Molecular Genetics of Lipoprotein (a)

Allan Gaw, M.B., Ch.B., Ph.D. (Glasgow)

Institute of Biochemistry
Royal Infirmary
Glasgow

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Acknowledgements

Learn of the skillful: he that teaches himself hath a fool for his master.

Benjamin Franklin, *Poor Richard's Almanack* 1733.

America's renaissance man, Ben Franklin, left us with many words of wisdom; none more so than the advice above. The M.D. degree is unsupervised but thankfully not un-advised. There were many 'skillful' from whom I have learned; who counselled me and led me away from my ignorance. The following list acknowledges those, whose contributions of various kinds made the completion of this work possible.

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Author's Declaration

The work presented in this thesis was performed solely by the author, except where the assistance of others is acknowledged.

Allan Gaw, December 1995

Dedication

Twenty-five years ago my father, Robert T. Gaw, died aged 51 from a stroke. His apolipoprotein (apo) (a) phenotype, unknown of course at the time, may be reconstructed from the apo(a) phenotypes of myself, my mother, and my siblings. This reveals that his apo(a) phenotype was most likely associated with a high plasma lipoprotein (a) [Lp(a)] level. As I write this thesis there is controversy in the literature on the contribution of an elevated plasma Lp(a) to the risk of cerebrovascular disease. Although my work does not primarily deal with this issue I hope in the future to extend our existing knowledge on this topic. It is therefore to the memory of my father that I dedicate this work.

Abstract

The work presented in this thesis aims to extend our understanding of the controlling mechanisms that influence the plasma concentration of lipoprotein(a) [Lp(a)]. The techniques of apo(a) genotyping and apo(a) phenotyping are used to study Lp(a) in different ethnic and genetic groups to achieve this end. In chapter 3 the effective use of a commercially available monoclonal antibody is demonstrated. Two primary antibodies were compared and apo(a) isoform analysis was performed using an immunoblotting method. When equal quantities of Lp(a) were loaded approximately equal signal intensities were obtained, irrespective of apo(a) isoform size. Serial dilutions of samples with known Lp(a) level and a single expressing apo(a) isoform were used to determine the detection limit of the system. With both monoclonals it was possible to detect 0.05 mg. dL^{-1} Lp(a).

Distributions of plasma Lp(a) concentrations exhibit marked inter-racial differences. Apo(a), the unique constituent of Lp(a), is highly polymorphic in length due to allelic variations in the number of kringle 4 (K-4)-encoding sequences. Plasma Lp(a) concentrations are inversely related to the number of K-4 repeats in the apo(a) alleles. To determine the contribution of this length variation to the inter-racial variation in plasma Lp(a) levels, the *APO(a)* allele size, glycoprotein size, and plasma Lp(a) concentrations in Caucasians, Chinese and African-Americans were compared in chapter 4. Caucasians and African-Americans had very different distributions of plasma Lp(a) concentrations yet there was no significant difference in the overall frequency distributions of their *APO(a)* alleles. Over the entire size spectrum of apo(a) alleles, the plasma Lp(a) levels were higher in African-Americans than in Caucasians. Conversely, Caucasians and Chinese had similar plasma Lp(a) concentrations but significantly different *APO(a)* allele size distributions. Therefore, inter-racial differences in the plasma concentrations of Lp(a) are not due to differences in the frequency distributions of *APO(a)* alleles. The relationship between *APO(a)* allele size and the presence of detectable plasma apo(a) protein in plasma were also examined. *APO(a)* alleles associated with no detectable plasma protein were not of uniformly large size, as had been expected, but were distributed over the entire size spectrum. From this analysis, it was concluded that there is no common "null" allele at the *APO(a)* locus.

The role of the low density lipoprotein (LDL) receptor in the control of plasma Lp(a) concentrations remains controversial. To investigate if a reduction in LDL receptor activity is associated with alteration in the plasma concentrations of Lp(a), the apo(a) phenotype and plasma lipid levels have been studied in families with familial hypercholesterolemia (FH). In normal Caucasian families it has previously been shown that 90% of the inter-individual variation in plasma Lp(a) levels is attributable to sequences linked to the *APO(a)* gene; this is reflected in the fact that sibling pairs who inherit *APO(a)* alleles identical by descent (ibd) had very similar plasma levels of Lp(a) (r=0.95). In chapter 5, plasma Lp(a) levels are compared in siblings from 9 families with FH. In the non-FH (n=15), and FH sibling pairs (n=10) the plasma Lp(a) levels were very similar. The plasma Lp(a) levels in the sibling pairs with the same apo(a) phenotype and with *APO(a)* alleles ibd in which one member had FH and one member did not (n=12), were significantly different [median plasma Lp(a) 22.0 vs. 12.0 mg. dL^{-1}, p=0.005], which would be expected if the LDL receptor played an important role in the control of plasma Lp(a) levels, as it does for LDL.

Apo B-67 is a truncated form of apoB previously described in a large Amish kindred. The mutation is associated with apoB levels that are 25% of normal, which result from decreased production of both apoB-100 and apoB-67. To determine the impact of decreased apoB-100 production on plasma Lp(a) levels families with this form of familial hypobetalipoproteinaemia (FHB) were studied. In chapter 6, plasma Lp(a) levels are compared in siblings from these FHB families. In the non-FHB (n=13), and FHB sibling pairs (n=6) the plasma Lp(a) levels were very similar. The plasma Lp(a) levels in the sibling pairs with the same apo(a) phenotype and with *APO(a)* alleles ibd in which one member had FHB and one member did not (n=13), were significantly different [median plasma Lp(a) 4.6 *vs.* 1.8 mg. dL^{-1}, p=0.003], which would be expected if the *APOB* gene played an important role in the control of plasma Lp(a) levels.

In chapter 7 an overall review of the major findings reported in this thesis is presented.

Chapter 1 Introduction

The factor present in the serum of positive human reactors was called Lp(a) factor, where Lp is an abbreviation for Lipo-protein.

Kåre Berg, 1963.

1.1 Introduction

Lipoprotein(a) [Lp(a)] is a cholesteryl-ester rich lipoprotein that resembles low density lipoprotein (LDL), but has distinctive structural, epidemiological, and genetic properties. Discovered and named by Berg in 1963, Lp(a) stimulated relatively little interest for almost 25 years despite early reports of its association with coronary heart disease (CHD). Now, however, there is a renewed and rapidly growing interest in this lipoprotein, due to mounting evidence linking high plasma concentrations of Lp(a) with increased risk of coronary and cerebrovascular atherosclerosis. In addition, the molecular genetic analysis of the *APO(a)* gene has provided new insight into the genetic control of Lp(a) levels.

1.2. Lp(a) and apo(a) structure

1.2.1 Lp(a) structure

Lp(a) has two components: a particle of LDL, to which is attached a large, hydrophilic, glycoprotein, apolipoprotein(a) [apo(a)] [Figure 1.1]. Apo(a) is attached to the apoB-100 of LDL by a single disulphide bridge (Gaubatz et al., 1983; Utermann & Weber, 1983; Sommer et al., 1991), but

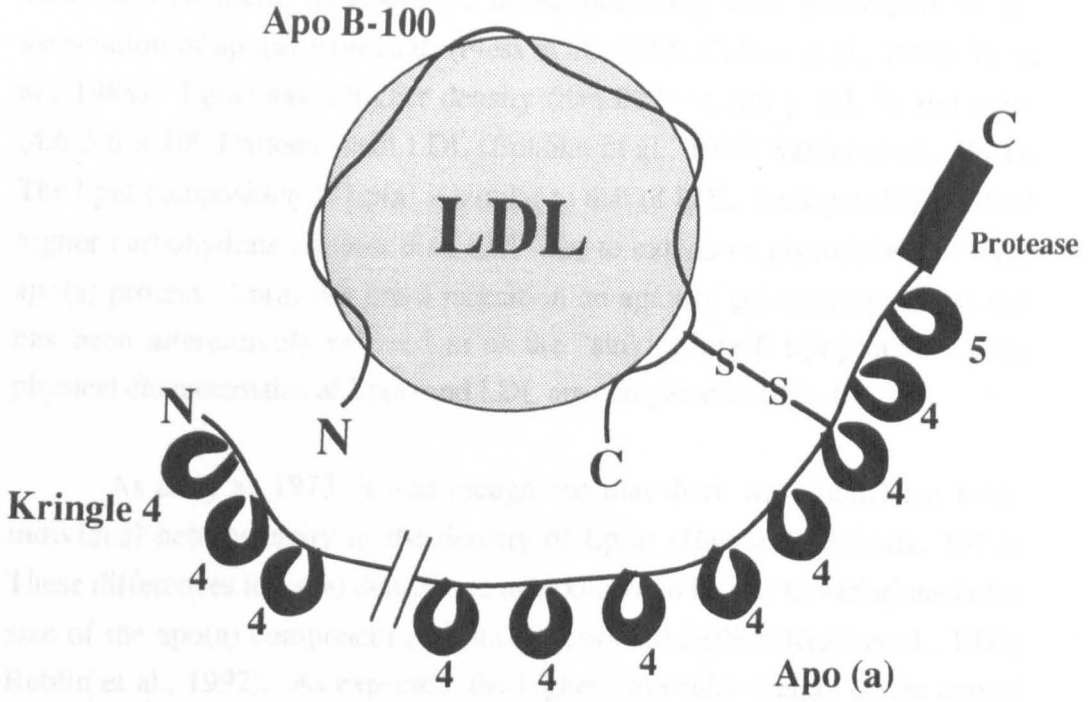


FIGURE 1.1. Structure of Lp(a)

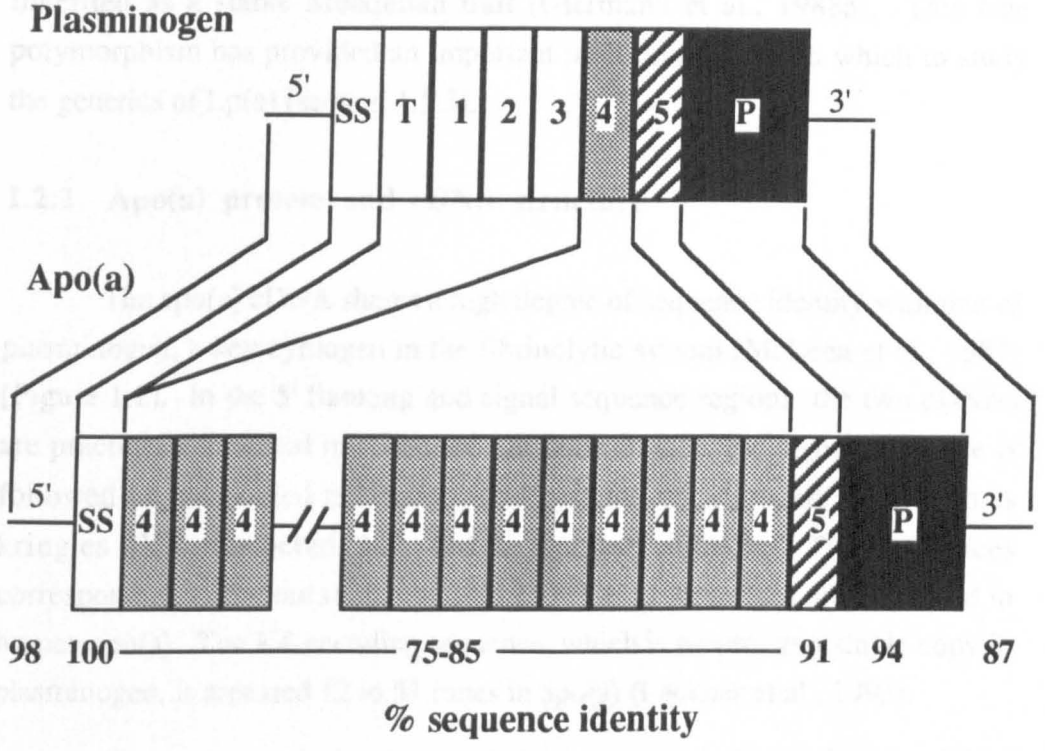


FIGURE 1.2.

Comparison of plasminogen and apo(a) cDNAs. SS-signal sequence; T - tail region; 1-5 - kringle 1 to kringle 5; P-protease domain. Adapted from McLean et al. (1987).

other non-covalent, hydrophobic interactions may also participate in the association of apo(a) with LDL (Fless et al., 1985; Chiesa et al., 1992; Ye et al., 1988). Lp(a) has a higher density (d=1.027 - 1.100 g. mL^{-1}) and mass (4.6-5.6 x 10^6 Daltons) than LDL (Enholm et al., 1972; Sattler et al., 1991). The lipid composition of Lp(a) is similar to that of LDL, but Lp(a) has a 4-fold higher carbohydrate content than LDL due to extensive glycosylation of the apo(a) protein. Lp(a) has pre-ß migration on agarose gel electrophoresis and has been alternatively referred to as the "sinking pre-ß lipoprotein". The physical characteristics of Lp(a) and LDL are compared in table 1.1.

As early as 1973, it was recognized that there was significant inter-individual heterogeneity in the density of Lp(a) (Harvie & Schultz, 1973). These differences in Lp(a) density are now known to be due to variations in the size of the apo(a) component of Lp(a) (Fless et al., 1984; Kraft et al., 1992; Reblin et al., 1992). As expected, the higher molecular weight of the apo(a) isoform, the denser the Lp(a) particle (Fless et al., 1984; Pfaffinger et al., 1991). It was Utermann (1989) who made the seminal observation that apo(a) varied in size over a wide range (400-700 kDa) and that the size variation was inherited as a stable Mendelian trait (Utermann et al., 1988a). This size polymorphism has provided an important molecular tool with which to study the genetics of Lp(a) [section 1.5.3].

1.2.2 Apo(a) protein and cDNA structure

The apo(a) cDNA shares a high degree of sequence identity with that of plasminogen, a key zymogen in the fibrinolytic system (McLean et al., 1987) [Figure 1.2]. In the 5' flanking and signal sequence regions, the two cDNAs are practically identical in sequence. In plasminogen, the signal sequence is followed by a so-called tail region, five cysteine-rich domains referred to as kringles (K), numbered 1-5, and a protease domain. The sequences corresponding to the tail region and K1 to K3 in plasminogen are not found in human apo(a). The K4-encoding sequence, which is present as a single copy in plasminogen, is repeated 12 to 51 times in apo(a) (Lackner et al., 1993).

In the single human apo(a) cDNA that has been characterized to date, there was a total of 37 K4 repeats (McLean et al., 1987). Twenty-four of the 37 repeats shared the same sequence (Type A repeats). Four repeats, the Type B repeats, differed from the Type A repeats by only 3 silent nucleotide

Table 1.1. Comparison of the physical characteristics of LDL and Lp(a)

	LDL	Lp(a)
Synonym	LpB	Sinking pre β
Electrophoretic mobility (agarose)	β	pre β_1
Buoyant density (g. mL^{-1})	1.019-1.063	1.027-1.100
Molecular mass (Da)	2.4×10^6	$4.6\text{-}5.6 \times 10^6$
Molecular diameter (Å)	210	250
Plasma $T_{0.5}$ (days)	2.5-3.0	3.0-3.5
Fractional catabolic rate (pools. day^{-1})	0.3-0.5	0.3
Protein (g. mol^{-1})	550,000	~850,000-1,250,000
Free cholesterol (mol. mol^{-1})	725	750
Cholesteryl ester (mol. mol^{-1})	1,900	2,000
Triglyceride (mol. mol^{-1})	220	350
Phospholipid (mol. mol^{-1})	1,000	1,110
Lipid : protein mass	3.5	2.2

Da, Dalton; Å, ångström
Adapted from MBewu & Durrington (1990).

substitutions. The remaining repeats, $K4_1$ and $K4_{30}$ - $K4_{37}$ differed from the Type A repeats by between 11 and 71 nucleotides.

Each 342 base pair K4 sequence encodes 114 amino acids including six conserved cysteine residues that form three intra-kringle disulphide linkages. The penultimate K4-like repeat, $K4_{36}$, contains a free cysteine residue thought to be the site of attachment of apo(a) to apoB-100 (McLean et al., 1987). The exact cysteine residue in apoB-100 to which apo(a) is bound has not been conclusively demonstrated. Since apo(a) does not associate with apoB48, the site of attachment probably resides within the C-terminal half of apoB-100. Physicochemical studies suggest that the residue responsible is at position 3734 (Coleman et al., 1990; Guevara et al., 1993a).

To prove that the single unpaired cysteine (position 4057) in $K4_{36}$ of apo(a) is involved in this covalent linkage with apoB-100, two groups have altered this residue by *in vitro* mutagenesis (Koschinsky et al 1993; Brunner et al 1993). When the cysteine was replaced with a serine, the resultant recombinant apo(a) glycoprotein failed to associate with human LDL *in vitro*. The final K4 repeat, $K4_{37}$, most closely resembles the K4 repeat of plasminogen. This repeat has been proposed as the site of attachment of apo(a) to lysine-rich proteins, such as fibrin (Guevara et al., 1993b; Scanu et al., 1993).

Each K4 repeat in apo(a), unlike plasminogen, contains a consensus sequence for the attachment of a single N-linked sugar, and six O-linked sugars. Exactly which of these potential glycosylation sites are used is not known. Approximately 30% of the mass of apo(a) is carbohydrate, which is 10-fold higher than the relative sugar content of plasminogen (Fless et al., 1986).

Immediately following the tandem array of K4 sequences, apo(a) contains a sequence that is homologous to K5 of plasminogen. In plasminogen, this region contains a cleavage site that permits activation by tissue-type plasminogen activator (tPA) or urokinase. Apo(a), however, is not activated because the amino acid sequence at the cleavage site is not conserved (McLean et al., 1987). Although the protease domain of apo(a) shares 88% amino acid identity with plasminogen, apo(a) has no known serine protease activity.

1.2.3 *APO(a)* gene structure

The *APO(a)* gene resides on the long arm of chromosome 6 (6q26-27) within 50 kb of the plasminogen gene and close to two other *APO(a)* - like genes (Murray et al., 1987; Malgaretti et al., 1992, Magnaghi et al., 1994) [Figure 1.3].

Although the apo(a) cDNA was cloned in 1987, the *APO(a)* gene and its 5' flanking region have only recently been isolated. The cloning and characterization of the *APO(a)* gene proved difficult because the gene is large, highly repetitive, and strikingly similar to genes encoding other members of the plasminogen gene family. A breakthrough was made by Malgaretti et al. (1992) who isolated a yeast artificial chromosome (YAC) with a ~470 kb insert that included the entire plasminogen gene as well as the 5' region of the *APO(a)* gene. The *APO(a)* and plasminogen genes are oriented in a head-to-head fashion within 50 kb of each other and are flanked by *APO(a)*- and plasminogen- like sequences, neither of which have yet been fully characterized (Magnaghi et al., 1994) [Figure 1.3]. However, it has been shown recently that one of these pseudogenes, the apo(a)-related gene C is transcribed in human liver (Byrne et al., 1994) and that loss of a splice donor site at a skipped exon in this gene leads to the transcription of a mRNA that encodes a protein consisting of a single kringle domain (Byrne et al., 1995). A total of 1.4 kb of the 5' flanking region of the *APO(a)* gene was sequenced and there was a high degree of similarity with the corresponding region of the plasminogen gene within the first 712 base pairs.

The 5' flanking region was also characterized by Wade et al. (1993) who noted multiple interleukin-6 responsive elements. This is of potential interest since Lp(a) has been proposed as an acute phase reactant (Maeda et al., 1989). Consensus sequences of a number of hepatocyte transcription elements, hepatocyte nuclear factor 1α (HNF-1α), C/EBP, and LF-A1, were also identified but it has not yet been determined which, if any, of these elements are functionally important *in vivo*, although positive regulation of *APO(a)* gene transcription has recently been shown *in vitro* to be dependent on the binding of HNF-1α to a regulatory element situated downstream of the mRNA start site (Wade et al., 1994). In addition, it is not known whether all the sequences necessary for *APO(a)* transcription and regulation are contained within the sequenced regions.

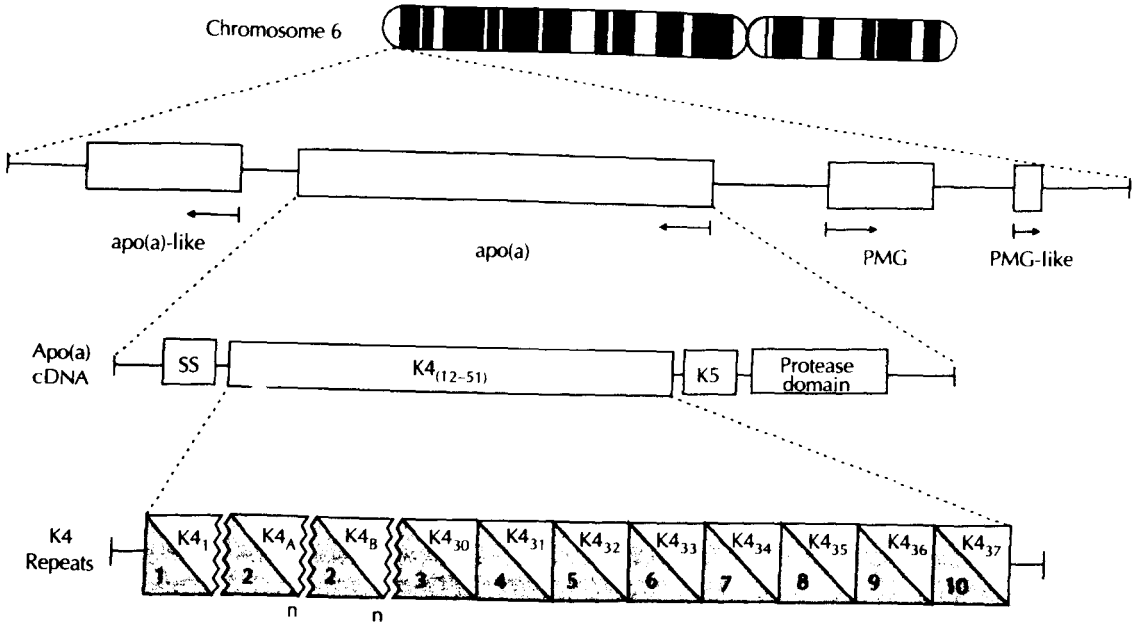


FIGURE 1.3.

Schematic of the *APO(a)* - plasminogen gene cluster and the kringle 4 (K4) region of the *APO(a)* gene. The *APO(a)* gene maps to the tip of the long arm of chromosome 6 (6q27), and is located within 50 kb of the plasminogen gene. The *APO(a)* gene has four structural domains: a signal sequence (SS), which is nearly identical to that of plasminogen; a K4 region, which contains 12 to 51 copies of the K4 repeat sequence; a single copy of kringle 5 (K5), which has alternatively been referred to as K11 and finally, the protease domain.

Two different nomenclatures have been used to designate the non-identical K4 repeats. The original numbering system put forth by McLean et al. (1987) or an alternative system in which the different types of K4 repeats are numbered consecutively (K1 - K10) (Morrisett et al., 1990). In the latter system, $K4_1$ is referred to as K1, the A and B repeats as K2 repeats, and so on. Reproduced from Gaw & Hobbs (1994).

In vitro studies using variable lengths of the 5' untranslated region of the *APO(a)* gene spliced to a luciferase reporter gene have revealed that only the portion of the *APO(a)* gene from position -98 to +130 is required to direct liver-specific transcription of the gene (Wade et al., 1994). Overexpression of HNF-1α in a HepG2 cell culture system resulted in significant stimulation of reporter gene transcription. This result suggests that positive transcriptional regulation of the *APO(a)* gene may be dependent on the binding of HNF-1α to a regulatory element situated downstream of the mRNA start site. Furthermore, Wade and his colleagues (1994) have hypothesised the existence of an as yet unidentified protein, which may down-regulate *APO(a)* transcription by binding to a discrete sequence within the 5' untranslated region.

However, the 5' untranslated regions of the apo(a) gene studied so far have relatively weak promoter activity and it remains to be seen whether the region identified by Wade and his colleagues (1993, 1994) is indeed the long sought after apo(a) promoter.

1.3 Metabolism

1.3.1 Synthesis of Lp(a)

Apo(a) is synthesized almost exclusively in the liver (Tomlinson et al., 1989) and over 95% of the apolipoprotein circulates in plasma coupled to lipoprotein particles (Gries et al., 1987). Metabolic studies suggest that Lp(a), unlike LDL, is not derived from a triglyceride-rich precursor particle (Krempler et al., 1979). However, lipoproteins that contain both apo(a) and apoE have been identified in both the VLDL and IDL density intervals using apoE immunoaffinity chromatography (Bard et al., 1992). If Lp(a) were initially secreted as part of VLDL it would be expected that apo(a) would accumulate in the VLDL or IDL fractions in individuals with lipoprotein lipase deficiency or Type III hyperlipoproteinaemia. Sandholzer et al. (1992a) have demonstrated that this is not the case.

Small amounts of apo(a) associate with triglyceride-rich particles, including chylomicron remnants and VLDL, after a high fat meal (Bersot et al., 1986; Pfaffinger et al. 1991; Cohn et al., 1991). The larger molecular weight forms of apo(a) appear to have a higher affinity for these particles (Pfaffinger et al., 1991). Neither the metabolic fate nor the pathological significance of these particles is known.

It is likely that apo(a) is secreted independently of apoB-100 and joins the LDL particle extracellularly. This hypothesis is supported by two lines of evidence. First, in a series of elegant biosynthetic studies of Lp(a) in cultured baboon hepatocytes White and her colleagues have demonstrated that there is no detectable intracellular apo(a)-apoB-100 complex (White et al., 1993), and that Lp(a) assembly can occur at the hepatocyte cell surface (White & Lanford, 1994). These workers found that newly synthesised apo(a) binds to the hepatocyte surface and from this location can be captured by apoB-containing lipoproteins to produce Lp(a). The second line of evidence comes from a series of important experiments conducted by Chiesa et al. (1992) who developed a mouse line expressing a human apo(a) transgene.

1.3.2 Development of an experimental animal model for Lp(a)

The elucidation of the metabolism of Lp(a) has been hindered by the lack of a convenient animal model. Plasma Lp(a) has a very unusual species distribution [see section 1.5.1] and none of the usual laboratory animals have circulating Lp(a). Therefore, major efforts have been made to develop a mouse model in which to study the metabolism, genetics, and atherogenicity of Lp(a).

Transgenic mice were recently engineered to express human apo(a) in plasma (Chiesa et al., 1992). In these mice, over 95% of the apo(a) circulated free of lipoproteins. An intact Lp(a) particle could be produced by infusion of human, but not mouse, LDL. From these studies it was clear that the development of a mouse expressing human Lp(a) would require the co-expression of human apo(a) together with human apoB-100. Recently, this goal has been achieved by crossing a mouse expressing human apoB with an apo(a) transgenic mouse (Linton et al., 1993; Callow et al., 1994). The effect of this cross on the migration of plasma apo(a) in a non-denaturing polyacrylamide gel is shown in Figure 1.4. In mice expressing only apo(a), the protein migrates rapidly, indicating that it is not attached to LDL. In the offspring that express both transgenes, the immunoreactive apo(a) is retarded, and travels at the same position as authentic human Lp(a). This result indicates that human apo(a) forms a stable complex with human apoB-100, but not mouse apoB-100.

These double transgenic mice are now being used for metabolic and atherogenicity studies (Callow et al., 1995) as well as possible pharmacological investigations into potential Lp(a) lowering drugs.

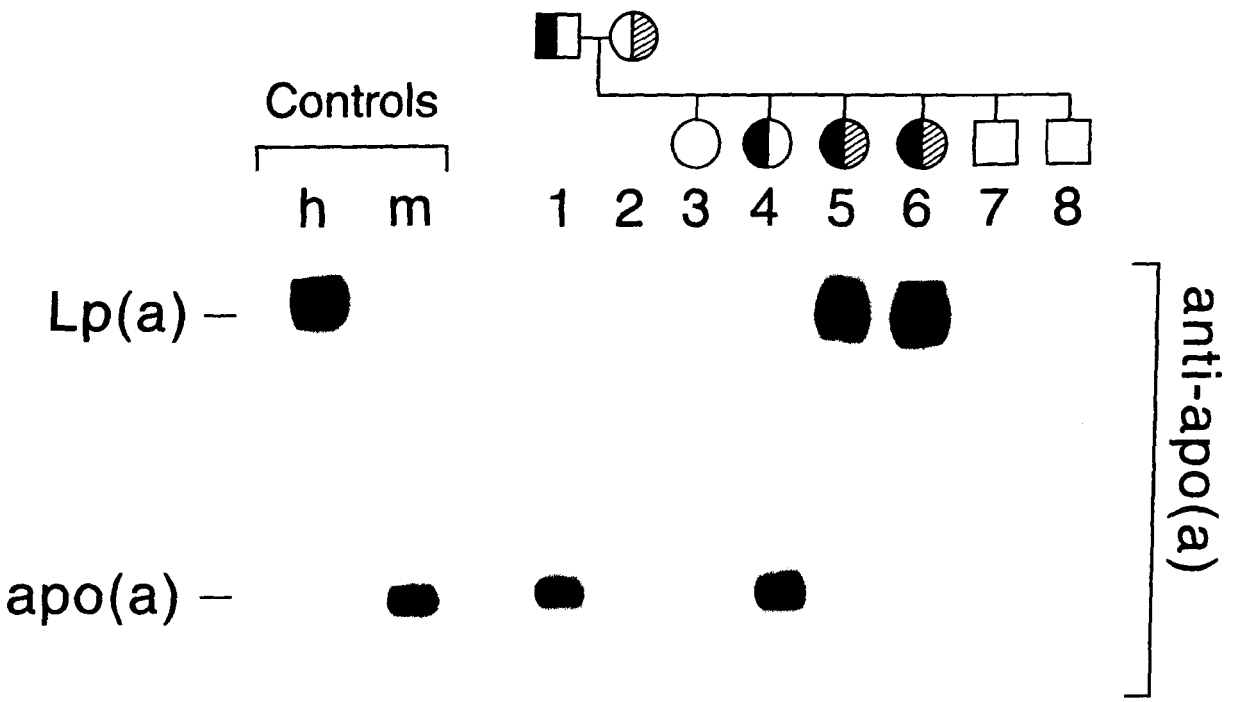


Figure 1.4.

Apo(a) distribution in plasma of mice expressing either or both human apo(a) and apoB transgenes. Plasma (1μl) from an apo(a) transgenic mouse (lane 1), an apoB transgenic mouse (lane 2), and their offspring (lanes 3-8) were loaded onto a 4% non-denaturing polyacrylamide gel. A total of 0.2 μl of human plasma (h) and 1 μl of apo(a) transgenic mouse plasma (m) were included on the gel as positive controls. After electrophoresis, immunoblotting was performed using an apo(a) specific monoclonal antibody $1A^2$, conjugated to horseradish peroxidase. This method has previously been shown to separate free apo(a) from lipoprotein bound apo(a). In the two offspring expressing both human apo(a) and apoB transgenes (lanes 5 and 6) the apo(a) migrated only a short distance into the gel, to the same level as human Lp(a), indicating the lipoprotein association of apo(a) in these mice. Modified from Linton et al (1993).

