of the triplicate analyses of aliquots per pool. In calculating the mean, outliers will be omitted. These are defined as single values differing by 40 or more percent of the mean of the two remaining replicates, should the CV of the two remaining replicates be less than 10%. The

average range, R, is then calculated by:

$$\bar{R} = \frac{dR}{N}$$

Where N is 15 or 30 days for preliminary or definite limits. Limits for the range are obtained by multiplication of R with the probability factor of 2.46 (warning limits) or 3.27 (control limits). An entire assay will be declared out of control and repeated, when:

1. x for two of the three control pools falls outside the control limits.

2. x values of two pools fall outside the warning limits on three successive days, or

3. R of two pools exceed the control limit.

An assay will be repeated in part, i.e., only those samples whose apparent apolipoprotein content falls in the range of the respective pool, when:

1. $\bar{x}$ of the respective pool falls outside the control limits.

2. x values of the respective pool fall outside the warning limits on three successive days, or

3. R of the respective pool exceeds the control limits.

Individual samples will be repeated when the CV of a given sample exceeds 18% of its mean value.

To maintain long term stability of analyses, overlapping analysis of newly prepared pools is performed in assays being controlled by the outgoing pools. To be prepared for changes in immunoreactivity of the primary pools upon storage, complimentary pools are stored. Temporary limits of these pools are established. Should there be any indication that immunoreactivity has changed within a given pool (i.e., drifts or shifts of pools, two subsequent analyses outside of the 2S limits), the backup pool covering the complementary range is analyzed in the next assay together with the questionable pool.

IV.D. QUALITY CONTROL FOR NEW ANALYSES IN SELECTED POPULATIONS

The quality control principles discussed above will be implemented for all other analytes proposed. When applicable, commercially available standards and control materials serve as

quality control materials in addition to locally-produced pooled human serum. This will include the proposed measurements of LpA-I, ApoC-III, free cholesterol and endothelin in commercial kits.

Quality control of apoA-IV and CETP measurements will be based on establishing standards

with known mass as determined by amino acid analysis and/or protein measurements. For phosphatidylcholine determinations, manufacturer standards with known phosphatidylcholine concentrations will be used for quality control purposes. The concept of implementing a strict quality control program is more difficult to apply to those tests of genetic analysis which provide yes or no answers. The performance of the respective assays will be monitored in control samples generated in our laboratory with known outcome. Quality control of mRNA abundance levels will be assured by using a control RNA pool.

References:

1. The Lipid Research Clinics. 1980. Population Studies Data Book, Vol. I, The Prevalence Study. NIH Publication No. 80-1527.
2. Warnick, G.R., J. Benderson, and W.V. Brown. 1984. Clin. Chem. 28, 1379-1388.
3. Gibson, J.C., A. Rubinstein, and M.W. Brown. 1984. Arteriosclerosis 4, 564a.

VI. SPECIMEN AND DATA PROCESSING

VI.A. SPECIMEN PROCESSING IN FIELD CENTERS, SHIPMENT TO CENTRAL LABORATORY, TEMPORARY AND LONG-TERM STORAGE

IV.A.1. Specimen Processing in Field Centers

The details of blood drawing and processing in field centers are described in ARIC Manual 7 for visits 1 and 2. For the measurements to be performed at the Central Lipid Laboratory, important steps include collecting blood into EDTA containing tubes (lavender-stoppered tubes containing 15 mg EDTA per 10 ml tube), keeping the collected blood at 4°C, and separating plasma by centrifugation at 4°C within one hour of blood collection. Two full 10 ml tubes are required for lipid measurements. Plasma is then separated and placed into 6 lavender sample tubes. The buffy coats from isolated whole blood components are aliquotted into two tubes. The following aliquots are prepared:

a. Three 1.5 ml aliquots (for measurement of plasma cholesterol, plasma TG, HDL-chol, glucose and repeats if necessary)

b. Four aliquots, each containing 1.5 ml of plasma for long-term storage. These aliquots may be prepared dividing the remaining plasma from the two lavender stoppered tubes into four equal aliquots.

c. Two aliquots, each containing 1.5 ml of the buffy coat of the two lavender stoppered tubes for DNA and RNA extraction.

The plasma and buffy coat aliquots are frozen at -70°C and placed into a pre-labeled 3"x 6" storage bag. Bags containing the aliquots of one plasma specimen are then placed into a shipping box kept at -70°C.

V.A.2. Shipment of Specimens and Receiving in the Central Lipid Laboratory

Shipping boxes are prepared according to the protocol outlined in Manual 7 and sent once a week (Monday or Tuesday) to the Central Lipid Laboratory. Shipments will generally contain a maximum of 30 bags. Shipments with over 30 bags require two shipping boxes. An ARIC batch shipping log containing a face sheet and a contents sheet, accompanies each shipping box. When the shipments arrive at the Central Lipid Laboratory, a technician records the time and date of arrival at the laboratory in the space provided on the face sheet. The condition of the total shipment with respect to temperature is also noted on the shipping log using the following codes:

00 = arrived frozen and unbroken
01 = arrived frozen, but vial broken
02 = thawed in transit, but vial not broken
03 = thawed in transit, and vial broken

The shipping log is then initialed by the receiving technician and maintained in the laboratory for inventory purposes. On the contents sheet, individual samples will be checked, and deficiencies with regard to volume, labeling, or temperature will be indicated. Samples are placed immediately into a -70°C freezer and are held there until processing which is completed within a 48 hour period.