1.3.3 Clearance of Lp(a)

Lp(a) turnover studies suggest that the rate of Lp(a) production, rather than catabolism, is the major determinant of the plasma Lp(a) concentration (Krempler et al., 1983; Rader et al., 1993). The predominant mechanism by which Lp(a) is cleared from the circulation remains controversial. Lp(a) can bind to, and be internalized via the LDL receptor, as demonstrated by the rapid catabolism of Lp(a) in mice overexpressing the LDL receptor transgene (Hofmann et al., 1990). However, the physiological significance of this observation is questionable. *In vitro* studies of LDL receptor mediated binding and uptake of Lp(a) by cultured cells have produced conflicting results (Floren et al., 1981; Hofmann et al., 1990; Krempler et al., 1983; Armstrong et al., 1985); this may in part be due to genetic heterogeneity in the Lp(a) particles, variations in the preparation and purity of the Lp(a), interactions between Lp(a) and LDL (Kostner, 1993), and differences in the cell types examined.

In vivo studies suggest that the LDL receptor probably does not play a major role in the catabolism of plasma Lp(a). Pharmacological agents that significantly modify LDL receptor activity, such as bile acid sequestrant resins or HMG-CoA reductase inhibitors, have no appreciable effect on the plasma concentration of Lp(a) (Vessby et al., 1982; Thiery et al., 1988; Jürgens et al., 1989; Kostner et al., 1989; Wiklund et al., 1990). Hypothyroidism, which is associated with a decrease in LDL receptor activity (Salter et al., 1991) and elevation in plasma concentrations of LDL, is not associated with a significant change in plasma Lp(a) levels (Klausen et al., 1992a). Moreover, the fractional catabolic rate (FCR) of ^{125}I-labelled Lp(a) is no different from normal in individuals heterozygous for an LDL receptor mutation (Knight et al., 1991).

Utermann et al. (1989) reported that individuals with familial hypercholesterolaemia (FH) had higher plasma concentrations of Lp(a) than non-FH individuals. These findings were supported by the results of other studies (Wiklund et al., 1990; Leitersdorf et al., 1991; Mbewu et al., 1991). However, more recent FH family studies suggest that the previously observed association between FH and higher plasma levels of Lp(a) was an artifact due to selection bias, as previously suggested (Scanu 1991). There was no association between FH and plasma concentration of Lp(a) in three human family studies (Ghiselli et al., 1992; Soutar et al., 1991; Hegele et al., 1990), as well as in a simian pedigree with FH (Neven et al., 1990).

1.4 Factors affecting Lp(a) concentrations

When Lp(a) was initially identified in 1963, it was thought to be present in only ~35% of all individuals (Berg, 1963). Harvie & Schultz (1970) were the first to suggest that Lp(a) was a quantitative trait and present in the plasma of most, if not all, individuals. Albers & Hazzard (1974) subsequently developed an immunoassay, which demonstrated that plasma Lp(a) concentrations vary over a very wide range.

Plasma levels of Lp(a) are relatively low at birth and gradually increase to adult levels over the first few months of life (Van Biervliet et al., 1991, Wang et al., 1992). Levels are similar in men and women and do not increase with age (Sandkamp et al., 1990), as do plasma concentrations of LDL-cholesterol. The one exception is postmenopausal women who have a ~15-50% higher plasma concentration of Lp(a) than premenopausal women (Heinrich et al., 1991; Jenner et al., 1993).

Unlike plasma LDL-cholesterol levels, the plasma concentrations of Lp(a) are remarkably stable in any given individual and are not significantly related to body mass index (Sundell et al., 1989), changes in weight (Giavarina et al. 1992, Corsetti et al., 1991), or exercise (Lobo et al., 1992, Mankowitz et al., 1992). Plasma levels of Lp(a) are also unaffected by changes in dietary composition (Albers et al., 1977; Brown et al., 1991) with one major exception. Mensink et al. (1992) reported that plasma Lp(a) levels were raised when *trans* C18:1 replaced either oleic, linoleic, or stearic acids in the diet. Nestel et al. (1992) compared diets containing up to 2.4 or 6.7 en% as *trans* fatty acids and found that plasma total cholesterol, LDL-cholesterol, and Lp(a) concentrations were higher on the *trans* fatty acid diets. In addition, heavy alcohol ingestion, especially if accompanied by alcoholic liver disease, lowers plasma Lp(a) levels (Marth et al., 1992).

Plasma Lp(a) concentrations do not correlate with levels of other lipoproteins (Boyer et al., 1990) or with indirect indicators of whole body cholesterol synthesis (Boomsma et al., 1993). Approximately one-third of the mass of the Lp(a) particle is cholesteryl-ester, so only very high plasma concentrations of Lp(a) significantly affect either the plasma level of total or LDL-cholesterol.

It has been suggested that Lp(a) is an acute phase reactant (Maeda et al., 1989). Lp(a) has been reported to increase dramatically in concentration after

Table 1.2 Published healthy adult plasma or serum Lp(a) levels in different ethnic groups.

Ethnic group	n	Lp(a) Mean (SD) (mg. dL^{-1})	Lp(a) Median (mg. dL^{-1})	Method	Reference
CAUCASIAN					
American	242	16.4	-	ELISA	Haffner et al. 1992a
American	134	16.3	-	EIA	Guyton et al. 1985
Tyrolean	279	14.1 (19.4)	-	EID	Sandholzer et al. 1991
Icelandic	184	13.5 (17.7)	-	EID	Sandholzer et al. 1991
Hungarian	202	8.3 (11.0)	-	EID	Sandholzer et al. 1991
Belgian	60	16.0	8.0	ELISA	Cobbaert+Kesteloot 1991
French	81	10.7 (15.2)	7.0	EIA	Parra et al. 1987
German	166	18.7 (23.1)	8.5	ELISA	Helmhold et al. 1991
AFRICAN					
African-Amer.	105	32.5	-	EIA	Guyton et al. 1985
Sudanese	105	45.7 (25.9)	-	EID	Sandholzer et al. 1991
Nigerian	60	17.8	13.4	ELISA	Cobbaert+Kesteloot 1991
Congoese	81	23.9 (20.2)	20.8	EIA	Parra et al. 1987
Seychellois	12,110	31.9 (36.2)	-	RIA	Bovet et al. 1991
Ghanaian	190	36.2 (31.5)	26.0	ELISA	Helmhold et al. 1991
San	67	21.1 (19.3)	15.2	ELISA	Helmhold et al. 1991
ASIAN					
Chinese (Sing)	112	7.2 (13.1)	-	EID	Sandholzer et al. 1991
Chinese	60	14.2	8.9	ELISA	Cobbaert+Kesteloot 1991
Chinese	88	22.9 (18.3)	15.3	ELISA	Helmhold et al. 1991
Korean	60	12.1	9.9	ELISA	Cobbaert+Kesteloot 1991
Korean	250	14.9	12.0	ELISA	Kim et al. 1992
Indian	143	20.1 (15.9)	-	EID	Sandholzer et al. 1991
Malay/Singap.	125	12.9 (17.9)	-	EID	Sandholzer et al. 1991
Japanese	34	13.1 (11.6)	-	ELISA	Yamasaki et al. 1992
Tibetan	60	9.2	4.9	ELISA	Cobbaert+Kesteloot 1991

EID, electroimmunodiffusion; EIA, electroimmunoassay; ELISA, enzyme linked immunosorbent assay; RIA, radioimmunoassay.

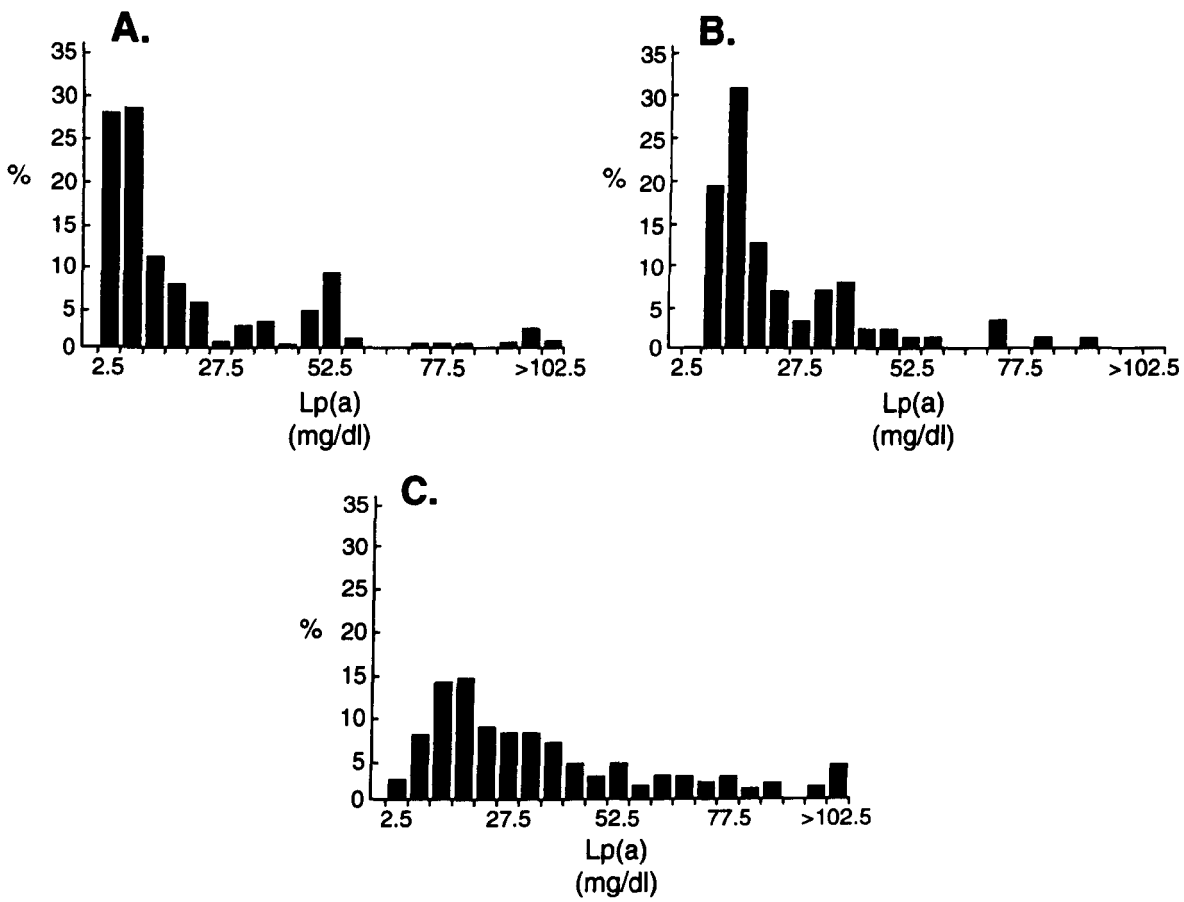


FIGURE 1.5.

The distribution of plasma Lp(a) concentrations in (A) Germans (B) Chinese and (C) Ghanaians. Adapted from Helmhold et al. (1991).

myocardial infarction and surgical operations, though no large controlled longitudinal studies have been performed to confirm this report.

1.4.1 Racial differences in plasma Lp(a) concentrations

In all populations studied to date, the plasma levels of Lp(a) vary over a wide range, from <0.1 mg. dL^{-1} to >100 mg. dL^{-1} (total Lp(a) mass). There are important racial differences in the distribution of plasma Lp(a) concentrations [Figure 1.5, Table 1.2]. In Caucasians the distribution of plasma Lp(a) concentrations are highly skewed towards lower levels (Sandholzer et al., 1991, Guyton et al., 1985), and greater than 65% of Caucasians have levels less than 20 mg. dL^{-1}. In African populations, the distribution of plasma Lp(a) levels tends to be more Gaussian (Guyton et al., 1985, Helmhold et al., 1991, Parra et al., 1987, Sandholzer et al., 1991, Bovet et al., 1991).

On average, individuals of African descent have an Lp(a) level that is 2-3 fold higher than Caucasians. In African-Americans there is some skewing in the distribution of plasma Lp(a) towards lower levels, which is most likely due to genetic admixture with Caucasians (Guyton et al., 1985; Chakraborty et al., 1992).

The distributions of plasma Lp(a) levels in Asian populations are very similar to those of Caucasians (Yamasaki et al., 1992, Cobbaert & Kesteloot, 1992; Kim et al., 1992), though two studies reported significantly lower plasma Lp(a) levels in Chinese (Sandholzer et al., 1991; Cobbaert & Kesteloot, 1992). These conflicting results may be due to regional ethnic variations within the Asian population or to methodological differences in the handling of the blood samples prior to analysis.

1.4.2 Plasma Lp(a) levels and renal disease

Chronic renal failure is associated with significant elevations in plasma Lp(a) levels. It is not clear how much of the elevation is due to renal insufficiency *per se* or to the dialysis procedure (Haffner et al., 1992b; Irish et al., 1992; Dieplinger et al., 1993). Plasma Lp(a) levels plunge over 50% within one week of renal transplantation (Black & Wilcken, 1992) presumably due to the associated improvement in renal function. However, plasma Lp(a) levels also fall after cardiac transplant (despite coincident elevations of LDL-cholesterol and HDL-cholesterol), suggesting that the administration of immunosuppressive agents could contribute to the reduction in plasma Lp(a) concentrations seen after renal transplantation (Farmer et al., 1991).

Plasma Lp(a) levels also tend to be elevated in patients with proteinuria (Black & Wilcken, 1992; Karádi et al., 1989). In nephrotic syndrome, the increase in plasma Lp(a) levels does not correlate with the levels of the other apoB-containing lipoproteins. In a small sample of patients with minimal change disease, there was a direct relationship between the plasma Lp(a) levels and the amount of protein excreted in the urine. Conversely, there was an inverse correlation between the plasma Lp(a) and the levels of proteinuria in the patients with membranoproliferative disease (Karádi et al., 1989). Unfortunately, the medications taken by the study patients were not reported, so the observed differences in plasma Lp(a) levels may be pharmacological effects.

1.4.3 Plasma Lp(a) levels and diabetes mellitus

Initial reports found plasma Lp(a) levels elevated in poorly controlled diabetics (Bruckert et al., 1990; Haffner et al., 1991; Levitsky et al., 1991; Joven & Vilella, 1991). Subsequent studies have not consistently demonstrated elevations in plasma Lp(a) levels in either Type I or Type II diabetes, in the absence of renal disease (Velho et al., 1993; Heller et al., 1993; Klausen et al., 1992b).

1.5 Evolution and genetics

1.5.1 Species distribution of Lp(a)

Lp(a) has an unusual species distribution. It is a major cholesterol-carrying lipoprotein of the European hedgehog (a hibernating insectivore) (Laplaud et al., 1988), yet it has not been found in any other mammals except old world monkeys (Azrolan et al., 1991) and great apes (Makino et al., 1989). The hedgehog *APO(a)* gene has not yet been cloned and characterized, but partial characterization of the 3' region of a rhesus monkey apo(a) cDNA revealed a structure very similar to that of the human, though the rhesus gene does not contain the K5 repeat (Tomlinson et al., 1989).

1.5.2 Evolution of the *APO(a)* gene

The *APO(a)* gene is located within 50 kb of the plasminogen gene and almost certainly evolved as the result of gene duplication followed by a subsequent deletional event and intragenic amplification. It is perplexing that the *APO(a)* gene is not present continuously in the evolutionary tree.

Presumably the gene has either evolved twice or has been regained after being deleted from the genome.

Sequence comparison between the human apo(a) and plasminogen cDNA in the 3' untranslated region suggests the two human genes diverged around the same time that the old and new world monkeys diverged: ~40 million years ago (McLean et al., 1987). However, Pesole and colleagues (1994) have more recently estimated the separation of the two genes by duplication to date back to ~90 million years ago, immediately before the radiation of the mammals. The strikingly high degree of sequence identity that has been maintained within regions of the *APO(a)* and plasminogen genes, especially at the 5' end, is likely due to frequent gene conversion events between the two closely linked loci.

Apo(a) is a member of a superfamily of plasma proteins and shares numerous protein motifs with proteins of the coagulation cascade, fibrinolytic system, as well as selected growth factors (Lawn, 1992). These shared sequence motifs have fueled much speculation on the physiological role of Lp(a) including a suggestion that it may play a role in wound repair by delivering cholesterol to regenerating tissues (Brown & Goldstein, 1987).

1.5.3 Human genetics of plasma Lp(a) concentration

Using qualitative assays, Lp(a) was shown to be an inherited trait with an apparent autosomal dominant inheritance pattern (Schultz et al., 1968; Heiberg & Berg, 1974; Berg & Mohr, 1963). When more sensitive assays were developed, it became clear that Lp(a) was a quantitative rather than qualitative trait (Schultz et al., 1974; Albers et al., 1974). Early family studies examining the segregation of plasma Lp(a) suggested a limited number of genes contributed to its plasma level (Sing et al., 1974; Morton et al., 1985; Hasstedt et al., 1983; Hewitt et al., 1982; Iselius et al., 1981). Qualitative analysis of plasma Lp(a) levels in twins revealed a strikingly high heritability index (0.98) (Hewitt et al., 1977). Later, quantitative analysis of plasma Lp(a) concentrations confirmed the high concordance of levels in identical twins (Austin et al., 1992; Boomsma et al., 1993).

The observation that the plasma level of Lp(a) tends to be inversely related to the size of the apo(a) isoforms (Utermann et al., 1987) thrust the *APO(a)* gene to the forefront as a potential major candidate gene for controlling plasma Lp(a) concentrations. Subsequently, plasma Lp(a) levels were shown

to co-segregate with a sequence polymorphism closely linked to the *APO(a)* gene, which further implicated the *APO(a)* locus as a major determinant of plasma Lp(a) concentrations (Drayna et al., 1988).

1.5.4 Apo(a) isoform determination

Various immunoblotting techniques have been developed to analyze the size of the apo(a) isoforms. SDS-polyacrylamide and agarose gel electrophoresis have both been used to size-fractionate reduced plasma proteins prior to immunoblotting with an apo(a)-specific antibody (Utermann et al., 1987; Kamboh et al., 1991). Initially, six apo(a) isoforms that ranged in molecular mass from 400-700 kDa were identified and classified according to their mobility relative to apoB-100 (~513 kDa): F (fast), B (migration similar to apoB-100), and S1-S4 (progressively slower migration than apoB-100) (Utermann et al., 1987). Approximately 49% of individuals had no identifiable apo(a) using this method and in only a minority of subjects could two apo(a) isoforms be detected (Utermann et al., 1987). With improved techniques, apo(a) was identified in almost every individual, but the isoforms were still not in Hardy-Weinberg equilibrium, which suggests that not all *APO(a)* gene products were detected equally (Gaubatz et al., 1991).

There are several technical problems associated with analyzing such a large glycoprotein by immunoblotting. First, the ability to size-fractionate large glycoproteins is limited using SDS-PAGE gels. Kamboh et al. (1991) reported greatly improved resolving capacity by using agarose gel electrophoresis for size fractionation. Optimization of the size-fractionation and immunoblotting procedures has resulted in the resolution of 34 isoforms ranging in size from 300 to 850 kDa (Lackner et al., 1993; Marcovina et al., 1993b) [Figure 1.6]. Second, isoforms associated with low levels of apo(a) were often not detected, especially in heterozygous individuals expressing different amounts of each isoform. Finally, isoforms of higher molecular weight do not transfer as well during immunoblotting and so may go undetected.

1.5.5 Apo(a) isoform size polymorphism and plasma concentration of Lp(a)

As previously noted, the apparent molecular mass of apo(a) isoforms tends to be inversely related to the plasma concentration of Lp(a) (Utermann et al., 1987) [Figure 1.7]. It has been estimated that approximately 42% of the

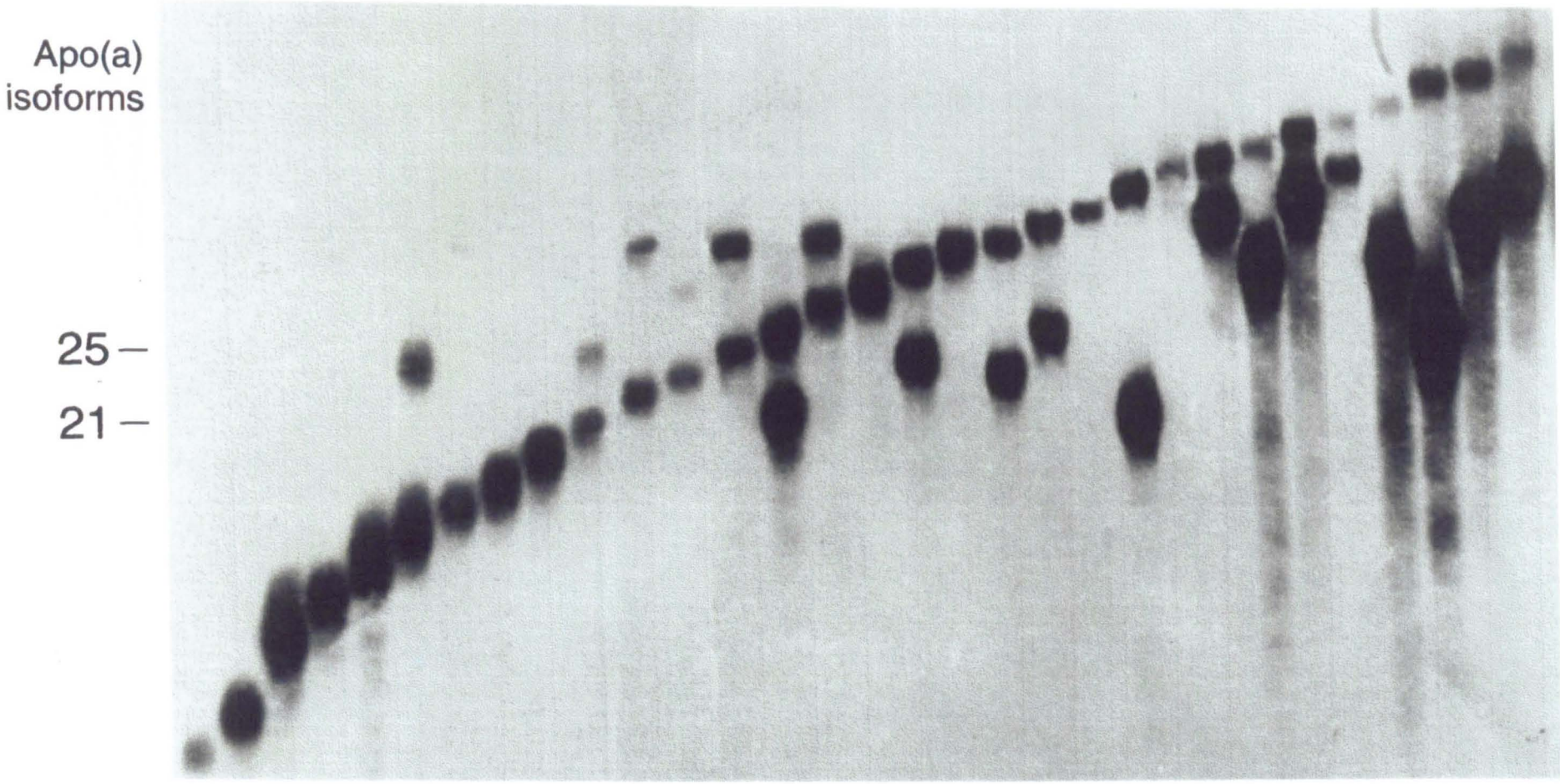


FIGURE 1.6.

Immunoblot analysis of apo(a) isoforms in human plasma. A total of 7 - 15 µl of plasma from each individual was subjected to electrophoresis on a horizontal 2% agarose gel. The plasma proteins were blotted onto a supported nitrocellulose membrane and immunoblotted with an apo(a) specific monoclonal antibody. The number of K4 repeats for each *APO(a)* allele is given. Reproduced from Lackner et al.(1993).

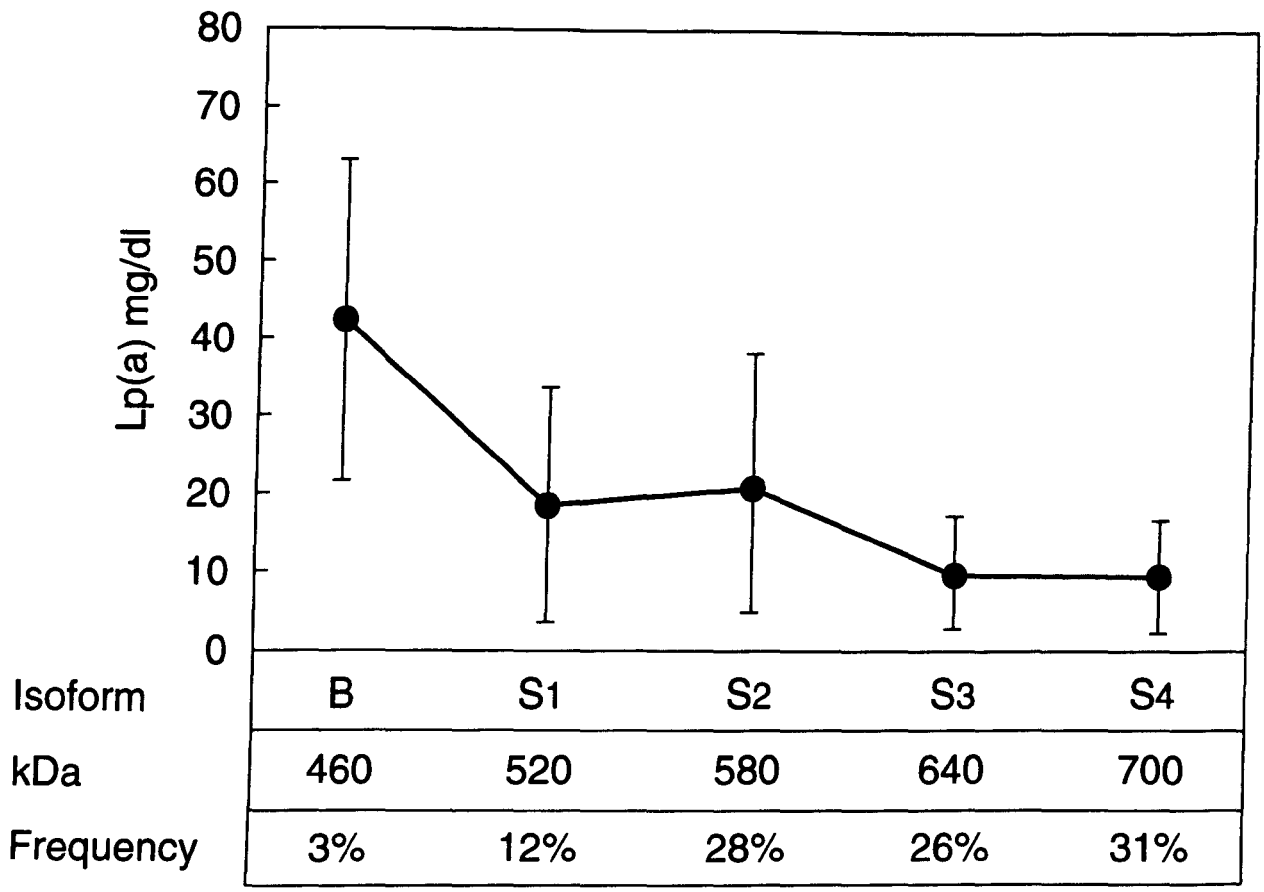


FIGURE 1.7.

Relationship between apo(a) isoform size and plasma concentration of Lp(a). Data from Utermann (1989).

variability in plasma Lp(a) level between unrelated individuals is attributable to the size polymorphism in the apo(a) protein (Boerwinkle et al., 1989; Gaubatz et al., 1990). The frequency distribution of isoforms in part accounts for the skewed distribution of plasma Lp(a) concentrations in the Caucasian population; the larger isoforms (S1-S4), which tend to be associated with lower plasma levels of Lp(a), are most common in Caucasian populations, whereas, the smaller isoforms (F and B), which are associated with higher plasma concentrations of Lp(a), are less common (Utermann et al., 1988b).

The mechanism responsible for this relationship is still not known, although there is some evidence that it is not due to differential catabolism. Apo(a) isoforms of varying size are apparently catabolized at similar rates (Rader et al., 1993). Nor can the relationship be completely explained by differences in hepatic mRNA levels. In primates, the amount of hepatic apo(a) mRNA tends to correlate with plasma concentration of Lp(a), but a number of exceptions have been described (Azrolan et al., 1991; Hixson et al., 1989). Interestingly, no correlation was found between apo(a) mRNA length and hepatic apo(a) mRNA levels. This suggests that factors other than the *APO(a)* gene size affect apo(a) mRNA levels (Azrolan et al., 1991; Hixson et al., 1989).

There are important exceptions to this relationship between apo(a) isoform size and plasma concentration of Lp(a). For example, the frequency distribution of apo(a) isoforms is not dramatically different when Caucasians are compared to Africans, despite the fact that Africans have higher plasma concentrations of Lp(a) (Sandholzer et al., 1991). Also, within populations, individuals with apo(a) isoforms of the same size (whether large or small) can have very different plasma levels of Lp(a) (Boerwinkle et al., 1989; Lackner et al., 1991).

1.5.6 Molecular analysis of apo(a) polymorphism

The molecular mechanism responsible for the size variation in apo(a) was first suggested by the structure of the apo(a) cDNA. McLean et al. (1987) hypothesized that the variations in the size of the apo(a) protein were due to differences in the numbers of K4 repeats in the *APO(a)* gene. In support of this suggestion, the size of the hepatic apo(a) mRNA was shown to correlate with the size of the apo(a) isoforms in rhesus monkeys, baboons, and man (Hixson et al., 1989; Azrolan et al., 1991; Koschinsky et al., 1990).

Direct proof that the apo(a) size variation was due to differences in the number of K4 repeats awaited the development of a method to analyze the *APO(a)* gene directly. Lackner et al. (1991, 1993) demonstrated that a *Kpn*I or *Hpa*I restriction fragment containing the common (i.e., Type A & B) K4 repeats varied in size between alleles. Size fractionation of the restriction fragments by pulsed-field gel electrophoresis and Southern blotting revealed that their size correlated directly with the size of the apo(a) isoforms. Thus, the major size variation in apo(a) isoforms was shown to be due to variations in the number of K4 repeats in the *APO(a)* gene and not to postranslational modifications. To date, 34 fragments of different sizes have been resolved using these methods (Lackner et al., 1993) [Figure 1.8]. Strikingly, 94% of individuals examined were heterozygous and thus had two *APO(a)* alleles of different sizes. In contrast to the observed distribution of apo(a) isoforms, the *APO(a)* allele frequencies, as determined by pulsed-field gel electrophoresis, were in Hardy-Weinberg equilibrium.

Individuals with *APO(a)* alleles of identical size can have very disparate plasma Lp(a) levels (Lackner et al., 1991). Family studies have shown that these differences are due to sequence differences linked to the *APO(a)* gene, other than the number of K4 repeats, and not to the effect of other genes or environmental factors (Boerwinkle et al., 1992).

1.5.7 Molecular mechanisms and implication of *APO(a)* polymorphism.

The *APO(a)* gene is amongst the most highly polymorphic loci yet described in the human genome outside the histocompatability complex. The high degree of length polymorphism in the *APO(a)* gene can be attributed to homologous recombination and unequal exchange. There is evidence that both inter- and intra-chromosomal exchanges occur frequently within the Type A and B kringle repeats (Cohen et al., 1993; Lackner et al., 1993). Only a single spontaneously generated *APO(a)* allele of new length has been identified and characterized at a molecular level thus far. The analysis was consistent with the allele being the result of sister chromatid exchange during mitosis, or a gene conversion event during meiosis (Lackner et al., 1993).

Not only are there many *APO(a)* alleles of different length, but it is now clear that *APO(a)* alleles of the same size do not necessarily have the same

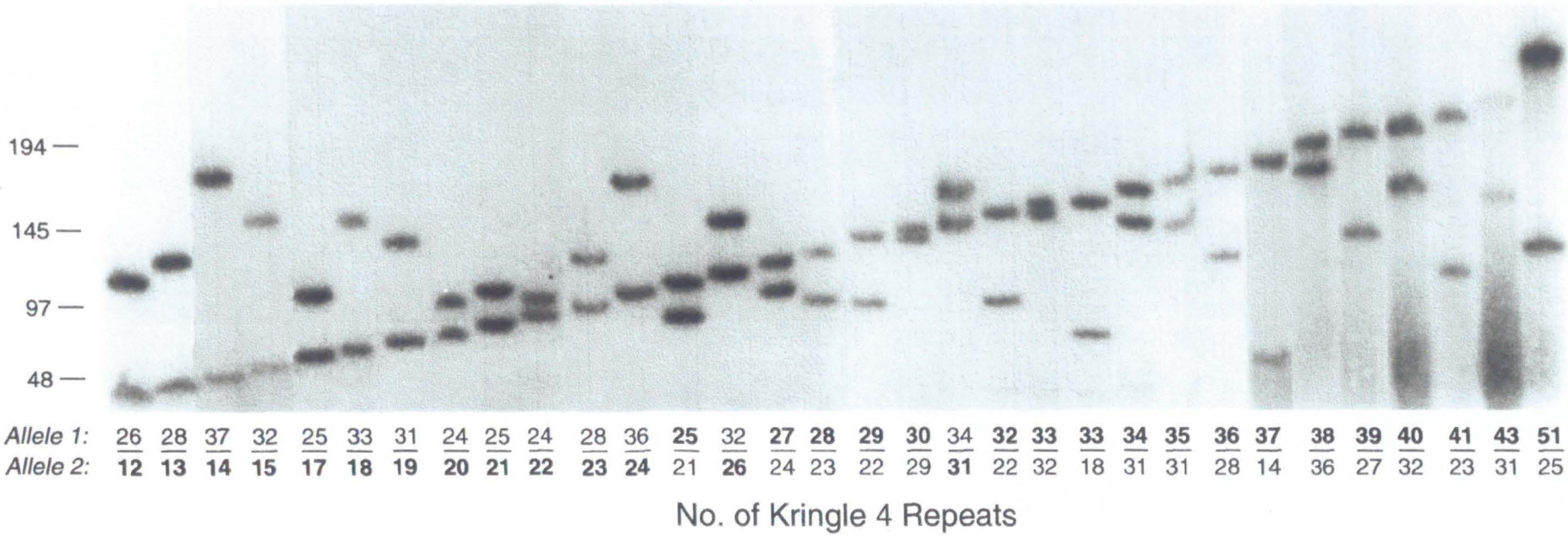


FIGURE 1.8.

Southern blot analysis of *Hpa*I-digested genomic DNA size-fractionated by pulsed-field gel electrophoresis. High molecular weight genomic DNA (1-2 µg) was digested with *Hpa*I and size-fractionated using pulsed-field electrophoresis. After transfer to a nylon membrane by Southern blotting, the filter was hybridized with a K4-*APO(a)*-specific probe. The *Hpa*I sites that define this fragment are located immediately 5' of the first exon of $K4_1$ and in the intron of $K4_{31}$. The fragment sizes vary between 28 to 250 kilobases depending on the total number of K4-encoding sequences in the *APO(a)* allele which have been estimated to range between 12 and 51 (*APO(a)K12-APO(a)K51*). Adapted from Lackner et al. (1993).

sequence (Cohen et al., 1993). Based on the analysis of both *APO(a)* gene structure and sequence, there are >100 identifiable different *APO(a)* alleles in the normal populations. This dramatically high degree of both sequence and length polymorphism in the *APO(a)* gene begs the question of whether the physiological role of apo(a) is vestigial [section 1.10].

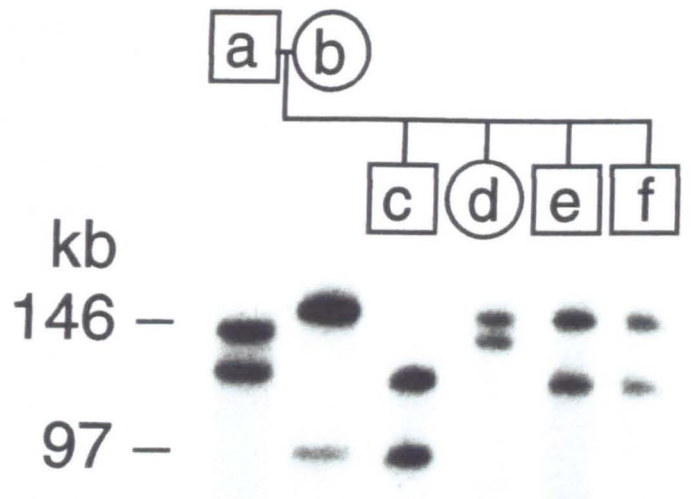
1.5.8 The *APO(a)* gene is a major determinant of plasma Lp(a) levels

The high degree of size polymorphism of the *APO(a)* gene allowed detailed segregation analysis of this gene in families [Figure 1.9]. If the *APO(a)* gene is an important determinant of plasma Lp(a) levels, then siblings who inherit the same *APO(a)* alleles from their parents would be expected to have similar plasma concentrations of Lp(a). Conversely, if genes other than the *APO(a)* gene significantly affect plasma Lp(a) levels, it would be expected that the levels of Lp(a) would vary between sibling pairs with identical *APO(a)*alleles. The degree of similarity in plasma Lp(a) levels in sibling pairs with identical *APO(a)* alleles (and only a 25% chance of having identical genes at any other locus) can be used to estimate the contribution of the *APO(a)* gene to plasma levels of Lp(a). Siblings who inherited the same *APO(a)* alleles from each parent had very similar plasma concentrations of Lp(a) (r=0.95) which was in dramatic contrast to siblings who inherited no *APO(a)* alleles in common (r =-0.23) (Boerwinkle et al., 1992). From this analysis, it was estimated that >90% of the inter-individual variation in plasma Lp(a) can be attributed to sequence differences at, or closely linked to the *APO(a)* locus (Boerwinkle et al., 1992).

The exact sequences at the *APO(a)* locus that determine the plasma concentration of Lp(a) have not been delineated. It is paradoxical that a single gene can predominate in the control of this widely varying quantitative trait. It is likely, given the polymorphic nature of the *APO(a)* gene, and the wide range in plasma concentrations of Lp(a), that the plasma Lp(a) levels are determined by a complex interaction of multiple sequence differences at the *APO(a)* locus.

1.6 Association of Lp(a) levels & atherosclerosis

The first paper to report an association between the presence of Lp(a) in plasma with coronary heart disease (CHD) was made shortly after the discovery of Lp(a) (Renninger et al., 1965). Later, Kostner et al. (1981) compared plasma levels of Lp(a) in 36 normolipaemic men (ranging in age from 40-60



Apo(a) Genotype	30/26	33/21	26/21	33/30	33/26	33/26
Lp(a) (mg/dl)	<1	32	28	<1	1	1

FIGURE 1.9.

Segregation of *APO(a)* alleles and Lp(a) plasma concentrations in a family. High molecular weight genomic DNA was digested with *Kpn*I and size-fractionated by pulsed-field gel electrophoresis as described (Lackner et al., 1993). The circles and squares denote females and males, respectively. The first two lanes of the autoradiogram show results of the analysis of the parents, and the subsequent four lanes demonstrate the Mendelian inheritance of the *Kpn*I restriction fragments to the offspring. Below the blot are the apo(a) genotypes and plasma Lp(a) concentrations. The latter are the mean of two separate determinations on different plasma samples by an ELISA assay. Adapted from Lackner et al. (1991).

years) with a history of a myocardial infarction to 55 age- and sex-matched controls, and found that a higher percentage of patients than controls had an Lp(a) level greater than 30 mg. dL^{-1} (44% vs. 26%). These early findings suggest that the risk conferred by high plasma levels of Lp(a) was independent of other lipoprotein-related risk factors. Numerous subsequent reports have confirmed the results of these earlier studies.

1.6.1 Epidemiology of Lp(a) and coronary atherosclerosis

Elevations of plasma levels of Lp(a) (over 20-30 mg. dL^{-1} or 0.07-0.1 μmol. L^{-1}) have been estimated to confer a 1.5- to 3- fold increased risk for the presence of coronary atherosclerosis in both Caucasian and Oriental men (Albers et al., 1977; Armstrong et al., 1986; Sandholzer et al., 1992b; Rhoads et al., 1986). This association is independent of the contribution of other plasma lipoprotein concentrations to coronary risk (Sandkamp et al., 1990). Moreover, plasma levels of Lp(a), together with a history of prior atherosclerotic events, were found to be the best predictors of subsequent vascular events (Cressman et al., 1992) in chronic haemodialysis patients.

The association between elevations in plasma Lp(a) and the presence of ischaemic heart disease is most robust in younger age groups. Rhoads et al. (1986) compared plasma Lp(a) concentrations in 303 Japanese-Hawaiian men with a history of myocardial infarction to those in 408 population-based controls, and found the odds ratio of having had a myocardial infarction was 2.5-fold higher in those men under age 60 who had a plasma Lp(a) level in the top quartile. In this study, the population attributable risk of having an MI, if the Lp(a) was in the top quartile, was 28% for men under 60 years, whereas it was only 5% for men older than 70.

1.6.2 Lp(a) and severity of CHD

Not only are high plasma concentrations of Lp(a) associated with the presence of CHD, but they are also directly related to its severity. This effect was found to be independent of the plasma concentration of LDL-cholesterol. Dahlén et al. (1986) evaluated 307 Caucasians and found a direct relationship between the level of plasma Lp(a) and the severity of CHD, as assessed by coronary angiography. Similarly, Budde and his colleagues (1994) studied 118 male patients undergoing coronary angiography and found that plasma Lp(a) levels correlated with number, severity and length-extension of atheromatous lesions. In women and men aged less than 55, the same direct relationship between plasma concentration of Lp(a) and severity of CHD held true;

however, the relationship was not observed in males over the age of 55. These findings again indicate that plasma Lp(a) levels are a better predictor of CHD in younger men.

1.6.3 Lp(a) and restenosis

Elevated plasma Lp(a) levels have been reported to be associated with an increased incidence of re-stenosis after coronary artery bypass surgery (Hoff et al., 1988) and percutaneous transluminal coronary angioplasty (PTCA) (Hearn et al., 1992). However, Johansson et al. (1992) found no relationship between restenosis after PTCA and plasma Lp(a) concentrations. A prospective study in which all subjects, irrespective of clinical outcome, are re-evaluated by angiography is required to determine if a high plasma level of Lp(a) is indeed a risk factor for restenosis.

1.6.4 Interaction of elevated plasma Lp(a) and LDL concentrations

Although an isolated increase in plasma Lp(a) concentration has been associated with an increase in risk of myocardial infarction (Kostner et al., 1981), it appears that this risk is compounded when coupled with elevated levels of LDL-cholesterol. Armstrong et al. (1986) measured plasma Lp(a) levels in a sample of 428 Caucasian men (age 40-60) with CHD (at least one major coronary vessel occluded >50%) documented by angiography as well as in 142 individuals who had no evidence of CHD. Individuals who had plasma Lp(a) levels over 30 mg. dL^{-1} had an odds ratio of having CHD of 2.7 when compared to those with an Lp(a) less than 5 mg. dL^{-1}. The odds ratio was not modified by other CHD risk factors, such as smoking, hypertension, or decreased plasma levels of HDL-cholesterol. Only plasma concentration of LDL-cholesterol significantly affected the odds ratio. In individuals with plasma Lp(a) concentrations >30 mg. dL^{-1}, and LDL-cholesterol levels greater than the median, the odds ratio rose from 2.7 to 6.3.

These results were supported by three studies that found that FH heterozygotes with CHD have significantly higher plasma Lp(a) levels than those without clinical evidence of CHD (Houlston et al., 1988; Seed et al., 1990; Wiklund et al., 1990). However, in one study in which there were age- and sex-matched controls, no significant relationship was found between the plasma Lp(a) levels and the presence or absence of CHD in FH heterozygotes

(Mbewu et al., 1991). Therefore, at this time it is debatable whether elevated levels of plasma Lp(a) are a prognostic indicator of ischaemic heart disease in patients with FH.

1.6.5 Lp(a) and family history of CHD

There is an association between the plasma level of Lp(a) and a parental history of CHD (Hoefler et al., 1988; Kostner & Grillhofer, 1991, and Durrington et al., 1988). Durrington and his colleagues (1988) compared 41 men who had suffered a myocardial infarction to 78 controls and found the best predictors of CHD were plasma concentrations of 1) apoB-100, 2) apoAI and 3) the presence of a positive parental history of myocardial infarction. The plasma Lp(a) level could be substituted for the parental history of myocardial infarction as a risk factor; no additional benefit was derived from knowledge of a parental history of myocardial infarctions if the plasma Lp(a) level was known. The results of their study are again consistent with the highly heritable nature of plasma Lp(a) levels.

1.6.6 Prospective studies of CHD and Lp(a)

Several prospective studies have examined the effect of plasma Lp(a) on the incidence of coronary events [Table 1.3]. In an early uncontrolled study, plasma Lp(a) levels were measured in 232 Swedish males (age 50-53) with no overt atherosclerotic disease. The patients were followed for 11 years, and there was a 2-3 fold higher incidence of myocardial infarction and stroke in those with high plasma concentrations of Lp(a) (Dahlén et al., 1991). A larger prospective case-control study of middle aged men (Rosengren et al., 1990) also revealed an association between elevated Lp(a) levels and coronary events.

Two other major prospective studies, however, failed to find an association between high plasma levels of Lp(a) and coronary events: the Helsinki Heart Study and the US Physicians' Health Study. In a subgroup analysis of the Helsinki Heart Study, 138 individuals who had suffered a myocardial infarction during the study were compared with 130 controls (Jauhiainen et al., 1991). There was no significant difference in the distribution of plasma Lp(a) concentrations in these two groups. The discrepant findings of this study remain to be explained, but it does not appear to be due to survival bias (i.e., the lack of association of cardiac events with plasma Lp(a) concentration is not explained by a bias incurred if individuals with high Lp(a) died before or during the course of the study and were therefore not represented in the analysis).

Table 1.3. Summary of controlled prospective studies of Lp(a) and CHD.

Study	CHD positive participants	Controls	Lp(a) association with CHD	Relative to lipids
Rosengren et al. (1990)	26 M*	109 M	Higher in affected participants P=0.01	Independent of TC
Jauhiainen et al. (1991)	138 M	130 M	No difference P=0.37	LDL also not different
Coleman et al. (1992)	51 F	201 F	Higher in affected participants P=0.15	No better than TC
Siggurdson et al. (1992)	104 M	1228 M	Higher in affected participants P<0.05	Independent of TC & apoB
Ridker et al. (1993)	296 M	296 M	No difference P=0.88	TC also not significantly different
Wald et al. (1994)	229 M	1145 M	Higher in affected participants P<0.05	Independent of TC
Schaefer et al. (1994)	233 M	390 M	Higher in affected participants P<0.02	Independent of LDL
Cremer et al. (1994)	107 M	5364 M	Higher in affected participants P<0.001	Lp(a) 5th most important risk factor after LDL-C, family history of MI, plasma fibrinogen, and HDL-C.

*M, male; F, female; TC, plasma total cholesterol concentration
Adapted from Rader et al. (1994).

The Helsinki Heart Study is not the only Finnish report that failed to demonstrate a relationship between plasma Lp(a) levels and CHD. When plasma Lp(a) levels in Finnish individuals with and without angiographic evidence of CHD were compared, no significant difference was found (Nieminen et al., 1992).

Another population in which plasma Lp(a) levels have not been shown to be a risk factor for CHD are individuals of African descent. On average, African-Americans have two-fold higher plasma concentrations than do Caucasians (Utermann, 1989; Guyton et al., 1985), and yet appear not to have a corresponding increase in incidence of CHD (National Center for Health Statistics, 1990; Keil et al., 1993). Sorrentino et al. (1992) found no significant difference in plasma concentration of Lp(a) in African-Americans with and without CHD, as documented by coronary angiography. Moreover, plasma levels of Lp(a) are good predictors of parental CHD in Caucasians, but not in African-American children (Srinivasan et al., 1991).

The lack of association between high plasma concentrations of Lp(a) and CHD in both Finns and African-Americans needs to be verified with additional studies. If confirmed, elucidation of the reason for this difference should provide insights into the mechanism responsible for the atherosclerotic effects of Lp(a). In the African-American population there may be apo(a) alleles of different sequence, which generate high plasma concentrations of Lp(a), but do not contain the epitope responsible for its atherogenicity. Alternatively, there may be another ethnic-specific factor(s) that protects African-Americans from the atherogenic effects of Lp(a).

The second major prospective case-control study that failed to find any evidence of association between plasma Lp(a) concentration and risk of future myocardial infarction was the US Physicians' Health Study (Ridker et al., 1993). While this was the largest prospective study of its kind to date, it has been the subject of criticism (Gaw, 1994).

Neither the racial breakdown nor the age range of the nested study group was reported. This is important because, as stated above [section 1.4.1], it has been well recognized that ethnic origin plays a significant role in the determination of plasma Lp(a) concentrations (Helmhold et al., 1991). The age distribution of the groups would not necessarily have affected the Lp(a) levels but may have significantly influenced the observed association between plasma

Lp(a) concentration and risk of myocardial infarction. Previous studies have reported that the association between plasma Lp(a) levels and risk of myocardial infarction is stronger in younger individuals (Rhoads et al., 1986). Plasma LDL-cholesterol levels of cases and controls were omitted from the paper and in view of the P value (0.06) for the comparison of plasma total cholesterol between the two groups it would have been interesting to see if the LDL-cholesterol levels were significantly different, as they should have been. If they were not, then the authors might have considered reporting this lack of association between another widely recognized lipid risk factor and myocardial infarction. Alternatively, it may be concluded that there was something atypical about their study group.

A similar sized prospective study of a cohort from the Lipid Research Clinics Coronary Primary Prevention Trial (Schaefer et al., 1994) did identify elevated plasma Lp(a) levels as an independent risk factor for CHD in hypercholesterolaemic Caucasian males.

The most impressive prospective study to date has been the Göttingen Risk Incidence and Prevalence Study (GRIPS) (Cremer et al., 1994). In this study approximately 6,000 males aged between 40-59.9 years with no overt evidence of CHD were followed for 5 years. The primary end-point in this study was myocardial infarction, fatal or non-fatal. Over the study period 107 subjects with myocardial infarction were recorded and compared with a control group of 5,364. These groups, as expected, differed significantly in plasma LDL-cholesterol (4.7 *vs.* 3.7 mmol. L^{-1}, $p<0.001$) and HDL-cholesterol (1.1 *vs.* 1.3 mmol. L^{-1}, $p<0.001$), but they also exhibited a statistically significant difference in plasma Lp(a) (18 *vs.* 9 mg. dL^{-1}, $p<0.001$). The association between plasma Lp(a) level and the incidence of myocardial infarction in GRIPS is shown in figure 1.10.

Using multivariate logistic regression analysis the authors of GRIPS were able to rank the different risk factors in order of importance. In this scheme plasma Lp(a) proved to be an important CHD risk factor ranking 5th behind LDL-cholesterol, family history of myocardial infarction, fibrinogen, and HDL-cholesterol.

Therefore, at the present time the balance of evidence is in favour of a positive and independent association between plasma Lp(a) concentration and risk of CHD [Table 1.3]. Taken together these studies do not completely clarify

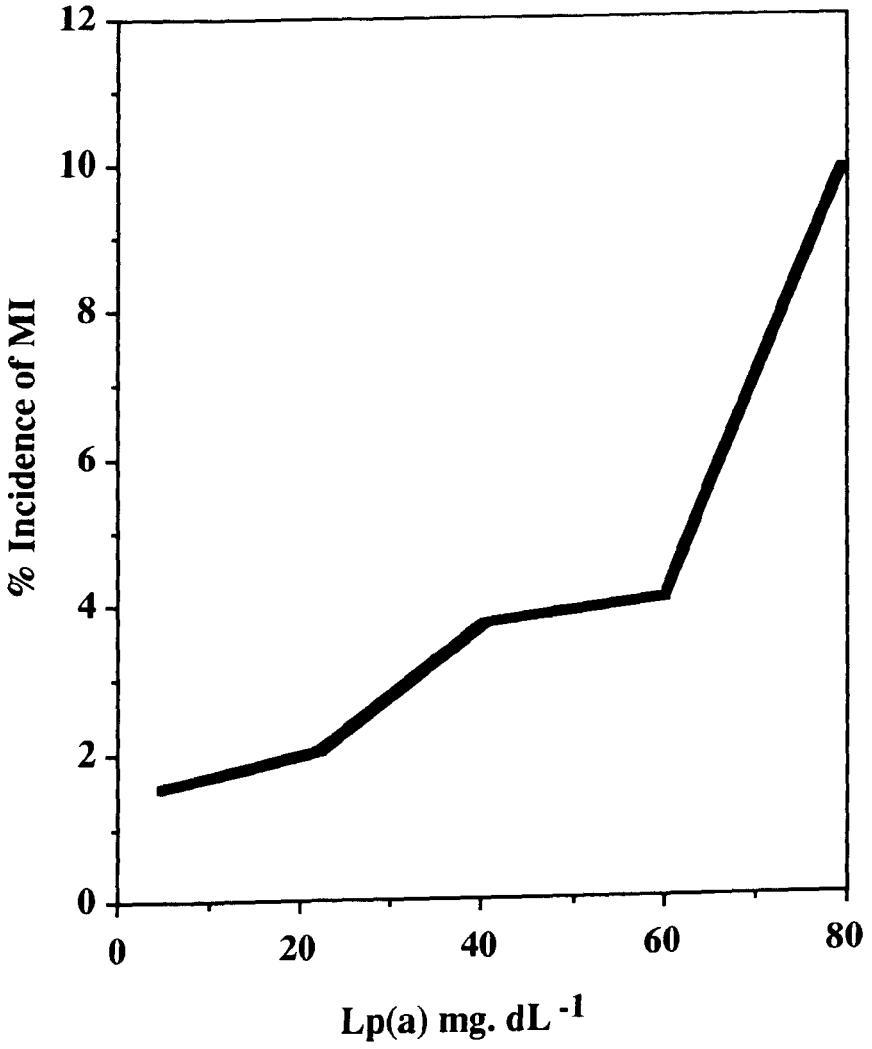


FIGURE 1.10.

Göttingen Risk Incidence and Prevalence Study (GRIPS). The impact of plasma Lp(a) concentration at baseline on the incidence of myocardial infarction during a 5 year follow up. Adapted from Cremer et al. (1994).

the association between plasma Lp(a) levels and CHD risk, but they do offer some important pointers, viz. patient characteristics such as gender, ethnic origin and age must be taken into account in any future study. The apparently discrepant findings from the prospective studies may at least in part be explained by the failure of some investigators to follow this advice.

1.6.7 Cerebrovascular and peripheral vascular disease and Lp(a)

High plasma concentrations of Lp(a) have been found to be associated with atherosclerotic involvement of vessels other than the coronary arteries, including both cerebral and peripheral vessels (Pedro-Botet et al., 1982; Jovicic et al., 1993; Cambillau et al., 1992; Koltringer & Jürgens, 1985; Murai et al., 1986; Norrgård et al., 1991). Plasma Lp(a) levels have been found to be significantly higher in individuals who have had strokes - either embolic or thrombotic (Woo et al., 1991).

1.7 Atherosclerosis & Lp(a)

The most direct evidence that apo(a) itself has an atherogenic role comes from the analysis of mice expressing the human apo(a) transgene. Fat-fed mice expressing human apo(a) had a 40-fold higher mean area of lipid staining in the aorta than did control animals. Importantly, only those animals maintained on a high fat diet developed lesions. Over 95% of the apo(a) in these mice circulated free of lipoproteins, yet in the atherosclerotic lesions the apo(a) not only co-localized with the lipid, but also with mouse apoB-100 (Lawn et al., 1992). Whether apo(a) forms a weak association with mouse lipoproteins and escorts them into the lesion or whether apo(a) binds first and then traps LDL has not been determined. The relevance of this result to man is unclear since there is little to no free apo(a) circulating in human plasma. Now that the double (apo(a)/apoB-100) transgenics are available (Linton et al 1993), the atherogenicity of whole Lp(a), has been studied in inbred mice (Callow et al., 1995). In this study, mice with plasma Lp(a) were compared to mice only expressing the apo(a) transgene and were found to have 2.5 times the number of proximal aortic fatty streak lesions and 8 times the number in non-transgenic control animals.

The early pathological studies of Walton et al., (1974) revealed that Lp(a) is present in human atherosclerotic lesions. Immunohistochemical studies of human aorta and venous saphenous grafts have demonstrated that Lp(a) is located predominantly in the extracellular subendothelial space. The apo(a) is

found largely intact and is associated with apoB-100 though free apo(a) and lipid poor apo(a)-apoB-100 complexes are also found (Pepin et al., 1991; Cushing et al., 1989; Rath et al., 1989). The amount of apo(a) in arterial biopsy specimens tends to correlate with plasma Lp(a) concentration (Rath et al., 1989; Nachman et al., 1991).

The mechanism by which Lp(a) traverses the endothelium is not known, but it has been shown to bind several components of the intimal matrix including glycosaminoglycans (Bihara-Varga et al., 1988), fibrinogen, crosslinked fibrin, and fibronectin (Harpel et al., 1989; Loscalzo et al., 1990; Rouy et al., 1992; Smith & Cochran, 1990; Salonen et al., 1989). Furthermore, reducing agents, including homocysteine, enhance the binding of Lp(a) to fibrin (Harpel et al., 1992).

The binding of Lp(a) to lysine rich proteins such as fibrin has received considerable attention (Guevara et al., 1993b; Scanu et al., 1993). As described above in section 1.2.2 this binding is thought to be mediated through the last K4 unit, $K4_{37}$, although other K4 units may be involved (Miles & Plow 1990).

The exact role Lp(a) plays in the development of arterial plaques has not been elucidated. *In vitro* studies have implicated Lp(a) as both a proatherogenic and antifibrinolytic agent. Lp(a) may be an innocent bystander that becomes trapped in the atherosclerotic lesion, thus contributing its cholesteryl-ester-rich cargo to the developing plaque. Alternatively, Lp(a) may interfere with the physiological role of plasminogen by molecular mimicry and thus promote thrombosis (Hajjar et al., 1989; Miles et al., 1989). These effects, for which there is only *in vitro* evidence, are summarized in Figure 1.11, and are listed below:.

1.Lp(a) may contribute to the development of foam cells in atherosclerotic lesions. Native Lp(a) and recombinant apo(a) bind *in vitro* to cultured mouse macrophages, but are only internalized if the macrophages are pre-loaded with cholesterol (Zioncheck et al., 1991; Bottalico et al., 1993). Native Lp(a) is not internalized through the macrophage scavenger receptor. However, if the lysine residues in Lp(a) are modified using malondialdehyde it becomes a ligand for this receptor (Haberland et al., 1992). Lp(a) can also undergo oxidation, though not as readily as LDL (Sattler et al., 1991).

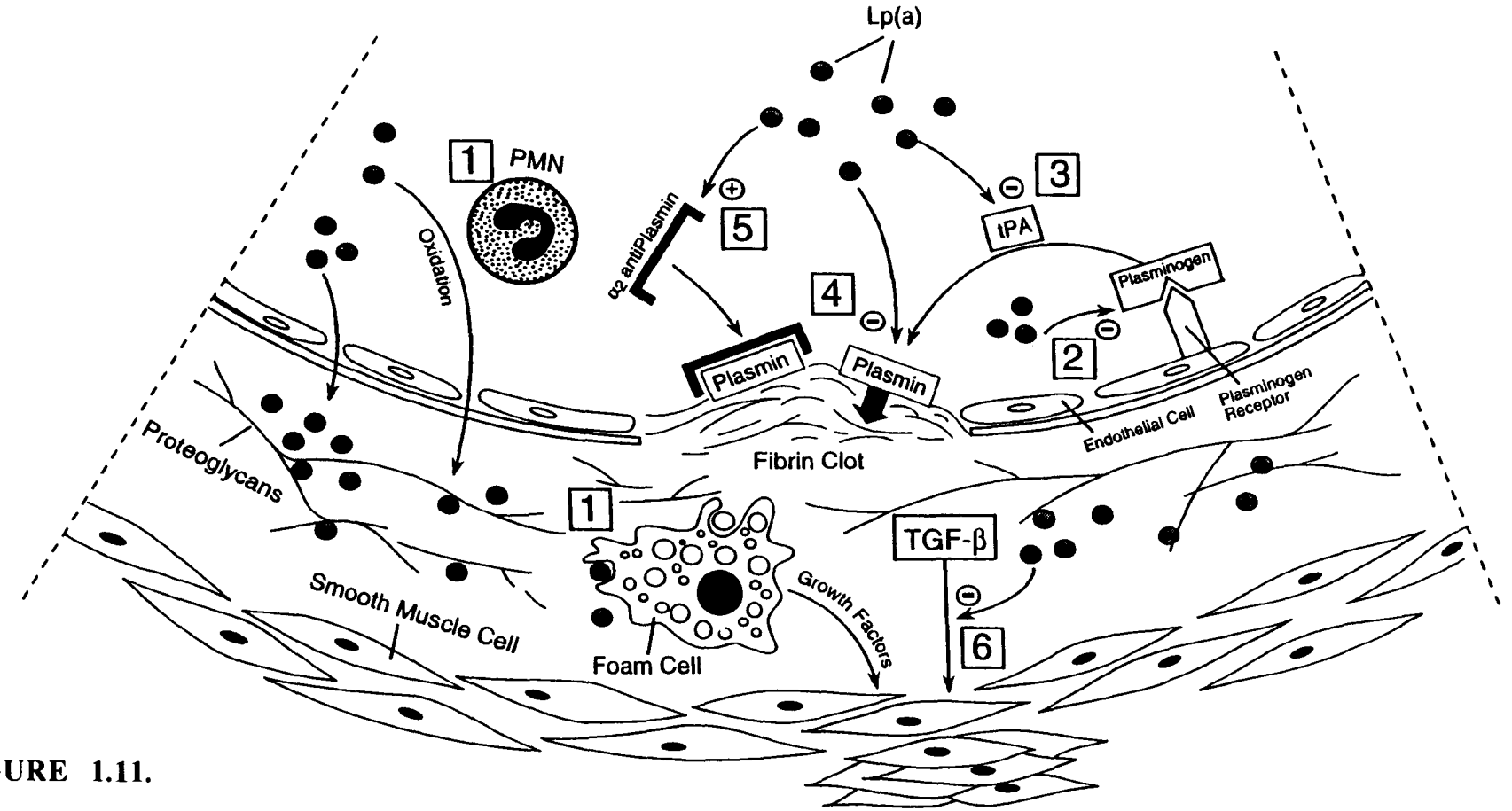


FIGURE 1.11.