V.A.3. Temporary and Long Term Freezing of Specimens

Upon arrival, one batch of samples is processed at a time. On the contents sheet, individual samples will be checked, and deficiencies with regard to volume, labeling, or temperature will be indicated. If any discrepancy is noted with regard to sample identification or number of donor specimens, the person processing the ARIC shipment reports this to the data coordinator who in turn notifies the appropriate field center in order to resolve the problem. All such interchanges are logged.

Sample bags are placed on ice and arranged sequentially according to the inventory sheet. A unique sequential Central Lipid Laboratory number is assigned to each ARIC sample. This

assignment facilitates sample processing and data production. The Lab ID numbers are contained on pre-printed self-adhesive labels and are placed both on the inventory sheets beside the appropriate ARIC I.D., and on the three plasma aliquots dedicated for routine lipid determinations and glucose analyses. These samples are transferred into boxes, labeled with the shipment number, and stored at -70°C on an assigned freezer shelf until ready to run.

Four plasma aliquots and two buffy coats are assigned for long term storage and are placed in standardized freezer boxes. These samples do not receive a Lipid Lab I.D. number. They are stored at -70°C in freezers dedicated solely for long term storage. For visits 3 and 4, long term storage has been computerized by the coordinating center. Thus, samples are stored according to the specific instructions of the DES Freezer Inventory Program. In addition, for each sample the identity of stored aliquots can be easily retrieved.

Documentation of specimens sent by each field center will be as follows: Specimen identification code will be logged manually into the computer. For each sample the identification code, arrival date, and a code for sample condition are entered into the Central Lipid Laboratory Data Entry System (DES).

V.B. DATA HANDLING

V.B.1. Lipid Laboratory Data

Documentation of specimens sent by each field center will be as follows: Specimen identification code will be logged manually into the computer. For each sample the identification code, arrival date, and a code for sample condition are entered into the Central Lipid Laboratory Data Entry System (DES).

A sample work sheet printed from the DES includes the Central Lipid Laboratory specimen number, the corresponding ARIC sample number, and test being performed. The following assays are performed on each specimen: plasma cholesterol, plasma triglyceride, HDL-cholesterol, and glucose. Following each assay, the results generated from each sample are recorded in the Central Lipid Laboratory DES. A medical alert level for triglyceride of 1000 mg/dL has been established. For glucose, the alert levels are $\geq$ 200 mg/dL or $\leq$ 60 mg/dL. The field centers are notified by telephone or facsimile if ARIC specimens equal or exceed the alert status levels.

V.B.2. Data Entry System

The DES System contains a number of editing features that are used according to the instructions of the coordinating center. These instructions are provided in the Data Management System User's Guide.

Archiving of laboratory data is performed periodically. A hard copy of raw data is available

which is printed by the analytical instrument. In the event of a computer problem all the information for the ARIC study can be entered manually into either a word processing program or Lotus file until the difficulty is corrected.

V.B.3. Internal Data Entry

V.B.3.1. Data Transfer from Peripheral Instruments to Host DES

The total cholesterol, TG, HDL-chol, and glucose data are generated from the Cobas autoanalyzer. The Cobas analyzer and the DES are not compatible for direct transfer of data. Thus, to eliminate error in the manual transfer of data, the Lab Supervisor and the data coordinator perform transcriptional checks of the stored data against Cobas paper printouts and laboratory worksheets.

V.B.3.2. Data Storage for New Case-Control Studies

Data from the new case-control studies will be transferred to commercially available databases compatible with IBM disk operating systems [Dbase, Quattro, etc.]. The principles of retaining hard copies of raw data as well as auditing of data, will apply to these studies. These data will be forwarded to the Coordinating Center on computer disks. Many of the tests proposed will be performed in relatively small samples compared to the entire cohort that will facilitate data entry and storage. Development and pilot testing of new data screens for each new test for the existing DES may be considered. Data entry is currently instrument-dependent in that only one station exists for the following activities: data entry; batch or sample I.D. revision, quality control review, worksheet printouts, paper reports, data disk generation, and other data archiving activities.

V.B.3.3. Data Transfer to the Coordinating Center

To meet the requirements of providing cholesterol, TG, HDL-chol, and glucose determinations to the Coordinating Center within one week, the following plan is in use: Specimens typically arrive on Tuesday of each week. Specimens are processed completely [labeled, entered into the computer, short-term storage, long-term storage, transcriptional checks against shipment logs] by the following Monday. Typically, weekly shipments of samples [ca 400 test results] require 2 working days to complete analyses, with data transfer [ARIC I.D., laboratory I.D., run number, technician I.D., test, problem codes] to the DES requiring an additional 2 to 3 days. Data transfer to the Coordinating Center is by computer disk via DHL. A paper copy is kept at this laboratory. In case of computer failure, the ARIC database is restored via a tape back-up system and transfer diskettes. The Coordinating Center is notified under these circumstances.

V.B.3.4. Data Transfer to the Field Centers

Data transferred to the field centers is retrieved from the DES database by the software provided. These data are transmitted electronically by the data coordinator via CC-Mail. A confirmation of receipt is required to avoid errors in communication.

V.B.3.4. External Quality Control Data Review

A timely review of the blinded duplicate sample data is necessary to maintain a credible quality control program. Such a program could interface with our current DES. In particular, the determination of monthly population means is one of the most important ways for the Central Lipid Lab to monitor long-term drift. Therefore, we request that the Coordinating Center provides such an analysis in a timely fashion.