In vitro effects of Lp(a) on atherosclerosis and fibrinolysis. This schematic shows a section of the artery wall and the possible effects of Lp(a) on cellular and humoral mediators of athero(thrombo)genesis. Numbers refer to text. (Section 1.7). PMN, polymorphonuclear cell; tPA, tissue-type plasminogen activator; TGFß, transforming growth factor-ß.

2. At high concentrations, Lp(a) competes *in vitro* with plasminogen binding to the surface of human umbilical vein endothelial cells, platelets, and a human monocyte cell line (Miles et al., 1989; Hajjar et al., 1989; Ezratty et al., 1993)
3. Lp(a) can compete *in vitro* with the activation of plasminogen by either streptokinase or tPA (Edelberg et al., 1990; Simon et al., 1991)
4. Lp(a) competes *in vitro* with plasminogen for binding to fibrinogen or fibrin (Edelberg et al., 1990; Rouy et al., 1992; Loscalzo et al. 1990; Harper & Saunders, 1981; Leerink et al., 1991)
5. Lp(a) promotes plasmin inhibition by α_2 antiplasmin (Edelberg & Pizzo, 1992).
6. Lp(a), by interfering with plasminogen activation, decreases the conversion of inactive transforming growth factor ß (TGFß) to active TGFß. TGFß acts as an antimitogen on smooth muscle cells. *In vitro* studies have demonstrated that additions of high concentrations of Lp(a) are associated with smooth muscle cell proliferation and migration (Kojima et al., 1991; Grainger et al., 1993).

Most of these *in vitro* effects require very high concentrations of Lp(a), which brings their physiological significance into question. It may be argued, however, that the concentrations of Lp(a) are much higher in the atherosclerotic lesion, and that plasma levels do not reflect these tissue concentrations.

There is little *in vivo* evidence that Lp(a) is prothrombotic. If Lp(a) interferes with plasminogen activation, it may be expected that thrombolytic therapy would be less effective in patients with high plasma concentrations of Lp(a), but this appears not to be the case (Von Hodenberg et al., 1991; Armstrong et al., 1991). Individuals with high plasma concentration of Lp(a) have not been noted to have a higher incidence of venous thrombosis.

A single study suggests that Lp(a) may be anti-thrombolytic *in vivo*. Moliterno et al. (1993) measured plasma Lp(a) concentrations in individuals who had sustained a myocardial infarction and had obtained coronary arteriograms at least one week after the event to assess the patency of the infarction-related artery. Prior studies have found that approximately half of all infarction-related arteries recanalize soon after the event (DeWood et al., 1980; Bertrand et al., 1979). Those survivors who failed to recanalize the occluded coronary artery had significantly higher plasma Lp(a) concentrations compared to those in which the artery had regained patency.

1.8 Clinical chemistry

1.8.1 Measurement of plasma Lp(a) concentrations

The measurement of plasma Lp(a) requires less stringent conditions for sample collection than other lipoproteins. It is not necessary that a fasting sample be collected, since Lp(a) concentrations are not significantly different in the fasting or fed state (Pfaffinger et al., 1991; Panteghini & Pagani, 1993). The plasma or serum can be stored at 4°C for up to two weeks without loss of antigenicity. Reports vary as to effects of long-term storage on the maintenance of the integrity of the Lp(a) particle, but some methods, such as radio-immunodiffusion assays, are particularly sensitive to the effects of long-term storage (Craig et al., 1992). It appears that storage at -20°C is equivalent to -70°C, and Lp(a) is stable for up to three months at both temperatures. It has been reported that serum Lp(a) concentrations are even stable in samples stored at -20°C for many years. Jauhianen and colleagues (1991) noted no significant reduction in the immunogenicity of the particle in the serum samples of the same individuals either assayed freshly or in samples stored at 5.5, 7.5 or 8.5 years.

A variety of methods have been developed to measure plasma Lp(a) concentrations, including quantitative immunoelectrophoresis (Berg, 1994), radio-immunoassay (Albers et al., 1977), radioimmunodiffusion (Albers & Hazzard, 1974), rate nephelometry (Gillery et al., 1993) fluorescent immunoassay (Jürgens et al., 1992, Kottke & Bren, 1994), and the most commonly used method, the enzyme-linked immunosorbent assay (ELISA) (Albers et al., 1990).

In most ELISA assay systems the first antibody, which is used to capture the Lp(a) particle, is usually a polyclonal antibody to apo(a). The second antibody, the detecting antibody, can either be an anti-apo(a) monoclonal antibody or an anti-human apoB-100 antibody. An advantage of using apoB-100 as the detecting antibody is that Lp(a) concentrations can then be expressed in molar units (i.e., number of particles), rather than total lipoprotein mass. Most assays have an intra-assay coefficient of variation (CV) of less than 5% and an inter-assay CV of ~10%.

As yet, there has been no consensus on the method used to express the results, and presently the plasma concentrations are reported as moles. L^{-1}, mg apo(a). dL^{-1}, or mg Lp(a). dL^{-1}. Given the high degree of polymorphism in

apo(a) mass, it may be argued that molar rather than mass units would be preferable.

Both monoclonal and polyclonal anti-Lp(a) antibodies may cross react with plasminogen or LDL. Any new antibody must be rigorously screened for these potential interactions and any new assay system must document this potential source of error (Berg, 1994). A further potential source of analytical error is the presence of interferants in the plasma. Pharmacological agents that alter the immunogenicity of Lp(a), such as thiols and cysteine-containing compounds, have been shown to interfere with the assay (Scanu et al., 1992).

The measurement of plasma Lp(a) concentrations is made more difficult by the highly polymorphic nature of apo(a) (Grinstead & Ellefson, 1988). It has not been conclusively demonstrated that any of the antibodies currently used react equally with all the apo(a) isoforms of different size. Many of the antibodies employed in the assays are directed against an epitope within the K4 sequences, which can vary in number over a 4-fold range. Efforts are being directed to develop antibodies to apo(a) that react with only one epitope in the apo(a) protein, such as in the protease domain.

There are no agreed primary reference materials or reference methods for the measurement of Lp(a) (März et al., 1993; Albers et al., 1990). A standard serum is needed for calibration and to permit comparisons between laboratories. The dissemination of a reference standard may be facilitated by using lyophilized samples, which have been stabilized by the addition of sucrose (Borgue et al., 1993) but this has yet to be fully validated. To address the many problems of standardisation (Labeur et al., 1994) the International Federation of Clinical Chemists has recently established a Working Group on Lp(a) Standardisation (personal communication, G. Siest, 1994).

Due to the highly skewed distribution of plasma Lp(a) concentrations, a population-based reference interval is not appropriate. Since there is no standardized method to measure plasma Lp(a) level or a unified approach to the expression of the results, there can be no consensus on the cut-off point above which an individual is deemed at risk.

1.8.2 Apo(a) isoform phenotyping and apo(a) genotyping

The pulsed-field gel analysis of the *APO(a)* gene, as described above, allows for the analysis of *APO(a)* alleles that produce little or no apo(a) protein and has clarified the relationship between *APO(a)* gene structure and the apo(a)

isoform size. Refinements of this analysis have indicated that the length of the *APO(a)* gene is even more variable than originally appreciated. The high degree of length polymorphism in the *APO(a)* gene is reflected by a similarly high degree of size polymorphism in the apo(a) glycoprotein. Most recent reviews refer to the work of Lackner et al. (1993) and Marcovina et al. (1993b) and cite the fact that 34 different apo(a) isoforms have been described, Undoubtedly, as the methodology improves and more subjects are analyzed, additional *APO(a)* alleles and isoforms will be identified and this number will increase.

There is currently no evidence, however, that the analysis of *APO(a)* gene structure provides useful additional information for decision making in patient care. Similarly, apo(a) phenotyping by immunoblot analysis has no significant role, as yet, in routine patient management.

1.9 Intervention

Cross-sectional studies, but not all prospective studies, have demonstrated that high concentrations of plasma Lp(a) are associated with coronary atherosclerosis in Caucasians, but no studies have demonstrated that lowering plasma concentrations of Lp(a) is associated with a reduction in CHD morbidity or mortality. Intervention studies will not be immediately forthcoming, since few available pharmacological agents lower Lp(a).

1.9.1 Pharmacological agents

Hypolipidaemic agents that interfere with lipoprotein synthesis have a more pronounced effect on plasma Lp(a) levels than those that accelerate the clearance of the lipoprotein. Agents that up-regulate the LDL receptor, such as bile acid sequestrant resins or HMG Co-A reductase inhibitors, significantly lower the plasma concentrations of LDL-cholesterol, but have little or no appreciable effect on the plasma concentration of Lp(a) (Vessby et al., 1982; Thiery et al., 1988; Jürgens et al., 1989; Kostner et al., 1989). Other agents that lower LDL and VLDL-cholesterol, such as the fibric acid derivatives and probucol, have no effect on Lp(a) (Albers et al., 1975; Maeda et al., 1989).

The administration of nicotinic acid interferes with the hepatic secretion of lipoproteins, causing a fall in not only plasma VLDL and LDL-cholesterol concentrations, but also Lp(a) (Gurakar et al., 1985; Carlson et al., 1989; Noma et al., 1990) [Figure 1.12]. Doses of nicotinic acid as low as 500 mg

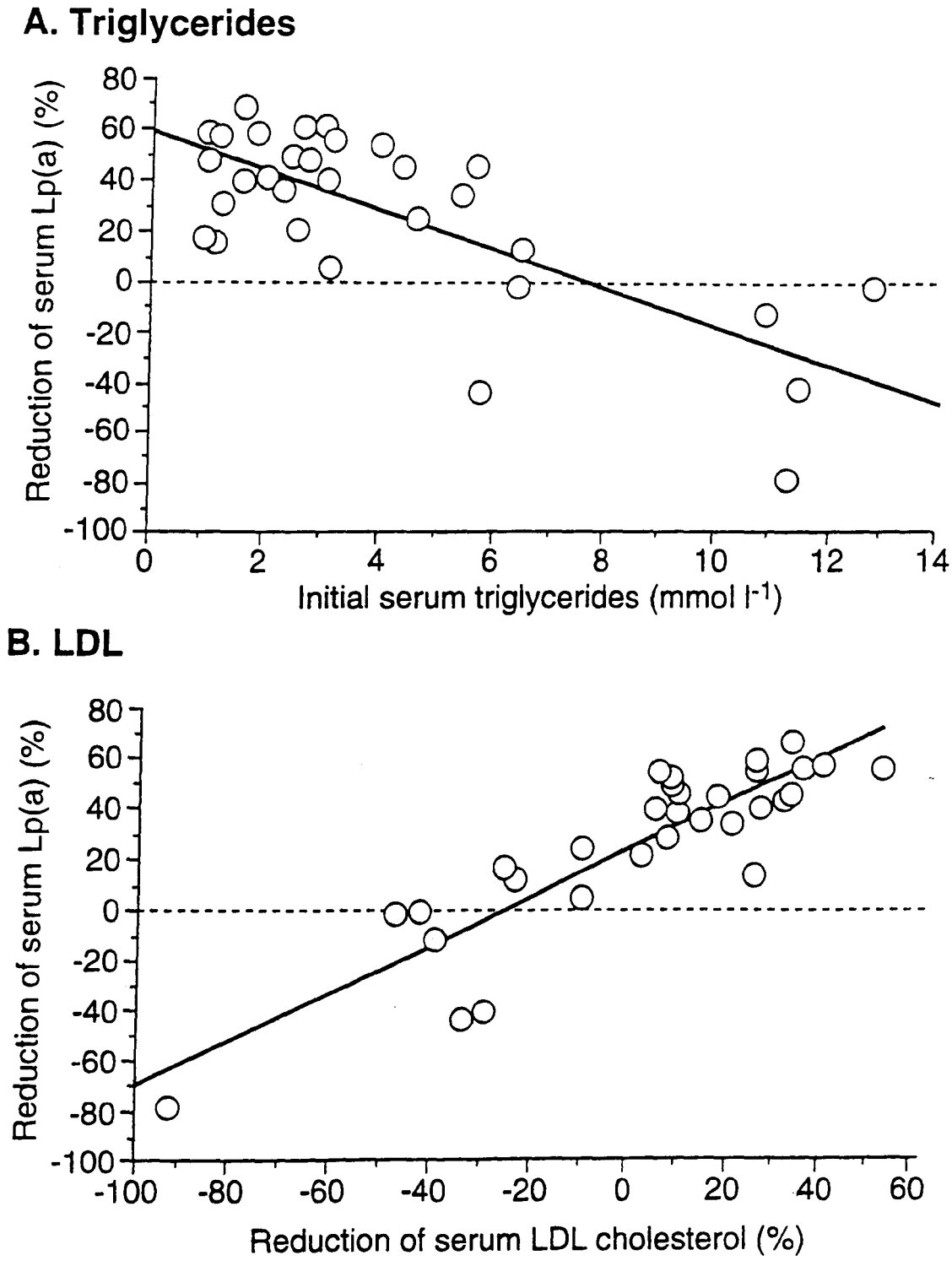


FIGURE 1.12.

Effects of nicotinic acid (4g. d^{-1}) on serum Lp(a) concentration. Reduction in serum Lp(a) is (A) inversely correlated with serum triglyceride concentration and (B) directly correlated with the percentage reduction of LDL-C. Data from Carlson et al. (1989)

(sustained-release) are associated with a significant fall in plasma Lp(a) concentration (Lepre et al., 1992). The degree to which the plasma Lp(a) concentration falls is proportional to the initial plasma Lp(a) level (Carlson et al., 1989); therefore, individuals with higher plasma levels have a more significant response to therapy. The decrease in plasma Lp(a) concentration tends to correlate with the decrease in plasma LDL-cholesterol. Paradoxically, in hypertriglyceridaemic individuals the plasma Lp(a) level may increase on nicotinic acid therapy.

For reasons that are not clear, administration of anabolic and androgenic steroids (stanozolol and danazol) to women (Albers et al., 1984; Crook et al., 1992; Hiraga et al., 1992; Henriksson et al., 1992) are associated with up to 50% lowering of plasma Lp(a) levels. High dose oestrogen administration to elderly men resulted in a ~50% lowering of plasma Lp(a) levels (Henriksson et al., 1992; Hiraga et al., 1992) though when lower doses were administered to women there was a less significant fall in plasma Lp(a) concentrations (Lobo et al., 1992). The effects of postmenopausal hormone replacement therapy on plasma Lp(a) levels have also been studied. Sacks et al. (1994) report beneficial falls in plasma Lp(a) of 14% and 16% with 0.625 and 1.25 mg of conjugated oestrogen respectively.

An initial report suggested that administration of N-acetylcysteine, a reducing agent, was associated with a marked reduction in plasma Lp(a) levels (Gavish & Breslow, 1991). The authors speculated that the mechanism by which this agent lowered plasma Lp(a) levels was by dissociating apo(a) from the LDL moiety. However, a follow-up study failed to confirm this result (Kroon et al., 1991). Whether or not it is advisable to use this strategy to lower plasma Lp(a) levels is not clear. It is not known how the free apo(a) is cleared and this moiety may be in itself particularly atherogenic (Lawn et al., 1992).

Thus, there are only three medications that have been convincingly shown to lower the plasma level of Lp(a) in some patients - nicotinic acid, oestrogens, and anabolic steroids. Since these medications all have multiple effects on the other classes of lipoproteins, it is not possible to lower the plasma Lp(a) concentration selectively without changing the levels of other lipoproteins. Therefore, it will be difficult to design a study to assess the independent effect of lowering plasma Lp(a) levels on coronary risk.

1.9.2 Plasmapheresis and Lp(a)

The plasma concentration of Lp(a), like LDL, can be dramatically lowered (30-50%) by either plasma exchange (Schenck et al., 1988) or LDL-apheresis (Armstrong et al., 1989; Ritter et al., 1990). Indeed the efficiency with which this procedure strips the plasma of Lp(a) has prompted Groß and colleagues (1994) to suggest it as a preparative method for isolating Lp(a). The therapeutic utility of this method to lower plasma levels of Lp(a) is questionable, however, given its inconvenience and expense.

1.9.3 Guidelines for therapeutic intervention

Despite numerous gaps in our knowledge and our relative inability to lower the plasma levels of this lipoprotein effectively, there is sufficient evidence to warrant measuring the plasma concentration of Lp(a) in Caucasian patients with ischaemic heart disease or with a strong family history CHD. It remains controversial whether Caucasians with elevated plasma Lp(a) levels, who are otherwise normolipaemic, should be treated with medications directed at lowering plasma levels of Lp(a). If a hyperlipidaemic individual also has a high plasma Lp(a) level, treatment with nicotinic acid might be preferable to other lipid-lowering medications. Oestrogen replacement therapy should be encouraged in post-menopausal women with high Lp(a) levels. Finally, in individuals with high plasma concentrations of Lp(a), the other known risk factors for ischaemic heart disease should be aggressively treated, especially any elevations in plasma LDL-cholesterol levels.

1.10 Lp(a) - molecular folly or design

The *APO(a)* locus is arguably the most highly polymorphic in the human genome outside of the histocompatibility complex. More than 100 identifiably distinct *APO(a)* alleles are present in the Caucasian population alone. The variability includes not only the number of kringle repeats, but also differences in sequence (Cohen et al., 1993). This high degree of polymorphism must raise the question as to whether the apo(a) protein performs any useful function.

To date, Lp(a) has no known physiological role. Individuals who have little to no Lp(a) in their plasma appear to be healthy. Moreover, most mammals have no detectable apo(a) protein or *APO(a)* gene, suggesting that

apo(a) does not have an essential function. However, it is possible that apo(a) has an intracellular or autocrine role and its presence in plasma is a consequence of cellular leakage. If this scenario were true, the association of apo(a) with LDL in plasma would simply be a marriage arranged blindly by the forces of physical chemistry. Alternatively, apo(a) may confer selective advantage in only certain populations. As stated above [section 1.4.1] individuals of African descent have 2-3 fold higher plasma levels of Lp(a) than either Caucasians or Chinese. These inter-ethnic differences may provide a clue as to a possible function for Lp(a). Lp(a) may perhaps provide protection against an infectious agent found in Africa, much in the same way that sickle cell trait provides selective advantage against falciparum malaria.

Alternatively, apo(a) may have no physiological relevance and simply be the result of a series of genetic and molecular mishaps. The plasminogen gene locus appears to be highly recombinogenic; giving rise not only to the *APO(a)* gene, but also to numerous other related genes. It is likely that the *APO(a)* gene is the product of a duplication event that may have occurred more than once, as evidenced by its presence in the hedgehog, the old world monkey lineage, but no intermediary species. Interestingly, in all animals that express apo(a), the protein is polymorphic in size, again attesting to the highly recombinogenic nature of this region of the genome.

Arguing in favour of a physiological role for Lp(a) is the high degree of sequence conservation in the coding regions of the *APO(a)* gene, excluding the common K4 and K5 regions. If apo(a) has a function, it is likely to be mediated by the sequences that are conserved among individuals and between species. For this reason, it would be instructive to compare the sequences of the unique K4 repeats and the protease domain in the hedgehog and man.

Thus, despite advances in our knowledge of Lp(a), important new pieces to the puzzle need to be put in place before "the mysteries of Lp(a)" as defined by Utermann (1989) can be solved.

1.11 General aims of this thesis

It will be clear from the above literature review that while much important and fundamental information exists on Lp(a) there are also considerable gaps. Most of the work presented in this thesis was performed in

the Department of Molecular Genetics, Dallas, Texas under the leadership of Drs. Michael S. Brown and Joseph L. Goldstein and in the team headed by Dr. Helen H. Hobbs. In these laboratories a major initiative is underway to understand the mechanisms that control plasma Lp(a) concentrations and the contribution of Lp(a) to atherogenesis. Much of this work is currently dominated by the study of murine models. My contribution to this work as presented in this thesis is concerned primarily with studies performed in human subjects.

In the following chapters I present a series of experiments performed using the techniques of high resolution apo(a) phenotyping in conjunction with apo(a) genotyping. As a preliminary, I present a study briefly characterising the monoclonal antibodies used in the apo(a) phenotyping studies and a description of developmental work performed to validate this method.

Next, a study is presented detailing a comparative analysis of the *APO(a)* gene, apo(a) glycoprotein and plasma concentrations of Lp(a) in three ethnic groups viz. Caucasians, Chinese, and African-Americans. This study uses the above methods to elucidate underlying mechanisms of plasma Lp(a) level control and to shed light on human evolutionary patterns.

The remainder of the experimental work focusses on the impact of candidate genes other than *APO(a)* on the plasma concentrations of Lp(a). Using sib-pair analyses the controversial effects of the LDL receptor gene were explored by studying kindreds with familial hypercholesterolaemia. Similarly, the effects of the apoB gene were studied in kindreds with hypobetalipoproteinaemia.

The thesis concludes with a discussion of the importance of the detailed characterisation of Lp(a). A case is made for the adoption of high resolution apo(a) phenotyping in future research initiatives aimed at the study of Lp(a), whether these be biochemical, metabolic, clinical or epidemiological.

Chapter 2 Materials and Methods

On my way to the Whim I slowly walked toward the Clare Bridge, staring up at the gothic pinnacles of the King's College Chapel that stood out sharply against the spring sky. I briefly stopped and looked over at the perfect Georgian features of the recently cleaned Gibbs Building, thinking that much of our success was due to the long uneventful periods when we walked among the colleges or unobtrusively read the new books that came into Heffer's Bookstore.

James D. Watson, (1962 Nobel Laureate)
The Double Helix 1968

Just do it

Joe Goldstein, (1985 Nobel Laureate)
Nike promotional slogan, and personal motto

2.1 Introduction

In this thesis four separate but related studies are presented. The general methods of plasma Lp(a) analysis, apo(a) phenotyping by high resolution immunoblotting, and apo(a) genotyping by pulsed-field gel electrophoresis and Southern blotting are described here in detail. Other methods, principally plasma lipid and lipoprotein analysis, apoB phenotyping and genotyping, and LDL receptor genotyping are crucial to the studies presented but were not performed by the author. These assays were performed by collaborators in the laboratories stated below and the methods used are outlined here for completeness.

2.2 Materials

All reagents used were of analytical grade and the names and addresses of all suppliers are shown in appendix 1 together with the manufacturers or suppliers of all hardware and software used in this work.

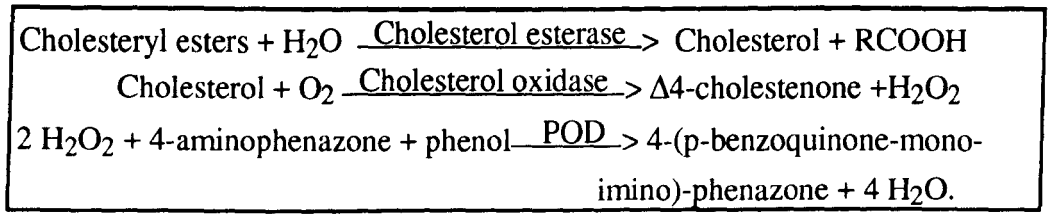
2.3 Plasma lipid and lipoprotein analysis

These were assayed using a variety of chemical, enzymatic, centrifugal and immunoassay techniques as described in detail below.

2.3.1 Plasma lipid assays

Total cholesterol and triglyceride were determined in fresh, whole plasma and lipoprotein preparations by enzymatic colorimetric assays according to the protocols of the Lipid Research Clinics Program (1982). For the subjects described in chapter 4 these assays were performed in the laboratory of Dr. Scott Grundy (University of Texas, Southwestern Medical Center, Dallas, TX), while those related to the work described in chapter 5 and 6 were performed in the laboratories of Dr. Paul Hopkins (Cardiovascular Genetics Research Center, University of Utah, Salt Lake City, UT) and Dr Francine Welty (Institute for Prevention of Cardiovascular Disease, Deaconess Hospital, Boston, MA) respectively.

Total cholesterol was assayed using the cholesterol oxidase method, the principal of which is shown below:



Triglyceride was assayed using an enzymatic method the principal of which is shown below:

Triglyceride+ 3 H_2O —Lipase→ glycerol + 3 RCOOH
glycerol + ATP —GK→ glycerol-3-phosphate+ ADP
glycerol-3-phosphate + O_2 —GPO→ dihydroxyacetone phosphate + H_2O_2
H_2O_2 + 4-aminophenazone + 4-chlorophenol —Peroxidase→ 4-(p-benzoquinone-mono-imino)-phenazone + $2H_2O$ + HCl.

The resultant colour changes were measured spectrophotometrically at 505 nm.

2.3.2 Beta quantification

Lipoprotein classes were prepared by a combination of ultracentrifugation and selective precipitation, using standard methodologies (Lipid Research Clinics Program 1982). VLDL were separated as a floating fraction. The infranatant was then treated with heparin/Mn^{2+} (at a final concentration of 1.3 g. mL^{-1} heparin and 0.092M Mn^{2+}) to precipitate LDL and leave HDL in solution (Warnick & Albers 1978). The cholesterol content of whole plasma, of the top (VLDL), and bottom (LDL and HDL) fractions, and of the heparin/Mn^{2+} supernatant (HDL) were measured as described above.

Ultracentrifugation.
Five mL of plasma was placed in an Ultra-clear tube (13x64 mm) (Beckman Instruments, Fullerton, CA) and overlayered with 2 mL of d 1.006 g. mL^{-1} solution. Tubes were capped and centrifuged overnight at 35,000 rpm (4°C) in a Beckman 50.4 rotor, then sliced 25 mm from the top and the supernatant collected into a 3 mL volumetric flask. The contents of the bottom fraction were transferred to a 5.0 mL volumetric flask, the tube washed with saline, the wash added to the flask, and the volume adjusted to 5.0 mL with 0.15M NaCl.

Precipitation of LDL.
An aliquot (1.0 mL) of the bottom fraction was placed in a Beckman centrifuge tube and 50 µL of precipitating reagent [9.56 g $MnCl_2.4\ H_2O$ + 1.05 g (approximately $5x10^5$ units) heparin sodium salt in 25 mL 0.15M NaCl] were added and mixed. The mixture was kept at 4°C for 15 min then centrifuged at 10,000 rpm for 30 min, and the supernatant separated immediately for cholesterol analysis.

2.4 Plasma Lp(a) assay

Plasma Lp(a) concentrations were determined in the different studies presented in this thesis using 3 diferent ELISA systems. This was done for two reasons. Firstly, during the course of this work the ELISA system used in the Dallas laboratory changed, and secondly Lp(a) measurements in the FH families from Utah had already been performed locally and were not repeated. It is important to note that at no time in this work is any comparison made between values for plasma Lp(a) concentrations obtained from more than one assay. Only comparisons of plasma Lp(a) concentrations measured by the same assay system are made. Therefore, the confounding effects of the lack of inter-assay standardisation, which may be considerable, are avoided.

2.4.1 Lp(a) ELISA 1

Plasma Lp(a) concentrations in the hypobetalipoproteinaemia family members were determined in the Northwest Lipid Research Laboratory, University of Washington, Seattle, WA according to the ELISA protocol of Marcovina and her colleagues (1995).

Blood was collected into vacutainers (Becton Dickinson, Lincoln Park, NJ) containing K_2EDTA as anti-coagulant to give a final concentration of 1 mg. mL^{-1}. Plasma was obtained from each of the blood samples by low speed centrifugation, 1000*g* (4°C) and stored at -80 °C. Approximately 7 days after collection the samples were shipped on dry ice by express courier to the Seattle laboratory. The ELISA measurements were performed as follows. First, flat-bottomed polystyrene microtitre plates (Nunc, Immunoplate Maxisorp, Intermountain Scientific, Bountiful, UT) were coated with 100µL of coating buffer (0.1M $NaHCO_3$, pH 9.6) containing 0.5µg MAb a-6. The plates were gently shaken for 1h at room temperature and then incubated overnight at 4°C. The unbound antibody was removed by washing the plates three times using an automated plate washer (Dynatech, Chantilly, VA) with phosphate buffered saline (PBS), pH 7.4. The remaining binding sites were blocked by the addition of 300µL PBS containing 30g. L^{-1} bovine serum albumin (BSA), incubated for 1h at room temperature and washed as before. The plates were then sealed and stored at 4°C until used. After equilibrating the plates at room temperature for 30min, 100µL of standard, quality controls or plasma samples, appropriately diluted in PBS containing 1g. L^{-1} BSA and 0.5mL. L^{-1} Tween

20, were added to the wells and incubated for 1h at 28°C in an incubator-shaker (Innova 4000, New Brunswick Scientific, Edison, NJ). After washing, 100μL of appropriate dilutions of either HRPO-conjugated MAb a-5 or MAb a-40, or a goat anti-human apoB, was added to each well and incubated as before for 1h at 28°C. Wells were washed and colour was developed by adding 100μL of substrate (hydrogen peroxide) and chromogen (O-phenylenediamine dihydrochloride). The enzyme reaction was allowed to proceed for 15 min at room temperature, stopped by adding 100μL of 1M H_2SO_4 and the absorbance read at 495nm using an automated microtitre plate reader (Molecular Devices, Menlo Park, CA). Standard curves were generated by plotting the absorbance against Lp(a) concentration by linear regression. Values for sample concentrations were obtained by interpolating their absorbance from the standard curve.

2.4.2 Lp(a) ELISA 2

Plasma Lp(a) concentrations in the FH family members were determined in the Cardiovascular Genetics Research Center, University of Utah, Salt Lake City, UT, using a commercially available ELISA kit: MACRA™ Lp(a) (Terumo Medical Corp. Elkton, MD). Briefly, this assay involves the incubation of plasma samples at room temperature in microtitre plate wells pre-coated with a capture monoclonal anti-Lp(a) antibody. The wells are washed and incubated with a polyclonal anti-Lp(a) antibody conjugated to horseradish peroxidase. The unbound enzyme conjugate is removed by washing the wells using an automated plate washer, as above. Following a 20min incubation with enzyme substrate (hydrogen peroxide) and chromogen (O-phenylenediamine dihydrochloride) the reaction was stopped with 2N H_2SO_4 and the absorbance read at 492nm using an automated plate reader, as above. Standard curves were prepared as above by plotting the absorbance against the Lp(a) concentration. The absorbance is directly proportional to the plasma Lp(a) concentration within the working range of the assay, which is quoted by the manufacturer as 0.8-80 mgLp(a). dL^{-1}.

2.4.3 Lp(a) ELISA 3

In the studies on inter-ethnic variation of Lp(a) concentration presented in chapter 4 , the Lp(a) assay system used was an ELISA described by Menzel

et al., (1990). This assay was performed by the commercial laboratory, GeneScreen (Dallas, TX).

Fasting venous blood was collected into vacutainers containing K_2EDTA as anti-coagulant to give a final concentration of 1 mg. mL^{-1}. Plasma was obtained from each of the blood samples by low speed centrifugation, 1000*g* (4°C) and stored at -80 °C and plasma Lp(a) concentrations were measured within two months of collection. The capture antibody used in the assay to coat the microtitre plate wells was a polyclonal rabbit anti-human Lp(a) antibody. The detection antibody was a mouse monoclonal anti-human apo(a) antibody, IgG-1A^2. The hybridoma cell line was provided by Dr. Gerd Utermann (University of Innsbruck, Innsbruck, Austria) and this was injected into mice in the Dallas laboratories where IgG-1A^2 was prepared and purified for use in-house and by GeneScreen. This antibody does not cross react with plasminogen and was conjugated to horseradish peroxidase before use using the protocol outlined in section 2.5 below. This antibody was also used in the apo(a) immunoblotting method to determine apo(a) phenotype.

2.5 Apo (a) isoform determination by immunoblotting

Apo(a) isoform analysis was performed using a modification of the immunoblotting method described by Kamboh et al. (1991) A 12.5 µl aliquot of plasma was mixed with 30 µl reducing buffer [1:2:10 ratio of ß-mercaptoethanol, 0.5% (w/v) bromophenol blue in 5% (v/v) glycerol, 5% (w/v) SDS] and boiled for 5 min. A 10 µl aliquot of this mixture was loaded onto a 2% agarose (Ultra Pure DNA Grade Agarose, Bio-Rad, Hercules, CA) gel (15 cm x 25 cm) in 90 mM Tris-HCl, 90 mM boric acid, 2 mM Na_2EDTA and 0.1% (w/v) SDS. A mixture of equal quantities of 5 apo(a) isoforms, with 14, 18, 24, 30 and 34 K-4 repeats, from fully characterized subjects were run on each gel as size standards.

Electrophoresis was performed in a horizontal gel apparatus (BRL Life Technologies, Gaithersburg, MD) in 45 mM Tris-HCl, 45 mM boric acid, 2 mM Na_2EDTA and 0.1% (w/v) SDS for 17 h at a constant 100 V at 4°C. After electrophoresis, proteins were transferred to a supported nitrocellulose membrane (Hybond^{TM}-C extra, Amersham, Arlington Heights, IL) by electroblotting using a Hoefer Transphor Cell (Hoefer Scientific Instruments, San Francisco, CA) at 90 V for 3 h in 10 mM Tris-HCl, 40 mM glycine and 5% (v/v) methanol at 4 °C. Prior to electroblotting the gel was soaked in pre-chilled

transfer buffer for 5-10 min and the membrane was soaked first in distilled water for 1 min and then pre-chilled transfer buffer for 5 min.

After electroblotting the membrane was incubated in blocking solution [TBS (0.5 M NaCl and 35 mM Tris-HCl, pH 7.4), 5% (w/v) powdered skimmed milk and 0.2% (v/v) Tween 20] on a rocking platform, for 1 h prior to a 30 min incubation in TBS plus 1% (v/v) NP-40 and either 34μg. $100mL^{-1}$ of $1A^2$ or 4F3. After incubation with the first antibody the membrane was washed four times for 10 min each in 100 ml washing buffer [TBS plus 1% (v/v) NP-40, 0.5% (w/v) deoxycholic acid and 0.1% (w/v) SDS] on a rocking platform.

The membrane was then incubated with a 1:10,000 dilution of an appropriate species-specific affinity purified secondary antibody conjugated to horseradish peroxidase. In the case of $1A^2$ and 4F3 this was a sheep anti-mouse peroxidase linked antibody (Cat # NA931 Amersham). The four cycle wash procedure was then repeated and the membrane was developed by using ECL™ Western blot detection reagents (Amersham) and subjected to autoradiography using Reflection film (Dupont, Willmington, DE) for 10 sec to 10 min.

The ECL™ Western blot detection system is a commercially available enhanced chemiluminescence system. The principle of the ECL™ Western blot detection system is illustrated in figure 2.1. Briefly the enhanced chemiluminescence is achieved by performing the oxidation of the cyclic diacylhydrazide, luminol, by horseradish peroxidase (HRPO) in the presence of chemical enhancers such as phenols. This has the effect of increasing the light emission by approximately 1,000 fold. The light signal produced by this reaction peaks after 5-20 min and decays slowly thereafter with a half-life of approximately 60 min. An example of the high resolution apo(a) phenotyping blot obtained by this method is shown in figure 1.6.

2.5.1 Conjugation of monoclonal antibodies

Mouse monoclonal antibody IgG-$1A^2$ used in the apo(a) immunoblotting system was conjugated to HRPO using the method of Nakane & Kawaoi (1974). This method joins the enzyme to the FC region of the IgG antibody using sodium m-periodate as a "linker" and was performed as follows:

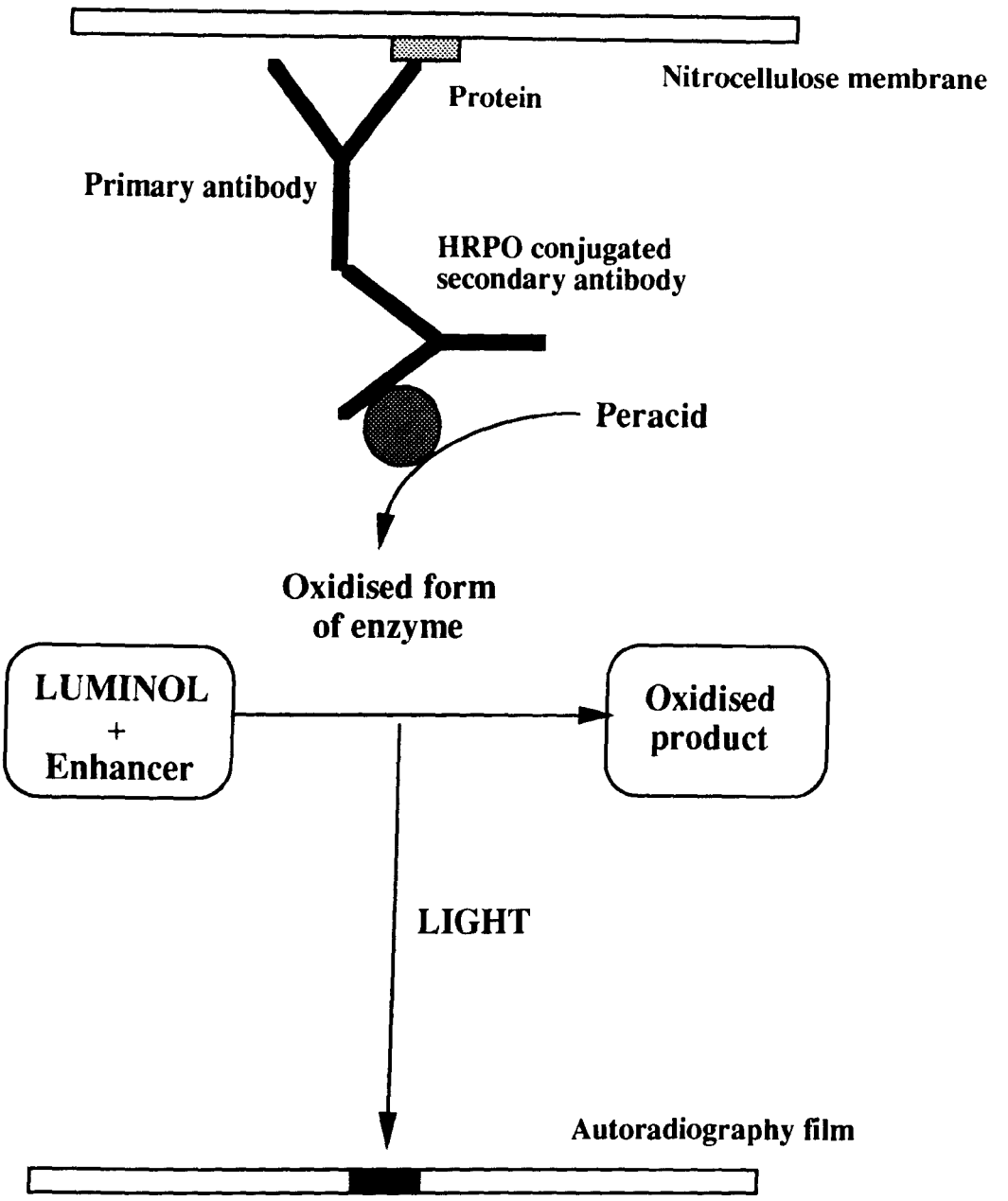


Figure 2.1

Principle of the ECL™ Western blot detection system. This system is a light emitting, non-radioactive method for detection of immobilised specific antigens, conjugated directly or indirectly with horseradish peroxidase (HRPO) -labelled antibodies. Adapted from ECL™ Western blot protocol booklet, Amersham International plc (1995).

a) Lyophilised IgG-1A^2 antibody (4mg) was dissolved in dH_2O (500µL) and dialysed against 10mM $NaHCO_3$ (pH 8.5) overnight at 4°C.

b) HRPO (2mg) was dissolved in dH_2O (500µL) to which was added 50µL 0.2M sodium m-periodate ($NaIO_4$). This mixture was then dialysed against 1mM sodium acetate (pH 4.5) overnight at 4°C.

c) After dialysis the pH of the antibody solution and of the HRPO solution were adjusted to 9.5 with 0.5N NaOH and 0.2M Na_2CO_3 respectively.

d) The two solutions were then combined and mixed on a rocking platform for 2h at room temperature.

e) The reaction was stopped by adding 50 µL sodium borohydride ($NaBH_4$) (4mg. mL^{-1}) and the solution allowed to stand for 2h at 4°C.

f) The solution was dialysed against 75mM Na_2HPO_4, 75mM NaCl and 18mM KH_2PO_4 overnight at 4°C.

g) The conjugate was tested using an optimisation ELISA and stored at 4°C for up to 6 months.

2.6 Apo(a) genotype determination by pulsed-field gel electrophoresis and genomic blotting

The sizes of the *APO(a)* alleles studied were confirmed by apo(a) genotyping using pulsed-field gel electrophoresis by a modification (Gaw et al., 1994) of the procedures originally described by Lackner et al. (1991).

2.6.1 Cellular agarose plug preparation

Leukocytes were isolated from whole blood using LeucoPrep tubes (Becton, Dickinson) as described by the manufacturer. The leukocytes were diluted in PBS to a final concentration of 2 x 10^7 cells. mL^{-1} and placed on ice. A 1% (w/v) solution of low melting point (LMP) agarose in PBS was prepared and cooled to 37°C. Equal volumes of LMP agarose and cell solutions were

mixed gently and 150µL of cellular-agarose mixture was injected into an ice-cold plug mold (Beckman). This mold was tightly wrapped in plastic film and incubated on ice for at least 30 min. The mold was unwrapped and the plugs were discharged by gentle shaking. The plugs were incubated in 0.5M Na_2EDTA (pH 8.0), 1% (w/v) SDS (Bio-Rad), 2 mg. mL^{-1} Proteinase K (Boehringer Mannheim) for 48h at 55°C. The plugs were washed with 0.04mg. mL^{-1} phenylmethylsulphonyl fluoride (PMSF, stock 40mg. mL^{-1} in isopropanol) in 1 x TE buffer (1mM Na_2 EDTA, 10 mM Tris-HCl, pH 8.0) at 55°C twice for 30min each. The plugs were stored in 0.5 M Na_2EDTA, (pH 8.0) at 4°C.

2.6.2 Restriction digest and electrophoresis

The agarose-cellular plug was washed twice for 30min in 1 x TE. A 3-4 mm segment was cut using a sterile razor blade and incubated twice for 2 h each with 100 units *Kpn*I or *Hpa*I (New England Biolabs, Beverly, MA) in 70 µL of the buffer suggested by the manufacturer (Lackner et al., 1991, Boerwinkle et al., 1992).

The plugs were rinsed with 1 x TE buffer and loaded into the wells of a 1% (w/v) agarose gel (without ethidium bromide). The wells were then sealed with molten 1% (w/v) agarose. The gel was subjected to transverse alternating gel electrophoresis using a GeneLine II™ apparatus (Beckman) containing 1 x TAFE II buffer (0.5mM EDTA[free acid], 4.35mM acetic acid, 10mMTris [pH 8.0]) pre-chilled to 14°C. The pulse times were 4 sec for 30 min followed by 6 sec pulses (150 volts/350 mAmps) for 18h. Low range PFG markers (New England Biolabs) were used as size standards.

After electrophoresis the gel was stained with 0.5µg. mL^{-1} ethidium bromide for 10min and photographed in UV light. The gel was rinsed with dH_2O for 10min and soaked in denaturation buffer (1.5M NaCl, 0.5M NaOH) twice for 30min. The size-fractionated DNA was blotted to a nylon membrane (ICN, Biomedicals, Irvine, CA) by southern blotting overnight in 10x SSC. After transfer the blot was baked for 2h at 80°C in a vacuum oven.

2.6.3 Single stranded M13 probe preparation

Lackner and her colleagues (1991) previously subcloned an *APO(a)*-specific K4 probe by amplifying the K4 encoding sequences from the human

APO(a) gene using the polymerase chain reaction (PCR). A single 4.2 kb band was indentified and this fragment was purified, digested with *Pst*I and cloned into *Pst*I restricted and dephosphorylated bacteriophage M13-mp18 DNA. The clones containing inserts were sequenced and one, designated MP-1 shared 15 bases of sequence identity with the lower strand of the apo(a) cDNA.

This probe was made in the Dept. of Molecular Genetics, Dallas, Texas and was made freely available to me. The probe was prepared according to standard protocols as shown in figure 2.2 (Sambrook et al, 1989) and radiolabelled prior to use according to the protocol of Church & Gilbert (1984).

2.6.4 Hybridisation

The filter was prehybridised for 2h at 42°C in a rotating incubator in the following buffer: 25mL 100% formamide, 5mL 100 x Denhardt's solution, 12.5mL 20 x SSPE, 5mL 10% (w/v) SDS (filtered), 1.5mL dH_2O, and 1mL denatured salmon sperm (100mg. mL^{-1}). The filter was then probed with 6 x 10^6 cpm. mL^{-1}) of MP-1, an [α–^{32}P]dCTP radiolabelled human apo(a) K4-specific single-stranded fragment at 42°C overnight as previously described (section 2.6.3 and Lackner et al., 1991).

2.6.5 Washing and exposure

The filter was washed in 500mL of 2 x SSC, 1% (w/v) SDS at room temperature for 15min on a shaker. The filter was then gently scrubbed with a sponge and placed in 1L 0.5 x SSC, 1% (w/v) SDS and incubated at 68°C for 30min. The filter was exposed to X-OMAT™ AR film (Eastman Kodak, Rochester, NY) with an intensifying screen (Lightening Plus Dupont Co., Wilmington, DE) for 16 h at -80°C. The *APO(a)* alleles were designated by the estimated number of kringle 4-encoding sequences per allele *(APO(a) K-12* to *APO(a) K-51* (Lackner et al., 1993). Examples of the Southern blots obtained by this method are shown in figures 1.8 and 1.9.

2.7 LDL receptor genetic analysis

The diagnoses of familial hypercholesterolaemia in the family members described in chapter 5 were made in the Cardiovascular Genetics Research Center, University of Utah, Salt Lake City, UT by Dr. Paul Hopkins and his

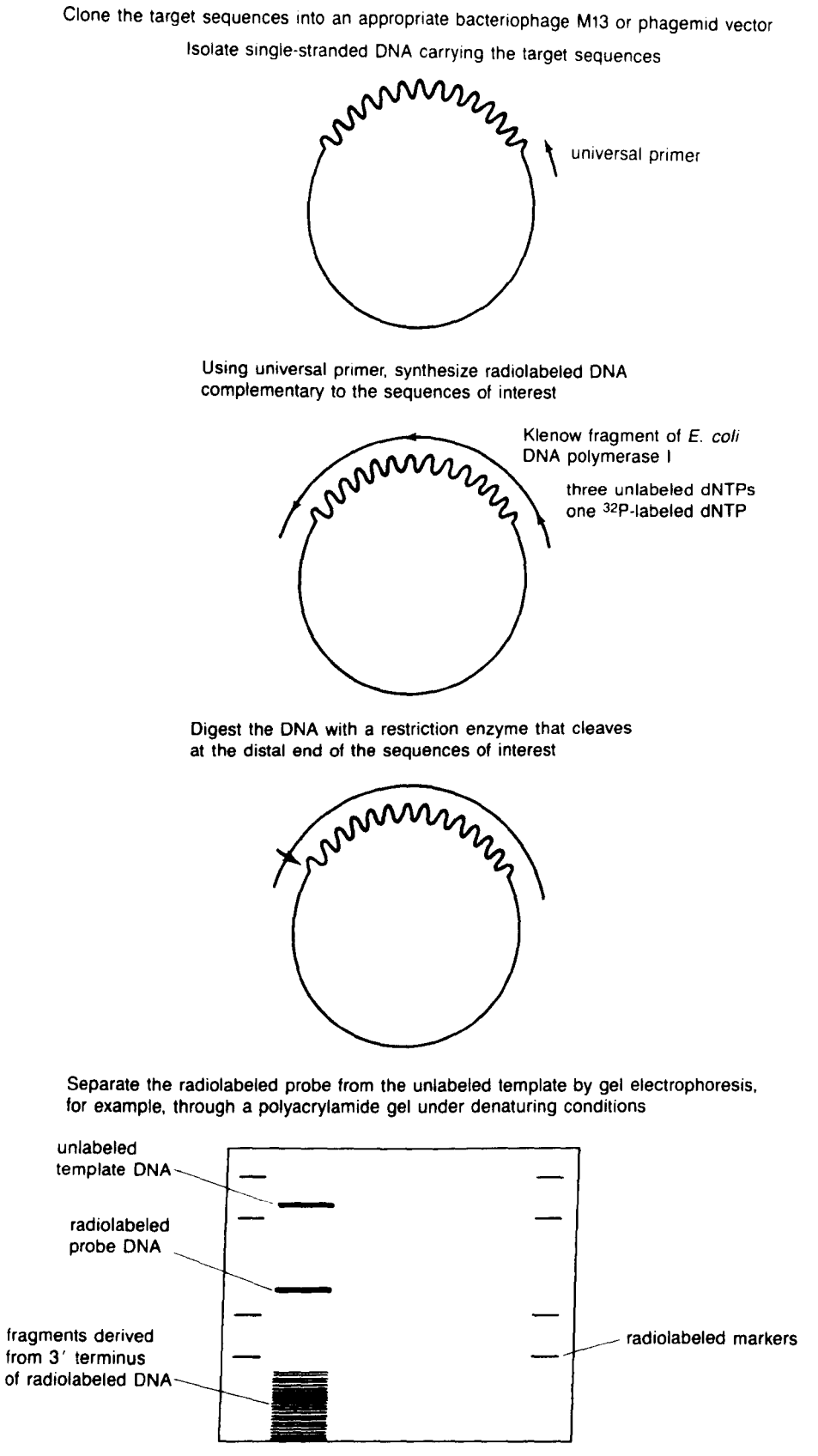


Figure 2.2.

Principle of the synthesis of probes from single-stranded DNA vectors such a M13. Adapted from Sambrook et al. (1989).

colleagues. The methods used were those detailed in Emi et al. (1991). Briefly these were as follows. Venous blood was collected after a 12-16 h fast and genomic DNA was prepared from peripheral lymphocytes as decribed by Cavanee et al. (1984). DNA polymorphisms at the LDL receptor locus were characterised by digesting genomic DNA with the restriction endonucleases *Nco* I, *Msp* I, or *Pvu* II (New England Biolabs). LDL receptor haplotypes were formed by combining the alleles of the three restriction fragment length polymorphisms (RFLPs). The LDL receptor cDNA clone pHHI was used to detect *Nco* I and *Msp* I diallelic RFLPs on Southern Blots (Cavanee et al., 1984; Kotze et al., 1987; Geisel et al., 1987). The other probe used for phenotyping was the LDL receptor genomic clone pRHP17, which detects a *Pvu* II diallelic RFLP (Hegele et al., 1988).

The plasma LDL-cholesterol levels of subjects were compared to the 95th percentile of their age and sex reference values (Lipid Research Clinics Program, 1980). Emi and colleagues (1991) have previously demonstrated that a high LDL-cholesterol phenotype segregated with these markers at the LDL receptor locus without recombination, with a maximum log of the odds score of 4.74, which corresponds to an odds ratio greater than 1 in 50,000.

2.8 Apo B genetic analysis

The diagnoses of hypobetalipoproteinaemia (apoB-67) in the family members described in chapter 6 were made in the Institute for Prevention of Cardiovascular Disease, Deaconess Hospital, Boston, MA by Dr. Francine Welty and her colleagues, using the following methods.

Venous blood samples were obtained after a 14h fast from family members. The blood was collected into vacutainers containing K_2EDTA as anti-coagulant to give a final concentration of 1 mg. mL^{-1}. Plasma was obtained from each of the blood samples by low speed centrifugation, 1000*g* (4^oC). Plasma total cholesterol, triglyceride, LDL-cholesterol and HDL cholesterol concentrations were measured according to LRC protocols as described above (section 2.3.2). The lipoproteins were further characterized and the patients' DNA analysed as described elsewhere (Welty et al., 1991).

The VLDL (d<1.006 g/ml), IDL (d 1.006-1.025 g/ml), LDL (d 1.025-1.063 g. mL^{-1}), and HDL (d 1.063-1.21 g. mL^{-1}) fractions were isolated from

fresh fasting venous plasma by ultracentrifugation (Havel et al., 1955). The total protein content of these fractions were determined by a protein assay using bovine serum albumin as a standard (Lowry et al., 1951). The apolipoprotein content of each lipoprotein fraction was then determined using 3-12% gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on gels stained with either 0.1% Coomasie Brilliant Blue R-250 or silver.

The SDS-PAGE revealed a truncated apoB species migrating identical to the apoB-67 previously described (Welty et al., 1991). To determine the precise mutation in the *APOB* gene responsible for the apoB-67 phenotype, an 842 bp segment of exon 26 of the *APOB* gene (apoB cDNA nucleotides 9013-9854) was amplified using PCR with the following oliginucleotide primers:

B67 5'-TGT CCA ATA AGA TCA ATA GCA AAC ACC TAA GA-3'
(apoB cDNA nucleotides 9004-9035)

B67-2 5'-CTG AAA AAT CTC ACG ACG AGC TCC CCA GGA CC-3'
(complemetary to apoB cDNA nucleotides 9832-9863)

Genomic DNA (0.5 μg) prepared from peripheral white blood cells was amplified using *Taq* polymerase (New England Biolabs) with denaturation, annealing and extension conditions of 96°C for 1min, 62°C for 1min, and 72°C for 2min for 3 cycles and then 96°C for 30sec, 62°C for 1min, and 72°C for 2min, for 27 cycles respectively.

DNA sequencing of the first apoB-67 kindred revealed a deletion of an adenine at cDNA 9327 (Welty et al., 1991). This frameshift mutation converted a lysine to an arginine, followed by a premature stop codon (TAG). Analysis of the genetic map revealed that this mutation deleted an *Mbo*II restriction site at cDNA 9339, 12 bp 3' of the apoB-67 mutation. To determine the precise mutation in the apoB gene responsible for apoB-67 in the other kindreds, the amplified DNA from the probands in each kindred was purified using column chromatography (Qiagen, Irvine, CA) and then digested with *Mbo*II (New England Biolabs) at 37°C overnight, in the buffer suggested by the manufacturer. The products were then subjected to electrophoresis on an 8% polyacrylamide gel for 3 h and stained with ethidium bromide.

After the mutation responsible for the apoB-67 phenotype was determined in each kindred, other family members were apoB phenotyped by

simply analysing freshly prepared lipoprotein samples by SDS-PAGE as described above.

2.9 Ethical considerations

All subjects gave informed written consent to the studies which met with the requirements of the Ethical Committees of the University of Texas Southwestern Medical Center, Dallas, Texas, USA, the Cardiovascular Genetics Research Center, University of Utah, Salt Lake City, UT, USA, or the Institute for Prevention of Cardiovascular Disease, Deaconess Hospital, Boston, MA, USA, as appropriate.

2.10 Statistical methods

In the ethnic studies described in chapter 4, the overall shape of the distribution of plasma Lp(a) concentrations was compared between populations using the two-sample Kolmogorov-Smirnov test (Conover, 1980). The distributions of plasma Lp(a), were clearly not normally distributed, therefore, non-parametric rank-sum tests and Kruskal-Wallis tests (Conover, 1980) were used to compare median Lp(a) levels between racial groups.

Because of the large number of apo(a) phenotypes relative to the numbers of individuals in this sample, traditional chi-square goodness-of-fit test and chi-square tests of independence were inappropriate. A goodness-of-fit test to Hardy-Weinberg expectations was carried out using a computerized permutation algorithm described by Guo & Thompson (1992). Comparisons of apo(a) isoform and phenotype frequencies among races were carried out using a Monte Carlo simulation method to derive p-values for Fisher's exact test (Guo & Thompson, 1989).

The relationship between the apo(a) polymorphism and plasma Lp(a) concentrations was analyzed in several ways. The contribution of the apo(a) genotypes to the inter-individual variance in plasma Lp(a) concentrations was estimated using the adjusted R^2 from an analysis of variance (Neter et al., 1990). The Spearman's rank correlation (Conover, 1980) between the sum of the number of K-4 repeats represented in an individual's genotype and their plasma Lp(a) level was calculated. These rank correlations were compared between populations using Fisher's z-transformation.

In the sibling pair analyses described in chapters 5 and 6 the plasma Lp(a) concentrations were not normally distributed but were highly skewed towards lower plasma levels. Therefore, a comparison of median plasma Lp(a) concentrations was made between sibling groups using the non-parametric test of significance, the Wilcoxon signed rank test. As previously used in a similar analysis by Boerwinkle et al (1992) all analyses were performed on the raw and square-root transformed data. For each analysis, the primary inferences were identical irrespective of which data format was used.

Even though the sibships described in chapters 5 and 6 were often larger than size two, the above methods have been shown to be valid when overlapping sibling pairs are analysed as though they were independent (Amos et al., 1989).

Chapter 3

Characterization of antibodies for use in high resolution apo(a) phenotyping by immunoblot analysis

The rapidity of change and the speed with which new situations are created follow the impetuous and heedless pace of man rather than the deliberate pace of nature.

Rachel Carson, *Silent Spring*, 1962.

3.1 Study Design

The studies described in this thesis rely in part on the quality of the high resolution immunoblotting technique used for apo(a) phenotyping. The preliminary study described in this chapter was designed to assess this assay system and in particular to answer the following questions:

a) What is the sensitivity of the assay?
b) In view of the highly polymorphic nature of the apo(a) glycoprotein is the detection of apo(a) proportional to the size of the number of K4 repeats in the apo(a) protein?
c) How does the IgG-1A^2 anti-Lp(a) antibody used in these studies compare with another commercially available anti-Lp(a) monoclonal?

3.2 Introduction

High resolution apo(a) phenotyping has been used in conjunction with apo(a) genotyping to help elucidate underlying mechanisms of plasma Lp(a) level control (Gaw et al., 1994). High resolution apo(a) phenotyping has also been reported with in-house monoclonal antibodies as an indirect means of assessing apo(a) allele frequencies in different populations (Marcovina et al., 1993b). With increasing interest in the Lp(a) field more laboratories are now establishing apo(a) phenotyping methods. Despite the use of such methods in a number of laboratories throughout the world a series of problems remain unanswered. Principle among these are the questions of specificity and sensitivity of the antibodies used and the availablity of good anti-apo(a) monoclonals that can be used in a high resolution system. To help answer these questions the following evaluation was performed to study the specificity and sensitivity of the antibody used in the other studies reported in this thesis comparing it with one of the few other available anti-Lp(a) monoclonal antibodies available commercially.

3.3 Study protocols

3.3.1 Subjects

From a survey of 500 individuals carried out in the University of Texas Southwestern Medical Centre at Dallas, a panel of 9 subjects were selected who had a single expressing *APO(a)* allele ranging in size from 14 - 34 K-4 repeats and who had a detectable plasma Lp(a) level. Blood sampling of human volunteers was approved by the University of Texas Southwestern Medical Center Institutional Review Board.

3.3.2 Plasma Lp(a) assay

Fasting venous blood samples were collected into Vacutainer Tubes (Becton Dickinson & Co. Rutherford, NJ) containing Na_2EDTA. Plasma was isolated by low speed centrifugation at 1000 x *g* for 15 min at 4°C, aliquotted and stored at -80 °C for up to six months until Lp(a) assay or apo(a) phenotyping were performed. Lp(a) levels have been shown to be stable up to 7 years in samples stored at -20 °C (Jauhiainen et al., 1991). Plasma Lp(a) concentrations were measured in the Northwest Lipid Research Laboratory, Seattle, WA using an in-house enzyme-linked immunosorbent sandwich assay

(ELISA) system described in chapter 2 and in detail elsewhere (Marcovina et al., 1995). This assay does not use either of the antibodies studied in the in the apo(a) phenotyping system as reported here.

3.3.3 Anti-Lp(a) monoclonal antibodies

Two primary antibodies were studied for characterisation and use in high resolution apo(a) phenotyping.

1. IgG-1A^2 is a mouse monoclonal developed in the laboratory of Dr. Gerd Utermann (University of Innsbrück, Austria) and kindly made available for the comparison, but which is now commercially available from Boehringer Mannheim. This antibody is directed against the common K-4 repeat, i.e., type A and type B K-4 repeat (Gaw et al., 1994) and has been used in a number of previous studies by ourselves and others for Lp(a) ELISA (Menzel et al 1990), and low (Utermann, 1989) and high resolution phenotyping (Lackner et al., 1993; Gaw et al., 1994).

2. 4F3 is a commercially available purified mouse monclonal antibody raised against human apo(a) (Cappel Cat #59408) previously only used for immunodetection of apo(a) in transgenic animals (Callow et al., 1994). The epitope in apo(a) to which this antibody is directed is not known. Because 4F3 only reacts with denatured apo(a) a potential site is in the K-4 repetivie region (Organon-Teknika/ Biotechnology Research Institute, Rockville MD, personal communication).

3.3.4 Apo(a) isoform determination by immunoblotting

Apo(a) isoform analysis was performed using a modification of the immunoblotting method described by Kamboh et al. (1991) exactly as described in chapter 2, methods section 2.5 above.

3.3.5 Apo(a) genotype determination by pulsed-field gel electrophoresis and genomic blotting

The sizes of the *APO(a)* alleles in those subjects used in this study were confirmed by apo(a) genotyping using pulsed-field gel electrophoresis by a modification of the procedures originally described by Lackner and her colleagues (1991), and exactly as described elsewhere (Gaw et al., 1994). The

APO(a) alleles were designated by the estimated number of K-4-encoding sequences per allele (*APO(a) K-12* to *APO(a) K-51*) (Lackner et al., 1993) as described in chapter 2.

3.4 RESULTS

3.4.1 Specificity/equivalence of response with different apo(a) isoforms

Plasma samples from the panel of 9 subjects ranging in plasma Lp(a) concentration from 5-88 mg. dL^{-1}, who had a single expressing apo(a) allele were diluted with mouse plasma to render all samples with an equivalent Lp(a) level (5 mg. dL^{-1}). Mouse plasma was used as the diluent because wild type mice have no plasma Lp(a). Equal volumes were loaded on to the SDS-agarose gel as described in the methods and after electrophoresis was immunoblotted with each of the antibodies.

Figure 1 shows the results of two different experiments using the same samples immunoblotted with either of the primary antibodies. Clearly, equal quantities of plasma Lp(a) yield approximately equal intensities of signal irrespective of apo(a) isoform size. This was also the case when different antibody dilutions were tried (data not shown).

Because the apo(a) protein is composed of a chain of very similar kringle-4 repeat domains and because the primary antibodies used are thought to have epitopes in these repeat regions it may have been expected that larger isoforms would have more binding sites and would therefore yield a relatively stronger signal, but this was not the case. Both antibodies showed an equivalence of signal when apo(a) isoforms of very different sizes were studied. The reasons for this remain to be determined by further studies of the primary antibodies, $1A^2$ and 4F3 .

3.4.2 Sensitivity

Serial dilutions of samples with known Lp(a) level and single expressing apo(a) isoform. Previously the detection limit of the $1A^2$ antibody in our immunoblotting system was reported as 0.1 mg. dL^{-1} of Lp(a) (Gaw et al., 1994).

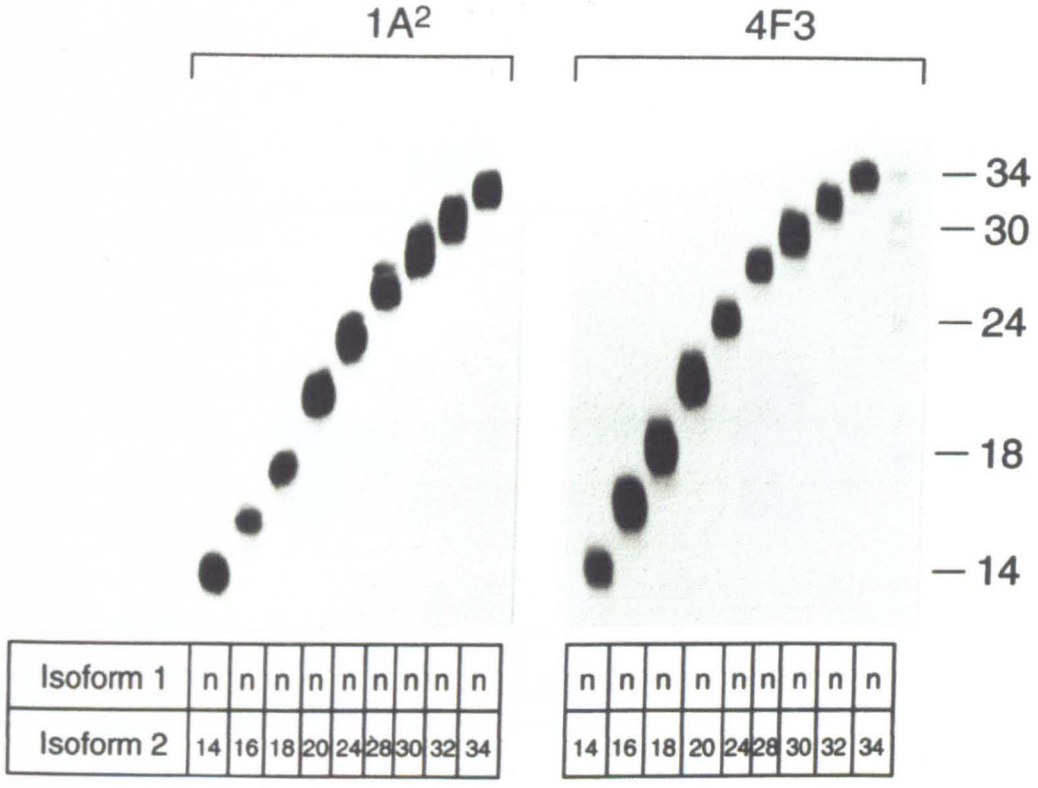


Figure 3.1.

Immunoblot analysis of apo(a) isoforms in human plasma. Plasma samples from 9 subjects with a single expressing apo(a) allele were diluted with mouse plasma to make the Lp(a) concentrations in each sample the same (5 mg. dL^{-1}). A total of 12.5 µl of each diluted plasma was subjected to electrophoresis on a horizontal 2% SDS-agarose gel. The plasma proteins were blotted onto a nitrocellulose membrane and immunoblotted with an apo(a) specific monoclonal antibody ($1A^2$ on the left panel and 4F3 on the right panel). The signals were developed using the ECL system with a 5 min exposure time. The number of kringle 4 repeats for each apo(a) isoform is given and 'n' denoted null allele. A mixture of equal quantities of 5 apo(a) isoforms, with 14, 18, 24, 30 and 34 K-4 repeats, from fully characterized subjects were run on each gel as size standards.

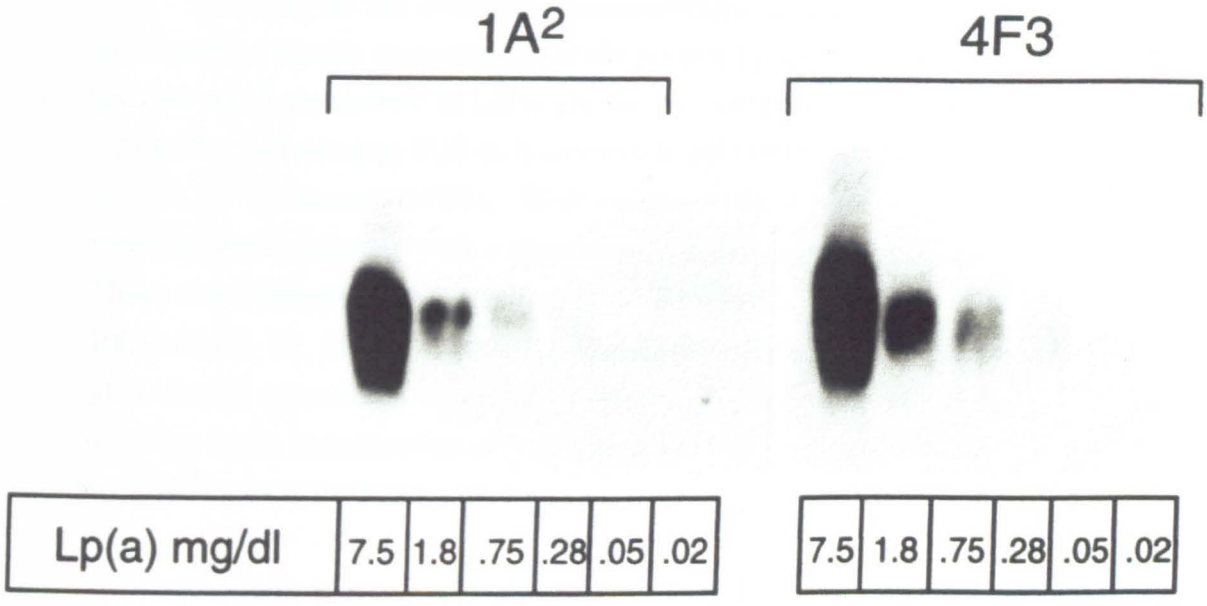


Figure 3.2.

Immunoblot analysis of apo(a) isoforms in serial dilutions of human plasma from a individual with a single expressing apo(a) allele. The sample was loaded neat (first lane in each panel with a concentration of 7.5 mg.dL^{-1}) and diluted with mouse plasma to yield Lp(a) concentrations of 1.8, 0.75, 0.28, 0.05 and 0.02 mg.dL^{-1}. A total of 12.5 µl of each diluted plasma was subjected to electrophoresis on a horizontal 2% SDS-agarose gel. The plasma proteins were blotted onto a nitrocellulose membrane and immunoblotted with an apo(a) specific monoclonal antibody (1A^2 on the left panel and 4F3 on the right panel). The signals were developed using the ECL™ Western blot detection system with a 16 hour exposure time.

With the very long exposures shown in figure 2 levels can be detected down to 0.05 mg. dL^{-1} Lp(a) using both $1A^2$ or 4F3 antibodies. In practice all blots are subjected to short and long (i.e., overnight) exposures in order to determine whether an isoform is present or not. The definition of the 'null allele' must, however, remain an operational one as discussed elsewhere (Gaw et al., 1994, and chapter 4).

3.5 DISCUSSION

Because of the enormous heterogeneity in the size of the apo(a) glycoprotein, simple measurement of the plasma Lp(a) concentration does not provide a full assessment of Lp(a) phenotype. Originally apo(a) phenotyping was performed using an SDS-polyacrylamide gel electrophoresis (SDS-PAGE) system by Utermann (1989). With their system 5 apo(a) isoforms were resolved which together with a putative null allele gave 6 apo(a) phenotypes. These were designated B (equalling apoB-100 mobility), F (faster than apoB-100) and S1, S2, S3 and S4 (slower than apoB-100 by varying degrees).This SDS -PAGE system was modified by others and the resolution was improved resulting in the identification of 9 (Soutar et al., 1991) then 28 (Perombelon et al., 1994) apo(a) isoforms.

Not until Kamboh and his colleagues (1991) suggested the use of an SDS-agarose matrix was it possible to resolve many more apo(a) isoforms. Using this system first 23 (Callow et al., 1994) then 34 apo(a) isoforms were resolved (Lackner et al., 1993, Marcovina et al., 1993b). Further screening and perhaps improved methodology will undoubtedly identify further apo(a) isoforms of different size. It should be noted that both high and low resolution apo(a) phenotyping systems only resolve apo(a) isoforms on the basis of size. A further level of heterogeneity was uncovered when apo(a) isoforms of the same size were found to be different in sequence (Cohen et al., 1993).

Although significant progress in our understanding of the biology of Lp(a) has been made using high resolution apo(a) phenotyping (Lackner et al., 1993; Gaw et al., 1994; Marcovina et al., 1993b), it remains to be seen whether this method has a place in the assessment of the patient by the clinical chemistry laboratory. However, one thing is certain: further research on the pathophysiological role of Lp(a) in human disease is needed. Such research performed without access to high resolution apo(a) phenotyping either alone or

in conjunction with apo(a) genotyping is likely to be severely limited in its impact and its relevance. The availability of good commercially produced antibodies for this purpose should make these assays more widely available for improved clinical research.

Chapter 4

Comparative Analysis of the APO(a) *Gene, Apo(a) Glycoprotein and Plasma Concentrations of Lp(a) in Three Ethnic Groups*

Here individuals of all nations are melted into a new race of men, whose labours and posterity will one day cause great changes in the world.

J. Hector St. John de Crèvecoeur, 1782.
Letters from an American Farmer

4.1 Study background

It has been previously estimated that in Caucasians, approximately 90% of the inter-individual variation in plasma Lp(a) levels is explained by sequence differences at, or closely linked, to the *APO(a)* locus (Boerwinkle et al., 1992). This finding, however, has not been extended to other racial groups to date, which is important since the distributions of plasma Lp(a) levels exhibit marked inter-racial variability.

In Caucasians and Chinese, the distribution of plasma Lp(a) is highly skewed towards lower levels (Utermann, 1989; Albers et al., 1990; Marcovina et al., 1993a, 1993b; Sandholzer et al., 1991). However, in Africans and African-Americans, the distribution curve of plasma Lp(a) levels has a more Gaussian shape (Scanu & Fless, 1990; Marcovina et al., 1993a, 1993b; Parra et al., 1987; Helmhold et al., 1991) and on average, African-Americans have 2-3

fold higher plasma concentrations of Lp(a) than either Caucasians or Chinese. The factors responsible for the racial differences in distribution of Lp(a) levels have not been identified, but previous studies have suggested that differences in apo(a) isoform size distributions are not solely responsible (Marcovina et al., 1993a, 1993b; Sandholzer et al., 1991; Helmhold et al., 1991). The use of apo(a) isoforms in such studies is problematic, however, because standard immunoblotting methods cannot detect protein products from all *APO(a)* alleles. In fact, in only one out of eleven ethnic groups, in which *APO(a)* allele frequencies have been calculated, were the alleles in Hardy-Weinberg equilibrium (Sandholzer et al., 1991; Helmhold et al., 1991).

In this study, high resolution apo(a) genotyping and phenotyping systems have been used to evaluate the relationship between *APO(a)* gene structure, apo(a) glycoprotein size, and plasma concentrations of Lp(a) in three racial groups: Caucasians, Chinese, and African-Americans. This is the first study in which *APO(a)* gene structure, apo(a) glycoprotein size and plasma Lp(a) levels have been simultaneously analyzed in any population.

4.2 Subject characteristics

Venous blood samples were obtained from 174 unrelated American Caucasians (86 men, 88 women), 101 unrelated Chinese (46 men, 55 women), and 106 unrelated African-Americans (20 men, 86 women). All subjects were selected at random from healthy individuals living in the Dallas-Fort Worth area. The only selection criteria were that all Caucasians and African-Americans had to be born in the United States and all Chinese had to be born in Mainland China or Taiwan. Four Caucasians, 17 Chinese and 12 African-Americans were post-menopausal at the time of sampling. A description of the characteristics of the three sample groups is given in Table 4.1, while the individual plasma Lp(a), apo(a) phenotype and apo(a) genotype of all subjects is given in appendix II. There was no significant difference in the ages, or plasma levels of total cholesterol or LDL-cholesterol of the 3 groups. The African-Americans had slightly lower plasma concentrations of triglyceride and higher concentrations of high density lipoprotein HDL-cholesterol than the other two racial groups.

4.3 Plasma Lp(a) distributions

The distribution of plasma Lp(a) concentrations in each of the three racial groups is shown in Figure 4.1. The characteristics of the distributions of plasma Lp(a) levels were compared in the three populations [Table 4.2]. In all populations, the plasma Lp(a) concentrations varied over a wide range. As reported previously, (Marcovina et al., 1993a, 1993b; Sandholzer et al., 1991; Helmhold et al., 1991), the plasma levels of Lp(a) were more highly skewed towards the lower range in the Chinese (skew=3.77) and Caucasians (skew=1.33) than in the African-Americans (skew=0.84). There was only a marginal statistical difference between the overall distribution of plasma Lp(a) levels (Kolmogorov-Smirnov, p=0.06) between the Chinese and Caucasians; the distributions in both populations were significantly different from the African-Americans. ($p<0.001$). In the Caucasians and Chinese, more than 45% of the individuals had plasma Lp(a) concentrations less than 9 mg. dL^{-1} whereas only 12% of the African-Americans had plasma Lp(a) levels in this range. In the African-Americans, the distribution of plasma Lp(a) concentrations was relatively uniform up to approximately 50 mg. dL^{-1}, whereas in the other two populations there was a dramatic decrease over this range [Figure 4.1].

Since plasma Lp(a) concentrations in the three populations were not normally distributed, the median rather than mean plasma Lp(a) concentrations was used to compare levels. There was no significant difference in median plasma Lp(a) levels in the Caucasian and Chinese populations (Kruskul-Wallis, p=0.21). In the African-Americans, the median plasma concentration of Lp(a) was, however, 2-3-fold higher than in the other two racial groups.

4.4 Analysis of *APO(a)* gene

The K-4 encoding region of the *APO(a)* alleles was analyzed using pulsed-field gel electrophoresis and genomic blotting (Lackner et al., 1993) as described in chapter 2. The alleles were classified according to the estimated total number of K-4 repeats contained within their sequences (*APO(a)12-APO(a)51*). The observed genotype frequencies in each of the racial groups was consistent with Hardy-Weinberg expectations (Guo & Thompson, 1992). The distributions of *APO(a)* allele sizes in the three groups are shown in Figure 4.2. In both the Caucasians and African-Americans, the distributions of *APO(a)* alleles were remarkably symmetrical and they were not significantly different from each other (Fisher exact test, p=0.17). However, the

Table 4.1

Description of the samples from each of the 3 populations

Race	n	Age (years)	Plasma levels (mmol. L^{-1}) TC*	TG	LDL-C	HDL-C
Caucasian	174	38 (9)	5.1 (1.6)	1.45 (1.05)	3.1 (0.9)	1.3 (0.4)
Chinese	101	44 (16)	4.9 (0.9)	1.59 (1.22)	3.0 (0.8)	1.3 (0.3)
African-American	106	39 (10)	5.1 (1.0)	1.13 (0.63)	3.3 (1.0)	1.4 (0.3)

*All values are given as means (SD).
TC total cholesterol; TG triglyceride; LDL-C low density lipoprotein cholesterol; HDL-C high density lipoprotein cholesterol.

Table 4.2

Description of plasma Lp(a) concentrations in U.S. Caucasians, Chinese and African-Americans.

Race	Mean	Median (mg. dL^{-1})	Standard Deviation	Skew	Kurtosis	Min. (mg. dL^{-1})	Max.
Caucasian	17.9	9.0	19.1	1.33	4.22	<1	88.0
Chinese	14.7	8.0	21.1	3.77	22.27	<1	156.0
African-American	37.7	33.0	26.9	0.84	3.33	<1	120.0

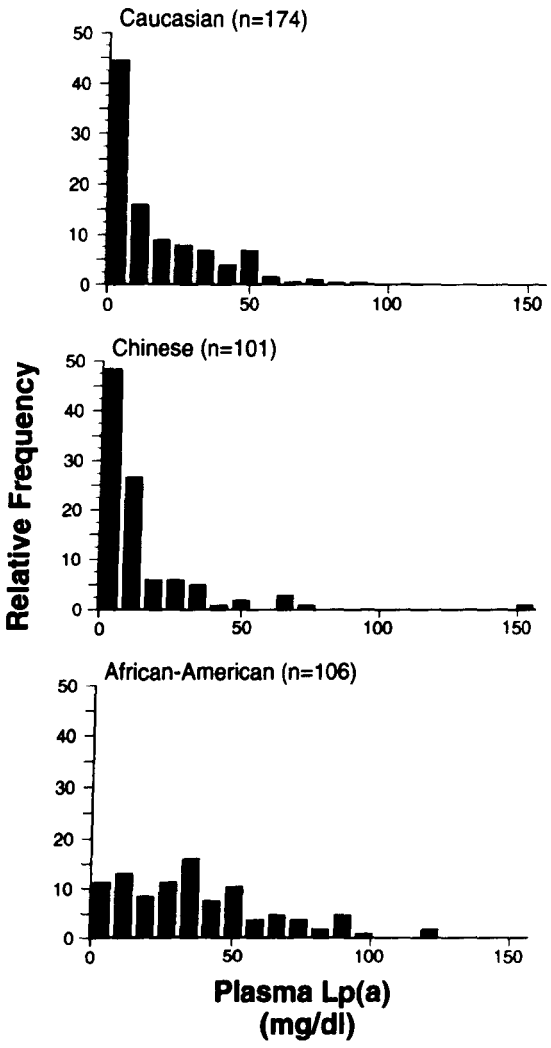


Figure 4.1

Frequency distributions of plasma Lp(a) concentrations in three populations. Plasma Lp(a) levels were measured in 381 subjects by a sandwich ELISA, as described in Section 2.4.3. The width of each bar corresponds to a plasma Lp(a) concentration interval of 7.8 mg. dL^{-1}. Figures in parentheses show number of subjects studied. Individual plasma Lp(a) levels for all subjects are shown in appendix II.

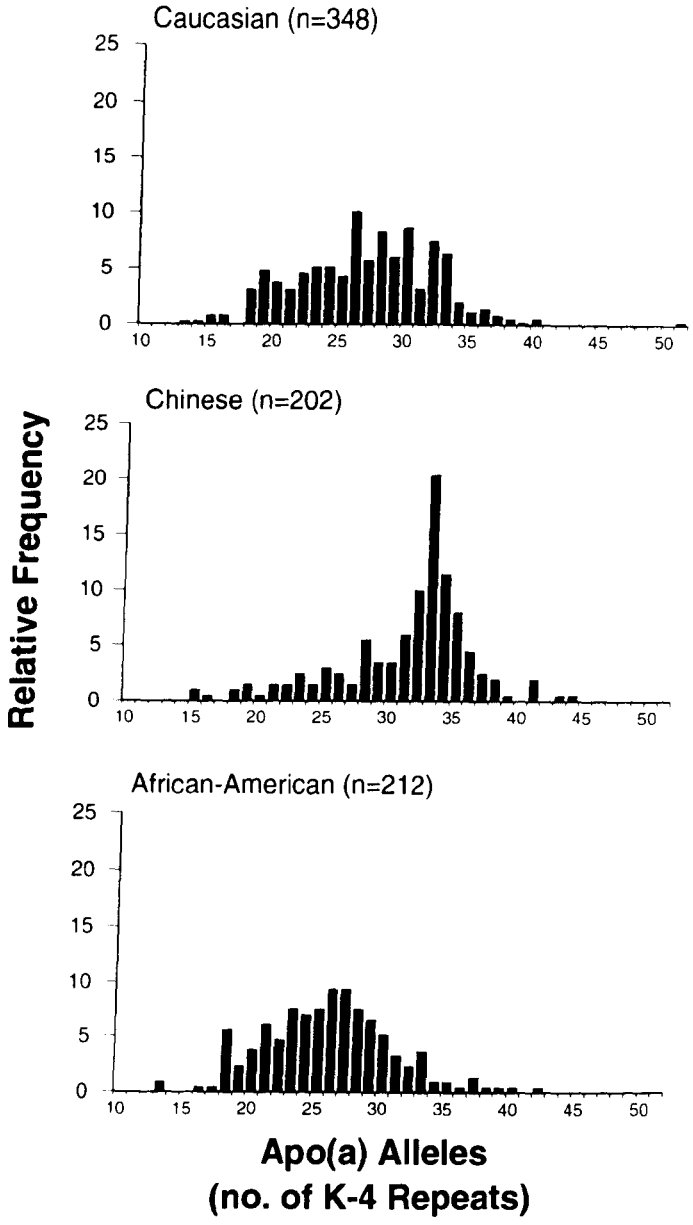


Figure 4.2

Frequency distributions of *APO(a)* alleles in three populations. *APO(a)* allele size (number of K-4 repeats) was estimated using pulsed-field gel electrophoresis and genomic blotting, as described in Section 2.6. Figures in parentheses show number of alleles studied. Individual *APO(a)* allele sizes for all subjects are shown in appendix II.

distributions of *APO(a)* alleles in the Caucasians and African-Americans were significantly different from the Chinese ($p<0.001$). In the Chinese population, the mean size of the apo(a) alleles was significantly larger than in the other two groups.

Due to the large number of different sized alleles in these populations, post hoc statistical comparisons of the frequencies of individual alleles must be viewed with caution. Nonetheless, some striking differences were apparent. A single *APO(a)* allele, *APO(a)-33*, comprised 20% of the alleles in the Chinese sample, whereas the most frequent allele in either the Caucasians or African-Americans contributed only 10% to the alleles in these populations. The high frequency of a single *APO(a)* allele in the Chinese suggests that the heterozygosity index in this population should be lower. This was indeed the case. The observed frequency of individuals homozygous for *APO(a)* alleles of identical size was highest in the Chinese (5.5%) and lowest in the Caucasians (2.9%) with the African-Americans having an intermediate level (4.7%).

4.5 Relationship between apo(a) genotype and plasma concentration of Lp(a)

The relationship between apo(a) genotypes and plasma concentrations of Lp(a) was examined in each population [Figure 4.3]. The apo(a) genotypes are represented on the floor (x and y axes) of the 3-D diagram. The vertical bars (z axis) represent the median plasma concentrations of Lp(a) for all individuals with a given apo(a) genotype. Although the patterns of apo(a) genotypes differed among the 3 populations, there was a similar inverse relationship between the size of the *APO(a)* alleles and the plasma concentration of Lp(a), as indicated by the decrease in the height of the bars from left to right. The Spearman rank correlation values for the relationship between the sum of the *APO(a)* allele sizes (i.e. total number of K-4 repeats) and the plasma levels of Lp(a) were remarkably similar in the three populations: -0.49 (Caucasians and African-Americans) and -0.53 (Chinese). The Chinese group had a significantly different "footprint pattern" on the floor of the graph from the other two populations, reflecting the difference in *APO(a)* allele frequencies (noted above). The Caucasians and African-Americans had more similar "footprint patterns", but had distinctly different "skyline patterns", that is, for almost all apo(a) genotypes, the African-Americans had higher median plasma Lp(a) levels than the Caucasians. While not apparent from this figure [4.3], it should be noted that the variance in plasma Lp(a) levels for each genotype was

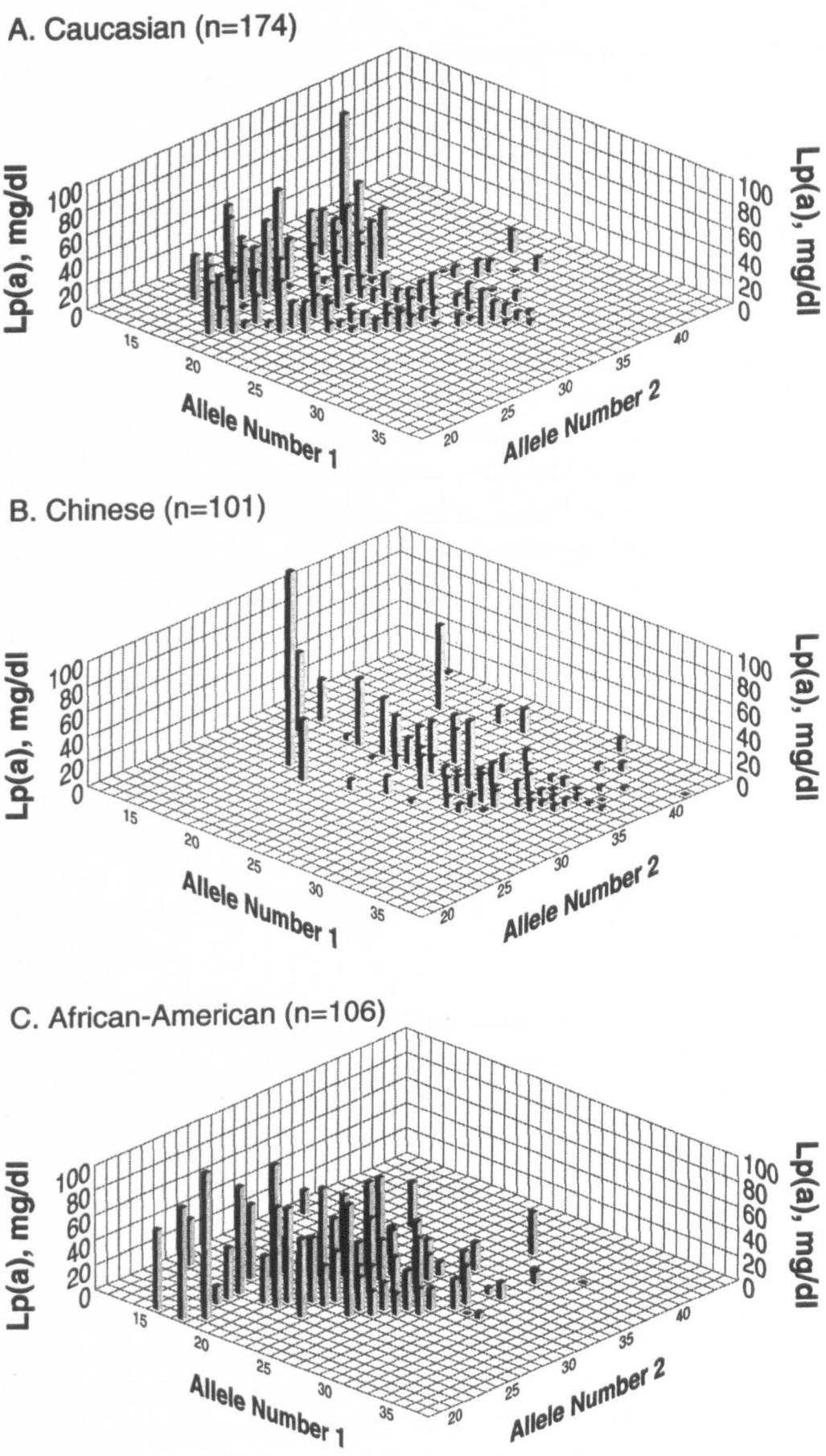


Figure 4.3

Relationship between apo(a) genotype and plasma Lp(a) concentration in three populations. In each 3D plot, the number of K-4 repeats of the smaller *APO(a)*allele (*Allele 1*) is given on the x-axis. The number of K-4 repeats in the larger *APO(a)* allele (*Allele 2*) is shown on the y-axis. The vertical bars (z-axis) represent the median plasma Lp(a) concentration of individuals with each genotype. Individual data for all subjects are shown in appendix II.

higher in African-Americans (s^2 = 664) than in the Caucasians (s^2 = 166) or Chinese (s^2 = 133).

The higher variance in plasma Lp(a) levels in the African-Americans is made more obvious if the total number of K-4 repeats is related to the plasma concentrations of Lp(a) [Figure 4.4]. In all three populations distribution of plasma Lp(a) levels *vs.* total number of K-4 repeats was triangular in appearance. The relationship between *APO(a)* allele size and plasma Lp(a) level is not linear over the entire range. The relationship appears to be biphasic; Caucasian and Chinese individuals with greater than approximately 65 K-4 repeats had uniformly low plasma concentrations of Lp(a) [Figure 4.4]. The variability in plasma Lp(a) concentrations was greater among individuals with fewer rather than more K4 repeats, and the variability was greatest in the African-Americans. This effect may be explained in part by the fact that those individuals with fewer K-4 repeats comprise more than one group of individuals: those with one large and one small *APO(a)* allele, or those with two medium sized alleles. In contrast, almost all the individuals with a larger total number of K-4 repeats e.g., > 65, constituted a more uniform group because their K-4 complement could only result from the addition of two large alleles, and not a very small plus a very large allele because such alleles (<12 or >51 K-4 repeats) were not observed in this study.

4.6 Relationship between *APO(a)* allele size and apo(a) glycoprotein

Next, the relationship between *APO(a)* allele size and the presence of detectable apo(a) glycoprotein in plasma was examined. The apo(a) isoforms were analyzed by immunoblotting, as described in chapter 2, in the entire Chinese group, in all but six of the African-Americans, and in 155 of the 174 Caucasians. Immunoblotting data were not obtained in 25 subjects (6 African-Americans and 19 Caucasians) because blood samples were obtained from these individuals prior to the development of the more sensitive immunoblotting technique. The alleles were classified into two groups based on whether or not there was clearly detectable plasma apo(a) protein present on immunoblotting. The analysis of serial dilutions of plasma samples of known Lp(a) concentration, reported in chapter 3, revealed that *APO(a)* alleles associated with a plasma concentration of Lp(a) $\geq$ 0.05 mg. dL^{-1} were invariably detectable. Only those *APO(a)* alleles in which no protein product was identified by immunoblotting (i.e., associated with plasma Lp(a) concentrations < 0.05 mg. dL^{-1}) are referred to as "null" alleles and are included in Figure 4.5.

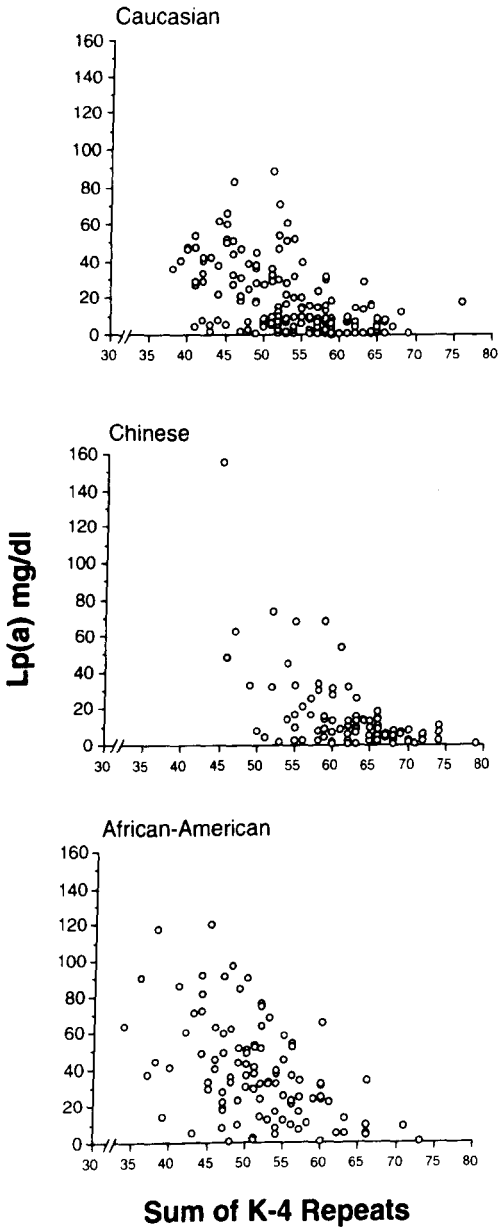


Figure 4.4

Relationship between the sum of K-4 repeats in the *APO(a)* alleles and plasma Lp(a) concentration in three populations. The total number of K-4 repeats from each individual, obtained by summing both *APO(a)* allele sizes, is plotted against the individual plasma Lp(a) concentrations.

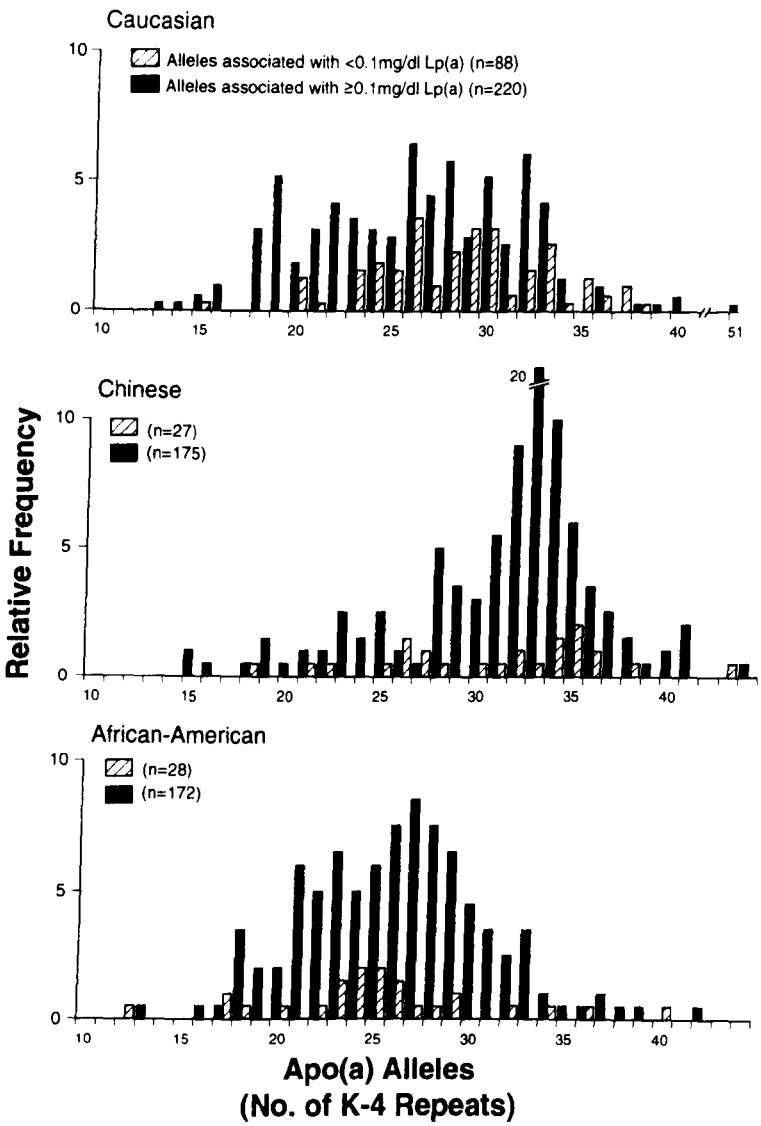


Figure 4.5

Frequency distributions of expressing and non-expressing *APO(a)* alleles in three populations. *APO(a)* allele and isoform sizes were both determined in each subject. The relative frequencies of *APO(a)* alleles associated with detectable protein [i.e. ≥ 0.05 mg. dL^{-1} plasma Lp(a)] and non-detectable protein [i.e. < 0.05 mg. dL^{-1} plasma Lp(a)] are plotted according to race. In all populations, the non-expressing alleles are spread throughout the entire spectrum of *APO(a)* allele sizes. Individual *APO(a)* allele and apo(a) isoform sizes for all subjects are shown in appendix II.

Since future refinement of the immunoblotting technique may reveal protein products that are presently undetectable, the designation of null alleles is qualified by using quotation marks.

The frequency of *APO(a)* alleles with undetectable protein products was highest in the Caucasians (28.6%), and was similar in the African-Americans and Chinese (14.0% and 13.4%, respectively). The frequency of individuals who had no detectable apo(a) protein by immunoblotting was 5.7% in the Caucasians, 2.0% in the Chinese and 2.8% in the African-Americans. In all three populations, the frequency distribution of alleles associated with trace to no detectable apo(a) protein [i.e. < 0.05 mg. dL^{-1} Lp(a)] was similar to the frequency distribution of alleles associated with detectable apo(a) protein [i.e. ≥ 0.05 mg. dL^{-1} Lp(a)]. Thus, despite the overall relationship between *APO(a)* allele size and plasma Lp(a) level in all three populations, the frequency of low expressing alleles was not skewed towards the larger alleles, as might have been expected based on the overall relationship between *APO(a)* allele size and plasma level of Lp(a).

4.7 Relationship between *APO(a)* allele size and plasma level of Lp(a)

Finally, the relationship between *APO(a)* allele size and plasma level of Lp(a) was examined in the subset of individuals who had one allele which expressed no, or only trace amounts of, immunodectable apo(a) protein. For this analysis, we assume that essentially all of the plasma Lp(a) measured by the ELISA is the product of a single expressing allele [Figure 4.6]. Again, in the Caucasians, there was a distinct triangular relationship between Lp(a) level and the size of the expressing allele. *APO(a)* alleles with less than 25 K-4 repeats showed much greater variability in the associated plasma Lp(a) levels. *APO(a)* alleles with greater than 28 K-4 repeats consistently were associated with plasma Lp(a) levels less than 10 mg. dL^{-1}. There were too few individuals with single expressing alleles in the Chinese or African Americans to make any definitive comments about the relationship to plasma Lp(a) level, although the pattern in both groups also appears to be triangular, especially in the African-American subset [Figure 4.6].

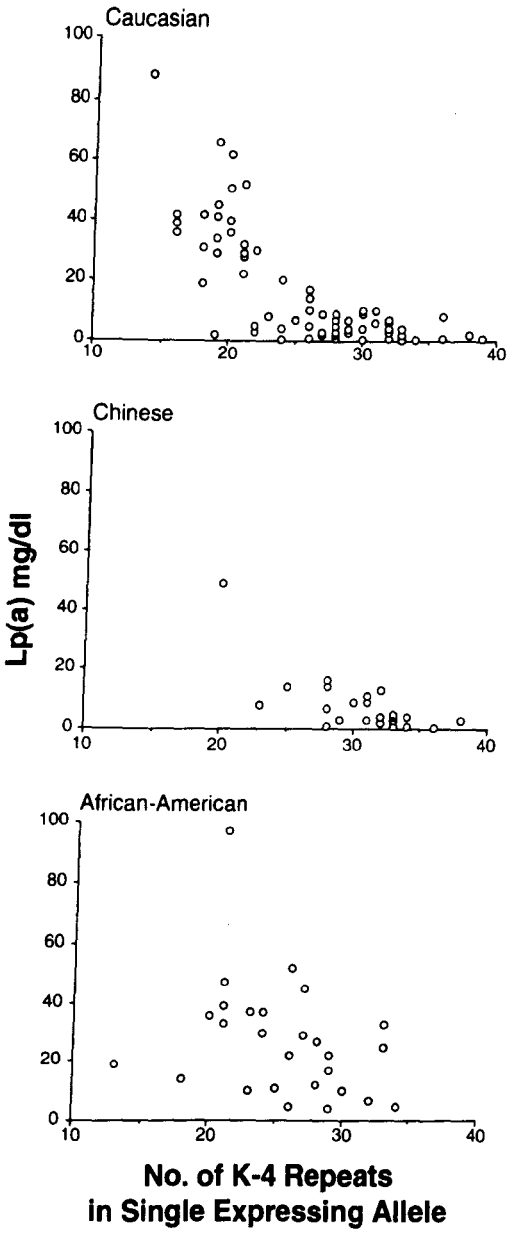


Figure 4.6

Relationship between the size of a single expressing *APO(a)* allele and plasma Lp(a) concentration in three populations. A number of individuals in each racial group had only a single expressing *APO(a)* allele (Caucasians, n=68; Chinese, n=23; African-Americans, n=27). The sizes (number of K-4 repeats) of these *APO(a)* alleles are plotted against the corresponding plasma Lp(a) concentrations revealing a similar pattern for each racial group as that illustrated in figure 4.4.

4.8 Discussion

4.8.1 Insights into the general control of plasma Lp(a) levels

This is the first study in which the *APO(a)* gene structure, apo(a) glycoprotein size, and plasma concentration of Lp(a) have been compared in any population. The apo(a) genotype frequencies in all three racial groups were in accordance with Hardy-Weinberg equilibrium, which is consistent with the samples studied being representative of the populations from which they were drawn. This is in contrast to the findings of previous studies that examined the size distribution of *APO(a)* alleles indirectly by examining the apo(a) isoforms (Sandholzer et al., 1991; Helmhold et al. 1991). In those studies, not all *APO(a)*allele protein products were detected, as indicated by the high estimated frequency of null alleles and the large number of subjects with only a single visible isoform. Direct analysis of the *APO(a)* gene obviates the problems associated with non-detection of *APO(a)* allele protein products when trying to assess the apo(a) genotype frequency. Consequently, in this study two *APO(a)* alleles of different sizes were observed in 97% of Caucasians, 95% of Chinese and 94% of African-Americans.

A major finding of this study is that the differences in plasma concentrations of Lp(a) between Caucasians, Chinese and African-Americans are not due to in differences in the *APO(a)* allele size distributions. This conclusion is evident from the following observations: 1) The Caucasians and Chinese had very similar distributions of plasma Lp(a) levels and yet had significantly different *APO(a)* allele distributions and 2) The African-Americans had a distinctly different plasma Lp(a) distribution from the other two groups, yet their *APO(a)* allele size distribution was not significantly different from the Caucasians. The similarity in the distribution of *APO(a)* alleles in Caucasians and African-Americans cannot be solely attributed to genetic admixture, since it has been estimated that only approximately 25% of the genes in African-Americans are Caucasian in origin (Chakraborty et al., 1992). Admixture may contribute to the observed modest skewing of African-American plasma Lp(a) concentrations to lower levels [Figure 4.1].

It is striking that the relationship between *APO(a)* allele size and plasma Lp(a) level was so similar in all three ethnic groups. The uniformity of this relationship suggests a fundamental underlying biological mechanism. Interestingly, we found that the relationship between *APO(a)* allele size and plasma level of Lp(a) was almost identical with previous reports based on the

analysis of the apo(a) isoforms (Marcovina et al., 1993a, 1993b; Sandholzer et al., 1991; Helmhold et al., 1991). The reason for this concordance has been elucidated by simultaneous analyses of the *APO(a)* gene and apo(a) isoforms. It had been anticipated, given the inverse relationship between *APO(a)* allele size and plasma Lp(a) level, that the *APO(a)* alleles associated with no detectable circulating apo(a) would have been of larger size, but this was not the case. The *APO(a)* alleles which were associated with little to no apo(a) protein had a very similar size distribution to those alleles with detectable protein products. Therefore, the *APO(a)* alleles undetected by immunoblotting in prior studies (Marcovina et al., 1993a, 1993b; Sandholzer et al., 1991; Helmhold et al., 1991) did not bias the relationship between *APO(a)* allele size and plasma Lp(a) level.

There are two possible explanations for the fact that *APO(a)* alleles associated with little or no plasma apo(a) protein are distributed throughout the entire size spectrum. First, the *APO(a)* alleles associated with very low concentrations of plasma protein may be the result of several different mutations that interfere with apo(a) synthesis or secretion. Given the cysteine-rich nature of this protein, it is likely that even rather conservative amino acid substitutions could disrupt proper folding and subsequent transport of this protein out of the hepatocyte (Amara, Cheng & Smith, 1992). Alternatively, there may be a few common mutations in the *APO(a)* gene which arose early in the gene's evolution and now are associated with *APO(a)* alleles of widely varying sizes due to subsequent recombination at the locus.

It is interesting that the plasma levels of Lp(a) in the Chinese population were not lower than the Caucasians, given the shift of the *APO(a)* alleles to the larger sizes [Figure 4.2]. This is probably due to the fact that a much larger percentage of *APO(a)* alleles in the Caucasian population were associated with little to no circulating apo(a) protein.

Despite the overall inverse relationship between *APO(a)* allele sizes and plasma concentrations of Lp(a) in all 3 populations, there were notable exceptions to this general trend. *APO(a)* alleles containing fewer K-4 repeats tend to be associated with higher plasma concentrations of Lp(a) but they can also be associated with little to no detectable plasma apo(a). There is considerably less variation in the plasma levels associated with *APO(a)* alleles at the high end of the size spectrum. In both the Caucasians and the Chinese, the *APO(a)* alleles containing more than 25 K-4 repeats were uniformly associated

with low plasma levels of Lp(a) (i.e. less than 10 mg. dL^{-1}) [Figure 4.6]. This is consistent with the observation that apo(a) isoforms of large size are processed inefficiently and secreted at very low levels in primary cultures of baboon hepatocytes (White et al., 1993, 1994). However, it must be noted that in the African-Americans, some very large alleles are associated with significant amounts of plasma Lp(a) protein (> 20 mg. dL^{-1}). Therefore, there is no intrinsic property of large apo(a) isoforms that precludes their synthesis and secretion.

A possible confounding factor in this study is the relative excess of women in the African-American sample. In all populations studied to date there has been no significant difference between plasma Lp(a) levels in men and women. The one exception is post-menopausal women where plasma concentrations of Lp(a) are increased by an average of 15% (Heinrich et al., 1991). To ensure that the higher plasma Lp(a) levels in the African-American population were not due to a relative excess of women in the sample, the median plasma Lp(a) levels were compared in the African-American men and women and were found not to be statistically different (30.5 men *vs.* 33.5 mg. dL^{-1} women). Similarly, when the sample was stratified to exclude African-American post-menopausal women and compared to the total female group of African-Americans, the median level was almost identical (32.5 *vs.* 33.5). Therefore, the results of this study are not biased significantly by the differences in sex ratios.

In previous studies, the contributions of *APO(a)* allele size polymorphism to inter-individual variations in plasma Lp(a) levels have been calculated. However, these calculations are based on the ratio between the variance in *APO(a)* allele size and the variance in plasma Lp(a) level. As noted previously, for almost every genotype, the plasma concentrations of Lp(a) vary much more in the African-Americans than in either of the two other populations. Accordingly, the size polymorphism appears to contribute much less to the inter-individual variability in plasma concentrations of Lp(a) in the African-Americans due to the greater variance in plasma Lp(a) levels in this population. Therefore, the significance of such calculations is not clear.

A compelling question that remains unanswered by this study is why African-Americans have higher plasma concentrations of Lp(a) than Caucasians despite the similar *APO(a)* allele distributions. We can definitively state that the higher plasma concentrations of Lp(a) in African-Americans are not due to a

higher frequency of *APO(a)* alleles with fewer K-4 repeats. Nor, do African-Americans have fewer *APO(a)* alleles associated with little or no circulating apo(a) protein. Rather, high Lp(a) levels in African-Americans appear to be due to a more general mechanism. For almost every apo(a) genotype, the African-Americans had higher median plasma levels of Lp(a) than did Caucasians or Chinese. To date, little is known about the genetics of plasma Lp(a) concentrations in African-Americans. In Caucasians, the *APO(a)* gene, or sequences linked to it, contribute > 90% to the inter-individual variation in plasma Lp(a) levels (Boerwinkle et al., 1992). The relative contribution of the *APO(a)* locus to plasma levels of Lp(a) in individuals of African descent has not been determined. The fact that the relationship between *APO(a)* allele size and plasma Lp(a) level is similar in the African-Americans and the Caucasians suggests that sequences at the *APO(a)* locus contribute importantly to plasma levels of Lp(a) in the African-American population. If indeed the *APO(a)* gene is a major determinant of plasma Lp(a) levels in African-Americans, there appear to be additional factors, genetic or non-genetic, that modify the effect of the *APO(a)* allele size in this group.

4.8.2 Insights into human evolution

The enormous polymorphism of the *APO(a)* gene offers us a useful marker with which to study other genetic and evolutionary problems. The evolution of the *APO(a)* gene itself has been the subject of much debate. Workers have attempted to resolve the discontinuity of the the *APO(a)* gene in the mammalian lineages while trying to date the duplication event that led to the separation of the ancestral plasminogen and *APO(a)* genes in antiquity. Early attempts placed this event 40 million years ago during primate evolution (McLean et al., 1987) but more detailed studies push the event back into a much more distant past, some 90 millon years ago, immediately before the radition of the mammals (Pesole et al., 1994).

The comparative analysis of the *APO(a)* gene and its product presented here may be used to examine a much more recent event, in geological terms; the evolution of man. By studying racial differences at a genetic level, as has been done here, it is possible to offer supportive evidence for the 'out of Africa' model of *Homo sapiens* evolution as set forth by Stringer and Andrews (1988).

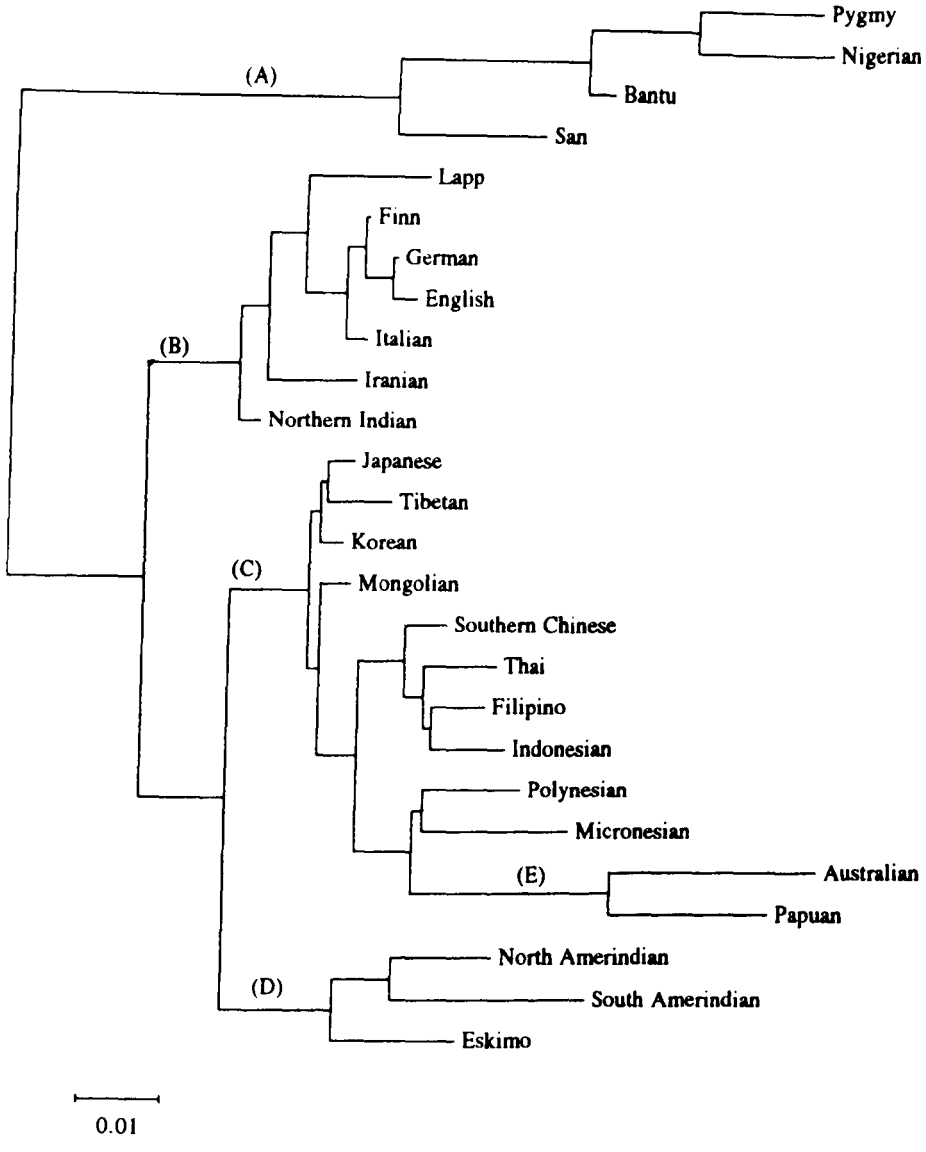


Figure 4.7

Phyllogenetic tree for 26 representative human populations. Major groups of human populations are Africans (A), Caucasians (B), Greater Asians (C), Amerindians (D) and Australopapuans (E). As noted in the main text the African-Americans in the present study represent an approximate genetic mix of African: Caucasian of 3:1. The American Caucasians in the study are all the descendants of West and East Europeans with little if any genetic admixture from other races. The Chinese in the study were all born in mainland China or Taiwan and again have little or no genetic admixture. The scale of each branch length is shown in D_A (modified Cavalli-Sforza units of genetic distance). Adapted fron Nei & Roychoudhury (1993).

In this theory it is proposed that *H. sapiens* originated approximately 200,000 years ago in Africa and later populated the globe by migration. A rival theory, the multiregional theory (Wolpoff et al., 1984) contends that *H. sapiens* evolved from a predecessor, *H. erectus* , simultaneously in several different areas in the world over the last one million years. Recent calculations of genetic distances between living human races (Nei & Roychoudhury, 1993) demonstrate a closer caucasoid/mongoloid than negroid/mongoloid relationship, with the caucasoid/negroid genetic distance intermediate between the two [Figure 4.7]. This suggests a population splitting in early human evolution between Africans and non-Africans and a further second level split later between Caucasians and other non-Africans, including Chinese.

In the present study, the greater similarity between Caucasian and African-American *APO(a)* allele distributions than that between Chinese and African-Americans is consistent with this phyllogeny. There is, as mentioned above, considerable genetic admixture between Caucasian and African-American populations and it has been estimated that approximately 25% of the African-American genome is in fact Caucasian (Chakraborty et al., 1992). However, if the gentic distances between West Africans and Western Europeans, as reported by Nei and Roychoudhury (1993) are corrected for this admixture rate, the African-American/Caucasian distance is still intermediate between the Caucasian/Chinese and the African-American/Chinese and the conclusion remains intact.

When plasma Lp(a) distributions are examined in this light it becomes clear that the same phyllogenetic tree derived from *APO(a)* allele distributions is not sustained. This apparent paradox has also been observed with many other protein and enzyme polymorphisms where a consistent negroid split from all other populations has been reported (Stringer & Andrews, 1988; Nei & Roychoudhury, 1993) but there is currently no accepted explanation. However, because the plasma Lp(a) phenotype is largely, though not exclusively attributable to the apo(a) length polymorphism, the distinctly different pattern of Lp(a) plasma concentrations in African-Americans in this study must mean that in this population other controlling factors, genetic and/or environmental are at play.

Chapter 5

The influence of LDL receptor gene defects on plasma Lp(a) levels

Democracy loosens social ties, but it tightens natural ones. At the same time as it separates citizens, it brings kindred closer together.

Alexis de Tocqueville, 1835.
Democracy in America

5.1 Study background

Many fundamental questions remain unanswered about the metabolism of Lp(a) largely because there are no stable cell lines or simple, accessible animal models that express the *APO(a)* gene in an authentic genomic context. Because Lp(a) is closely related in structure to LDL, the metabolism of LDL has been used as a paradigm to study the metabolic control of Lp(a). The LDL receptor plays a pivotal role in determining the plasma LDL concentration. Considerable efforts have therefore been made to define the role of the LDL receptor in the control of plasma Lp(a) concentrations. Many factors, both genetic and non-genetic impact on the function of the LDL receptor. The activity of this transmembrane protein not only effects the clearance, but also the production rates of LDL (Gaw et al., 1993). Thus, mutations in this protein are associated with dramatic elevations in plasma concentrations of LDL-cholesterol and are the cause of the autosomal codominant disorder familial hypercholesterolemia (FH) (Goldstein & Brown, 1989). To date over 150 different mutations of the LDL receptor gene have been identified (Hobbs et al.,

1992). FH heterozygotes have a two-three fold increase in plasma LDL-cholesterol levels associated with tendon xanthomas and premature coronary heart disease. FH homozygotes may have as much as a six-fold elevation in plasma LDL-cholesterol and often develop clinical atherosclerosis in childhood (Goldstein & Brown, 1983).

Metabolic and cell culture studies have provided conflicting results as to whether the LDL receptor binds and internalizes Lp(a). *In vitro* studies of LDL receptor mediated binding and uptake of Lp(a) by cultured cells have produced equivocal results (Floren et al., 1981, Hofmann et al., 1990, Krempler et al 1983, Armstrong et al., 1985). Krempler et al., (1983) demonstrated that Lp(a) may be specifically bound with high affinity to human fibroblast LDL receptors, but added that the affinity and maximal binding capacity of Lp(a) was lower than that of LDL. Most recently Snyder and her colleagues (1994) demonstrated variable binding characteristics of Lp(a) in different cell types and concluded that in fibroblasts Lp(a) and LDL had equal affinities, but because of markedly different physiological plasma concentrations it would be unlikely that the hepatic LDL receptor could contribute significantly to the degradation of Lp(a) *in vivo*.

In contrast to these *in vitro* studies, analysis of Lp(a) metabolism in mice expressing a human LDL receptor transgene revealed rapid catabolism of native human Lp(a) (Hofmann et al., 1990). Thus, Lp(a) can bind to and be internalized by the LDL receptor *in vivo*, but the physiological relevance of these studies remain to be determined.

Another approach that has been taken to examine the role of the LDL receptor in Lp(a) metabolism is to analyse the plasma levels of Lp(a) in FH subjects. Utermann and his colleagues (1989) reported that FH heterozygotes have 2-3 fold higher plasma levels of Lp(a). These findings were supported by the results from other groups (Wiklund et al., 1990; Leitersdorf et al., 1991; Mbewu et al., 1991). However, all these studies were flawed since there was no control for the possible effect of differences in apo(a) isoform distributions between the two groups being compared. Also, in some cases the sample of patients with FH were from a lipid clinic and the control sample was not. Thus, there may have been selection bias as those FH patients with high plasma Lp(a) levels may have overt CHD and may be more likely to be seen in lipid clinics. In addition, analysis of a large rhesus monkey pedigree with FH (Neven et al.,

1990) disclosed no difference in the plasma levels of Lp(a) between the affected and unaffected animals.

In humans, recent evidence suggests that the LDL receptor may not play a major role in the catabolism of plasma Lp(a). Up-regulation of the LDL receptor by pharmacological agents such as bile acid sequestrant resins or HMG-CoA reductase inhibitors, has no appreciable effect on the plasma concentration of Lp(a) (Vessby et al., 1982, Thiery et al., 1988, Jürgens et al., 1989, Kostner et al., 1989, Wiklund et al., 1990, Hunninghake et al., 1993), however, these studies are relatively small and there is considerable inter-individual variation in response to therapy. Hypothyroidism, which is associated with a decrease in LDL receptor activity (Salter et al., 1991) and elevated plasma LDL-cholesterol levels, is not associated with a significant change in plasma Lp(a) levels (Klausen et al., 1992a).

In normal individuals, kinetic studies have revealed that the plasma level of Lp(a) appears to correlate with the production rate, rather than the degradation rate of Lp(a) (Rader et al., 1993) and the fractional catabolic rates of ^{125}I-labelled Lp(a) in FH heterozygous individuals, who have 50% of normal LDL receptor activity, and normal indivduals were not significantly different (Knight et al., 1991).

In addition, three human family studies have failed to demonstrate a significant difference in the plasma levels of Lp(a) of FH heterozygotes when compared to their unaffected relatives (Soutar et al., 1991, Ghiselli et al., 1992, Hegele et al., 1990). In two of these studies (Ghiselli et al., 1992, Hegele et al 1990), the plasma Lp(a) levels were compared in family members who did or did not have FH. In both studies there was no control for the effect of the *APO(a)* allele size on the plasma level of Lp(a). In the other study (Soutar et al., 1991) the apo(a) isoforms were analyzed and it was demonstrated that the size distribution of apo(a) isoforms between the FH and non-FH groups were similar and there was no difference between the plasma levels of Lp(a) of the two groups. It is still possible, however, that the lack of difference in this study is due to variations at the *APO(a)* locus not revealed by simple sizing of the apo(a) isoforms.

Apo(a) isoforms of the same size can be associated with very different levels of plasma Lp(a) (Cohen et al., 1993). Therefore, even if there is a similar distribution of apo(a) isoforms sizes in the comparison groups as was

the case in the study by Soutar and her colleagues (1991), this does not control for inter-individual differences in plasma Lp(a) due to the *APO(a)* gene. The only way to show rigorously that the *APO(a)* allele distributions between the two groups are similiar is to compare Lp(a) levels in family members with *APO(a)* alleles identical by descent (ibd). By examining *APO(a)* alleles that are identical in sequence (i.e., sibling pairs who inherit alleles ibd), the effect of a decrease in LDL receptor activity on plasma levels of Lp(a) can be unequivocally determined.

In this study such sibling-pair analyses of FH and non-FH siblings were used to examine the hypothesis that the LDL receptor plays a key role in the control of plasma Lp(a) levels. The results show that mutations in the LDL receptor do influence the plasma levels of Lp(a).

5.2 Methods

The general methods used in this study are described in detail in chapter 2. Here, specific details of the protocol and genetic analysis are described.

5.2.1 Subjects

Venous blood samples were obtained from 162 subjects from 9 large well-defined FH kindreds from Utah. The plasma was separated and the leukocytes isolated for DNA extraction. Lipid and lipoprotein assays were performed on fresh plasma aliquots, obtained by low speed centrifugation (1000 x*g*, 4°C) , using Lipid Research Clinics protocols (1982) as described in chapter 2. The plasma Lp(a) was measured using a commercial kit Macra Lp(a) (Strategic Diagnostics Inc.).

Some of these large families with well-defined FH have been used in other studies and their pedigrees have been published elsewhere (Emi et al., 1991). The diagnosis of FH was confirmed by following the co-segregation of DNA polymorphisms at the LDL receptor locus with the hypercholesterolaemia as described in chapter 2. In each kindred at least one family member had clinical evidence of tendon xanthomata. The plasma lipid and lipoprotein levels of each family member used in the sibling pair analysis are given in appendix III. The apo(a) isoforms were sized, based on their migration in an SDS-agarose gel relative to standards, as decribed in section 2.5 in chapter 2. From

the screening of 162 subjects in the FH kindreds 51 informative sibling pairs were identified. The characteristics of these sibling pairs are shown in tables 5.1, 5.2 and 5.3.

5.2.2 Statistical Methods

Plasma Lp(a) concentrations are not normally distributed but are highly skewed towards lower plasma levels. Therefore, a comparison of median plasma Lp(a) concentrations was made between sibling groups using the non-parametric test of significance, the Wilcoxon signed rank test. As previously used in a similar analysis by Boerwinkle and his colleagues (1992) all analyses were performed on the raw and square-root transformed data. For each analysis, the primary inferences were identical irrespective of which data format was used and only the raw data are presented here.

Even though the sibships were often larger than size two, the above method has been shown to be valid when overlapping sibling pairs are analysed as though they were independent (Amos et al., 1989).

5.2.3 Apo(a) phenotyping vs. apo(a) genotyping

In the present study high resolution apo(a) phenotyping is used as a surrogate for apo(a) genotyping. This approach provides equally valid information on *APO(a)* alleles in all but one situation. This exception is important and merits further discussion. Where a parent has an apparent (n,X) phenotype and all the offspring have inherited only the 'X' allele it is not possible to differentiate the parent's genotype as a (n,X) or an (X,X). If the parent is indeed a homozygote with two *APO(a)* alleles of equal size, but which are not necessarily identical, it is not possible to state unequivocally that two offspring, such as subjects 3 and 5 in figure 5.1, have inherited identical *APO(a)* alleles.

However, as only approximately 3% of the Caucasian population are homozygotes with two *APO(a)* alleles of the same size (chapter 2 , section 4.8.1), virtually all of the parents asigned as (n,X) will indeed be (n,X) and not (X,X).

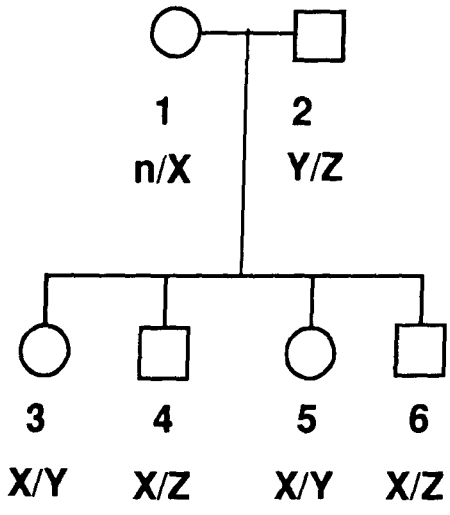


Figure 5.1

Hypothetical pedigree showing apo(a) phenotypes. Circles denote females and squares denote males. The numbers below each symbol denote the subjects' identification numbers. n designates a null allele while X, Y and Z designate expressed isoforms of different sizes.

The analyses were also performed on the subgroup of sibling pairs in which *APO(a)* alleles were unequivocally inherited identical by descent (normal/normal, n=2; FH/FH, n=8; normal/FH, n=12).

5.3 Results

From the screening of 162 subjects in the FH kindreds 51 informative sibling pairs were identified. The characteristics of these subjects are shown in appendix III. Two examples of the apo(a) phenotyping gels for two of the FH familes studied are shown in figures 5.2 and 5.3.

Fifteen pairs of normal siblings who had inherited the same apo(a) phenotype from their parents were identified from the FH families. As expected, there was no significant difference between the plasma Lp(a) levels of the siblings in this group (12.9 mg. dL^{-1} *vs.* 13.2 mg. dL^{-1}, p=0.47). Only two of these sibling pairs had *APO(a)* alleles ibd and in view of the small number these were not further analysed.

Ten pairs of siblings both of whom had inherited a defective LDL receptor allele from one of their parents and who were consequently FH heterozygotes, were also identified from these families. These FH siblings had inherited the same apo(a) phenotype from their parents. Again there was no significant difference between the plasma Lp(a) levels of the siblings in this group (29.5 mg. dL^{-1} *vs.* 23.0 mg. dL^{-1}, p=0.50). When the 8 sibling pairs who had had *APO(a)* alleles ibd were examined separately this conclusion was upheld.

Twenty-six sibling pairs were identified in which one sibling was an FH heterozygote, while the other was normal with respect to LDL receptor function, but both of whom had inherited the same apo(a) phenotype from their parents. These sibling pairs were used to evaluate the impact of reduced LDL receptor function on the plasma Lp(a) concentration. When the median plasma Lp(a) level in the FH heterozygotes was compared to that of their normal siblings the result just failed to reach statitisical significance (21.5 mg. dL^{-1} *vs.* 19.0 mg. dL^{-1}, p=0.08). With a more rigorous analysis including only those sibling pairs who had inherited *APO(a)* alleles unequivocally ibd (n=12), the difference was now strongly significant (22.0 mg. dL^{-1} *vs.* 12.0 mg. dL^{-1}, p=0.005).

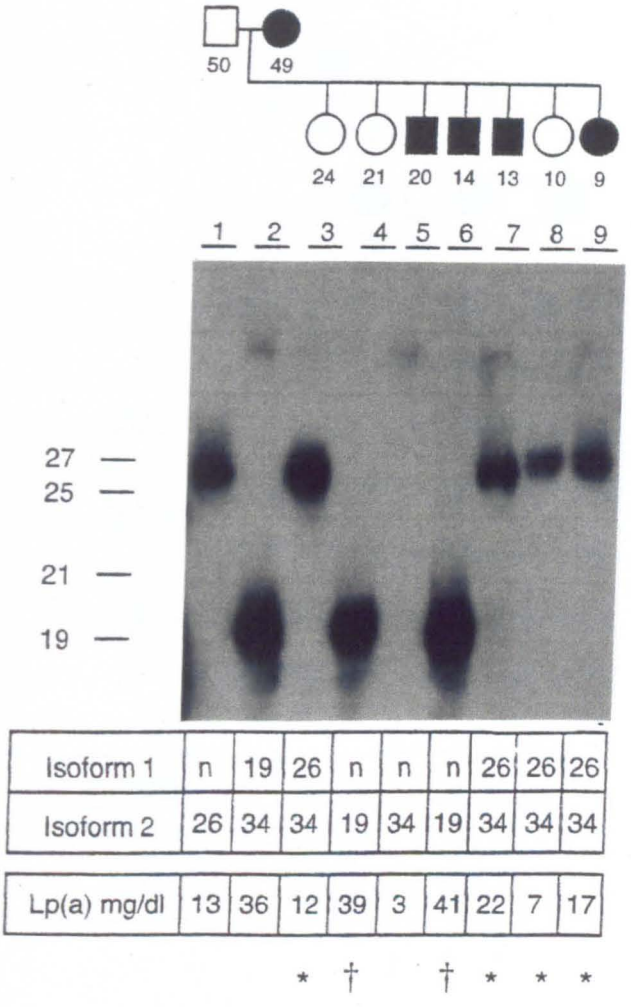


Figure 5.2

Example 1 of apo(a) immunoblot showing the inheritance of apo(a) isoforms in family 653-4. The gel was run, blotted and developed exactly as described in chapter 2 section 2.5. The exposure time was 5 min. Sibling pairs with identical apo(a) phenotypes and *APO(a)* alleles ibd are shown in lanes 3, 7, 8 and 9 (*) and lanes 4 and 6 (†). Size markers shown on the left side of the gel are in numbers of K4 repeats. In the pedigrees the numbers denote the ages of the family members in years at the time of sampling. The squares and circles represent males and females, respectively, while open symbols and shaded symbols represent normal and FH, respectively. The characteristics of the family members and the full pedigree are shown in appendix III.

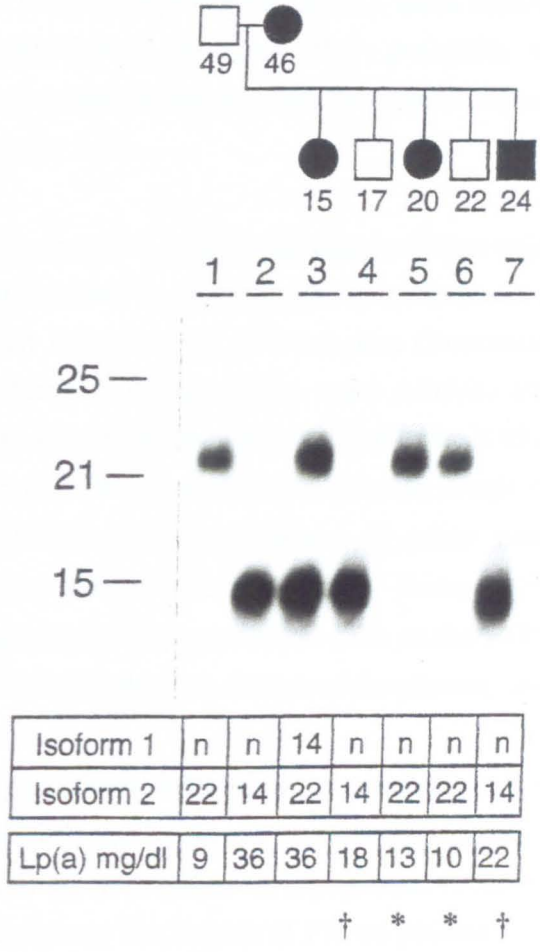


Figure 5.3

Example 2 of apo(a) immunoblot showing the inheritance of apo(a) isoforms in family 26. The gel was run, blotted and developed exactly as described in chapter 2 section 2.5. The exposure time was 5 min. Two sibling pairs with identical apo(a) phenotypes and *APO(a)* alleles ibd are shown in lanes 4 and 7 (†) and lanes 5 and 6 (*). Size markers shown on the left side of the gel are in numbers of K4 repeats. In the pedigrees the numbers denote the ages of the family members in years at the time of sampling. The squares and circles represent males and females, respectively, while open symbols and shaded symbols represent normal and FH, respectively. The characteristics of the family members and the full pedigree are shown in appendix III.

5.4 Discussion

In this study, plasma Lp(a) levels are compared in siblings from 9 families with FH. In the non-FH (n=15), and FH sibling pairs (n=10) the plasma Lp(a) levels were very similar. The plasma Lp(a) levels in the sibling pairs with the same apo(a) phenotype and with *APO(a)* alleles ibd in which one member had FH and one member did not (n=12), were significantly different [median plasma Lp(a) 22.0 vs. 12.0 mg. dL^{-1}, p=0.005], which would be expected if the LDL receptor played an important role in the control of plasma Lp(a) levels, as it does for LDL.

As described in chapter 1, it has been estimated that approximately 90% of the inter-individual variation in plasma Lp(a) concentrations is attributable to sequences at, or closely linked to, the *APO(a)* gene (Boerwinkle et al, 1992). In family studies, siblings who inherit the same *APO(a)* alleles from their parents have strikingly similar plasma levels of Lp(a) (r=0.95), whereas those who shared no *APO(a)* alleles had different plasma levels (r=-0.23). This would not be expected if normal variations in other genes contributed importantly to the plasma concentrations of Lp(a). However, major defects in key genes controlling lipoprotein metabolism such as the LDL receptor have been proposed as additional controlling factors of the plasma Lp(a) level.

Attempts to explain the variable extent of atherosclerosis in subjects with FH have focussed on the adverse impact of environmental influences, as well as the influence of an LDL receptor defect on lipoprotein levels other than LDL (Seed et al 1990). Studies on the impact of FH on plasma Lp(a) levels have been conflicting. Initial observations that FH heterozygotes had increases in plasma Lp(a) approaching three-fold over normal controls (Utermann et al 1989) have been supported by other workers who studied FH heterozygotes and age and sex matched controls (Wiklund et al 1990) or unaffected first degree relatives (Mbewu et a 1991). Other groups have, however, reported that there is no difference in the plasma Lp(a) level between FH heterozygotes and their unaffected siblings when low resolution apo(a) phenotyping is used to match subjects and controls (Soutar et al 1991). As a corollary Knight and his colleagues (1991) found that the clearance rates for trace-labelled Lp(a) were the same in subjects with FH and normal controls.

In the present study the influence of the *APO(a)* gene, which has previously been shown to be the principle determinant of plasma Lp(a) levels

(Boerwinkle et al 1992), has been normalized by examining sibling pairs who share *APO(a)* alleles that are identical by descent. The influence of a second gene, the LDL receptor can therefore be examined in relative isolation. When 26 FH/non-FH sibling pairs were examined in this way there was a significant difference in the plasma Lp(a) levels between the two groups (p=0.005).

These results suggest that the LDL receptor is playing a role in the control of plasma Lp(a) levels. The level of this control remains unclear and there are two distinct mechanisms that may be invoked to explain this phenomenon.

1. The LDL receptor may play an important physiological role in the clearance of Lp(a) from the plasma.

2. The LDL receptor is known to be an important controlling factor in determining the plasma LDL concentration. Dysfunction of the LDL receptor may then increase the available LDL substrate for Lp(a) assembly and indirectly increase plasma Lp(a) levels.

The pool of any plasma constituent is determined by the combined effects of its production rate and its catabolic rate. Previous published discussions on the influence of the LDL receptor on plasma Lp(a) have focussed on the putative clearance of Lp(a) from the circulation via the LDL receptor. There is clear *in vitro* evidence that the LDL receptor can indeed bind to and internalise the Lp(a) particle (Krempler et al., 1983; Snyder et al., 1994). Furthermore, *in vivo* studies with mice over-expressing a human LDL receptor transgene have demonstrated that Lp(a) clearance in this situation can be increased (Hofmann et al., 1990).

However, human metabolic studies have demonstrated that it is the production rate rather than the catabolic rate that primarily determines the plasma Lp(a) level. Here, we must consider a more indirect mechanism by which defective LDL receptor function may impact upon plasma Lp(a) concentrations.

From the work of White and her colleagues (1993, 1994) we know that Lp(a) assembly most likely takes place extracellularly on the hepatocyte surface. This assembly requires the interaction of the secreted, and possibly immmobilised, apo(a) glycoprotein with free LDL particles in the extracellular

fluid (ECF). This interaction, in common with all chemical reactions, must be bound by the laws of mass action as shown below:

$$\text{Apo(a)} + \text{LDL} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Lp(a)}$$

If we assume the apo(a) secretion rate to be invariate because it is directly dependent on the length of the apo(a) isoform, which in turn is determined by the length of the *APO(a)* gene, then the only factor that can increase k_1 would be the concentration of LDL in the ECF. If this LDL concentration is increased k_1 will be increased and the mass action equation will be pushed to the right in favour of Lp(a) production. In FH the plasma and ECF LDL concentrations are increased. Therefore, we may hypothesise that in this condition the plasma Lp(a) concentration will be higher, not because of any effect on Lp(a) clearance by the defective LDL receptor, but rather an in indirect effect of the elevated LDL pool which leads to an increased availability of one of the two components for Lp(a) assembly.

Further supportive evidence for the role of the plasma LDL pool as a controlling factor of the Lp(a) production rate comes from groups who have examined plasma Lp(a) concentrations in subjects with familial combined hyperlipidaemia (Fonda et al., 1993) and in patients with type IIa hyperlipoproteinaemia (Bartens et al., 1995). In both these studies those subjects with elevated plasma cholesterol levels had higher plasma Lp(a) levels when compared to controls. However, it is important to note that in neither of these studies was the contribution of the *APO(a)* gene polymorphism completely controlled and the results must remain tentative.

Clearly, this hypothesis is consistent with some but not all of the available evidence. We know that the Lp(a) production rate is more important than its clearance rate in determining the plasma Lp(a) level, but we would also expect k_1 to be lowered by decreasing the plasma LDL level by pharmacological means such as statin therapy. There are no good data to support an Lp(a) lowering effect of the statins although as noted above the available studies are relatively small and there is a degree of inter-individual variability in therapeutic response. When the LDL-C concentrations of the normal and FH siblings are compared there is an average difference of 50% (Mean (SEM) for normals 2.7

(0.1) mmol. L^{-1} vs. for FH 5.4 (0.2)mmol. L^{-1}). Simvastatin, the most potent of the currently available statins, will lower LDL-C by approximately 35% (Scandinavian Simvastatin Survival Study Group, 1994). Perhaps in order to alter the kinetics of Lp(a) assembly significantly we may have to lower plasma cholesterol by a similar percentage to its corresponding increase in FH. Currently, the only available therapy to do this is LDL apheresis. This proceedure also directly removes Lp(a) from the plasma (Groß et al., 1994) and would therefore be unhelpful in trying to ascertain the effect of dramatic LDL lowering on plasma Lp(a) concentration. Recently, phase III trials of a second generation statin called atorvastatin, have been reported (Nawrocki et al. 1995) revealing very impressive LDL-C reductions of more than 60%. Further testing of the hypothesis presented here for plasma Lp(a) level control may soon be possible by lowering LDL-C levels by this novel pharmacological modality.

An alternative way to test this hypothesis would be to examine the effects of a genetically controlled reduction in plasma LDL levels on plasma Lp(a) levels. This experiment may be performed by examining families with the condition, hypobetalipoproteinaemia, in a similar fashion to that described here. This proposed study has now been performed and is the subject of chapter 6.

Table 5.1

Comparison of plasma Lp(a) levels in normal sibling pairs with the same apo(a) phenotype.

Sibling pair ID numbers	Apo(a) phenotype	*APO(a)* alleles ibd	Plasma Lp(a) mg. dL^{-1}		WSR
			Normal	Normal	
26-397/399	17/34		21.0	33.0	
26-394/395	32/34		2.0	4.0	
653-1-268/272	n/28	+	14.0	14.0	
653-2-102/104	n/30		4.0	5.0	
653-2-102/105	n/30		4.0	5.0	
653-2-105/104	n/30		5.0	5.0	
625-30/42	28/39		8.0	12.0	
625-52/59	26/28		17.0	22.0	
625-52/69	26/28		17.0	20.0	
625-52/70	26/28		17.0	16.0	
625-59/69	26/28		22.0	20.0	
625-59/70	26/28		22.0	16.0	
625-69/70	26/28		20.0	16.0	
71002076-265/266	n/27		9.0	3.0	
653-4-103/109	26/34	+	12.0	7.0	
Median			12.9	13.2	P=0.47 (NS)

ibd: identical by descent
WSR: Wilcoxon Signed Rank Test

Table 5.2

Comparison of plasma Lp(a) levels in FH sibling pairs with the same apo(a) phenotype.

Sibling pair ID numbers	Apo(a) phenotype	*APO(a)* alleles ibd	Plasma Lp(a) mg. dL^{-1}		WSR
			FH	FH	
659-125/127	n/20	+	47.0	42.0	
659-139/140	15/24		56.0	62.0	
659-143/147	n/20	+	4.0	4.0	
653-1-81/83	21/28	+	10.0	18.0	
653-1-1/41	20/26	+	31.0	10.0	
653-1-1/5	20/26	+	31.0	28.0	
653-1-5/41	20/26	+	28.0	10.0	
653-1-28/29	n/20	+	24.0	28.0	
653-4-108/110	26/34	+	22.0	17.0	
26-496/527	22/30		57.0	87.0	
Median			29.5	23.0	P=0.50 (NS)
Median (only ibd pairs)			26.0	17.5	P=0.12 (NS)

ibd: identical by descent
WSR: Wilcoxon Signed Rank Test

Table 5.3

Comparison of plasma Lp(a) levels in normal and FH sibling pairs (i.e. one sibling with FH and one normal) with the same apo(a) phenotype.

Sibling pair ID numbers	Apo(a) phenotype	*APO(a)* alleles ibd	Plasma Lp(a) mg. dL^{-1}		WSR
			FH	Normal	
625-64/52	26/28		20.0	17.0	
659-152/151	n/21		21.0	36.0	
659-61/63	n/15	+	45.0	37.0	
659-144/145	15/20	+	40.0	45.0	
26-393/397	17/34		29.0	21.0	
26-393/399	17/34		29.0	33.0	
625-64/59	26/28		20.0	22.0	
625-64/69	26/28		20.0	20.0	
625-64/70	26/28		20.0	16.0	
653-4-108/103	26/34	+	22.0	12.0	
653-4-110/103	26/34	+	17.0	12.0	
653-4-107/104	n/19	+	41.0	39.0	
653-1-12/24	20/26		37.0	60.0	
653-1-31/30	n/19	+	3.0	2.0	
653-5-102/109	29/31	+	11.0	7.0	
653-6-105/103	20/33	+	63.0	46.0	
71002076-264/265	n/27		14.0	9.0	
71002076-264/266	n/27		14.0	3.0	
26-726/721	n/n		1.0	1.0	
26-719/723	n/20		35.0	56.0	
26-527/497	22/30		87.0	64.0	
26-496/497	22/30		57.0	64.0	
26-343/342	n/22	+	13.0	10.0	
26-341/344	n/14	+	22.0	18.0	
653-4-108/109	26/34	+	22.0	7.0	
653-4-110/109	26/34	+	17.0	7.0	
Median			21.5	19.0	P=0.08 (NS)
Median (only ibd pairs)			22.0	12.0	P=0.005

ibd: identical by descent
WSR: Wilcoxon Signed Rank Test

Chapter 6

The influence of defects in the APOB*gene on plasma Lp(a) levels*

Let us read and recollect and impress upon our souls the views and ends of our own immediate forefathers in exchanging their native country for a dreary, inhospitable wilderness.

John Adams, 1765.
A Dissertation on the Canon

6.1 Study Outline

Familial hypobetalipoproteinaemia (FHB) is an autosomal codominant disorder characterized by abnormally low circulating LDL-cholesterol concentrations, which can be caused by mutations in the *APOB* gene (Young et al., 1988, Collins et al., 1988). Approximately 25 mutations in the *APOB* gene resulting in FHB have been described (Linton et al., 1993). FHB heterozygotes typically have LDL-cholesterol and apoB levels 25-50% of normal (Linton et al., 1993) and due to the low levels of atherogenic lipoproteins may be protected from the development of coronary artery disease (Kahn & Glueck, 1978).

As detailed in chapter 1, apoB is one of the two main proteins in Lp(a). The *APO(a)* gene accounts for > 90% of the variation seen in plasma Lp(a) concentrations (Boerwinkle et al., 1992), but relatively little is known about post-transcriptional and post-translational control of Lp(a) levels. The impact of

APOB gene mutations on plasma Lp(a) levels have not been previously studied and the apoB-67 mutation described here provides a unique opportunity to examine the relationship between decreased production of apoB-100 and Lp(a) levels.

ApoB-67 is a truncated form of apoB previously reported in a large Amish kindred in which affected family members have plasma apoB and LDL-C levels approximately 25% of normal, low plasma triglyceride levels, elevated plasma HDL cholesterol levels and the absence of CHD (Welty et al., 1991). The production rates of both apoB-67 and apoB-100 in these FHB subjects is <10% of normal subjects, however, catabolism of apoB-100 in both the VLDL and LDL fractions is identical in both groups. Because these FHB subjects catabolize apoB-100 at normal rates, the association between decreased production of apoB-100 and Lp(a) levels should be independent of LDL receptor activity. As there is only approximately one quarter of the normal amount of apoB-100 in the FHB subjects to complex with apo(a), it is hypothesised that Lp(a) levels would be lower in the FHB subjects when compared to their normal siblings

In the present study the influence of the *APO(a)* gene, which has previously been shown to be the main determinant of plasma Lp(a) levels (Boerwinkle et al., 1992), has been normalized by examining sibling pairs who share *APO(a)* alleles that are identical by descent. The influence of a second gene, *APOB*, can therefore be examined in relative isolation. When FHB/normal sibling pairs were examined it was clear that those subjects who had inherited the apoB defect had significantly lower plasma Lp(a) levels.

6.2 Methods

The general methods used in this study are described in detail in chapter 2. Here, specific details of the protocol and genetic analysis are described.

6.2.1 Subjects

Venous blood samples were obtained from 80 subjects from large well-defined FHB kindreds from Ohio. The plasma was separated and the leukocytes isolated for DNA extraction. Lipid and lipoprotein assays were

performed on fresh plasma aliquots, obtained by low speed centrifugation (1000 x*g*, 4°C) , using Lipid Research Clinics protocols (1982) as described in chapter 2. The plasma Lp(a) was measured using ELISA 1 as described in section 2.4.1.

Some of these large families with well-defined FHB have been used in other studies and some of their pedigrees have been published elsewhere (Welty et al., 1991). The diagnosis of FHB was confirmed in the proband of each family by RFLP analysis as described in chapter 2. Thereafter, the presence or absence of the mutation in other family members was assessed by analysing freshly prepared lipoprotein samples for the presence of apoB-67 by SDS-PAGE. The plasma lipid and lipoprotein levels of each family member used in the sibling pair analysis are given in appendix IV. The apo(a) isoforms were sized, based on their migration in an SDS-agarose gel relative to standards, as described in section 2.5 in chapter 2. From the screening of 80 subjects in the FH kindreds 33 informative sibling pairs were identified. The characteristics of these sibling pairs are shown in tables 6.1, 6.2 and 6.3.

6.2.2 Statistical Methods

The statistical methods used in this study were exactly as described in chapter 5 section 5.2.2.

6.3 Results

From the screening of 80 subjects in the FHB kindreds 33 informative sibling pairs were identified. The characteristics of these subjects are shown in appendix IV. Two examples of the apo(a) phenotyping gels for two of the FHB families studied are shown in figures 6.1 and 6.2.

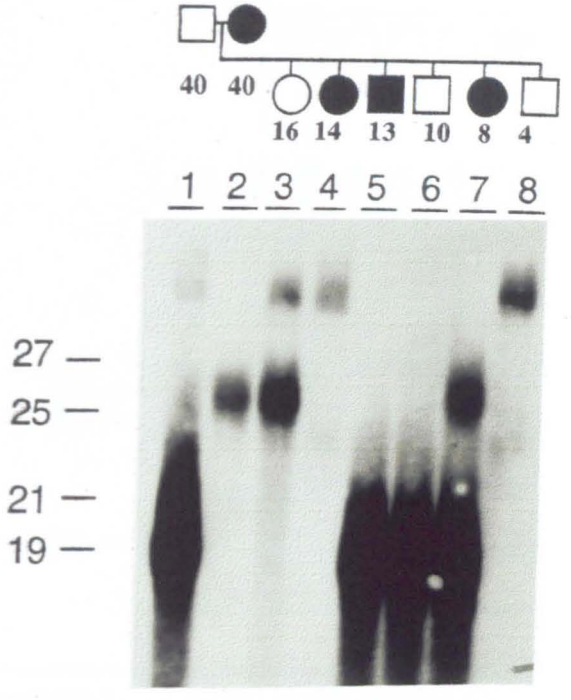
Thirteen pairs of normal siblings who had inherited the same apo(a) phenotype from their parents were identified from the FHB families. As expected, there was no significant difference between the plasma Lp(a) levels of the siblings in this group (22.8 mg. dL^{-1} *vs.* 19.6 mg. dL^{-1}, p=0.15). Twelve of these sibling pairs had *APO(a)* alleles ibd and when only these groups were included in the analysis the conclusion was the same: no significant difference (45.5 mg. dL^{-1} *vs.* 47.9 mg. dL^{-1}, p=0.11).

Six pairs of siblings both of whom had inherited a defective *APOB* allele from one of their parents and who were consequently FHB heterozygotes, were also identified from these families. These FHB siblings had inherited the same apo(a) phenotype from their parents. Again there was no significant difference between the plasma Lp(a) levels of the siblings in this group (11.3 mg. dL^{-1} *vs.* 10.8 mg. dL^{-1}, p=0.25). Only 5 of these sibling pairs unequivocally had *APO(a)* alleles ibd and because of this small number this group could not be examined separately. However, simple inspection of the median Lp(a) levels in this group shows no difference (18.6 mg. dL^{-1} *vs.* 18.6 mg. dL^{-1}).

Fourteen sibling pairs were identified in which one sibling was an FHB heterozygote, while the other was normal with respect to apoB function, but both of whom had inherited the same apo(a) phenotype from their parents. These sibling pairs were used to evaluate the impact of reduced apoB-100 production on the plasma Lp(a) concentration. When the median plasma Lp(a) level in the FHB heterozygotes was compared to that of their normal siblings there was a highly significant difference (4.8 mg. dL^{-1} *vs.* 1.8 mg. dL^{-1}, p=0.002). Once again, when only those sibling pairs who had inherited *APO(a)* alleles unequivocally ibd (n=13), the difference was equally significant (4.6 mg. dL^{-1} *vs.* 1.8 mg. dL^{-1}, p=0.003).

6.4 Discussion

In this study, plasma Lp(a) levels were compared in 33 sibling pairs identified from the study of 80 subjects in large Amish pedigrees with FHB from Ohio in which an apoB-100 mutation has been identified and thoroughly characterized (Welty et al., 1991). The *APOB* mutation causing FHB in these families is a frameshift that results in the formation of a truncated apoB-100 that contains approximately 67% of the apoB sequence. The mutation results in the production of only trace amounts of circulating apoB-67 and these individuals have markedly reduced levels of circulating apoB-100. In the non-FHB (n=13), and FHB sibling pairs (n=6) the plasma Lp(a) levels were very similar. The plasma Lp(a) levels in the sibling pairs with the same apo(a) phenotype and with *APO(a)* alleles ibd in which one member had FHB and one member did not (n=13), were significantly different [median plasma Lp(a) 4.6 *vs.* 1.8 mg. dL^{-1}, p=0.003], which would be expected if the *APOB* gene played an important role in the control of plasma Lp(a) levels.

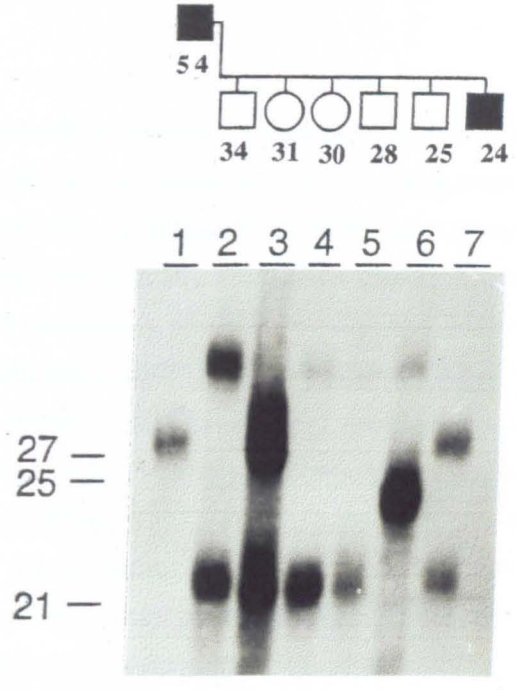


Isoform 1	19	25	26	25	19	19	19	25
Isoform 2	34	26	34	34	25	26	26	34
Lp(a) mg/dl	47	5	8	1	51	98	64	3

* † † *

Figure 6.1

Example 1 of apo(a) immunoblot showing the inheritance of apo(a) isoforms in family 145. The gel was run, blotted and developed exactly as described in chapter 2 section 2.5. The exposure time was 10 min. Two informative sibling pairs with identical apo(a) phenotypes and *APO(a)* alleles ibd are shown in lanes 4 and 8 (*) and lanes 6 and 7 (†). Size markers shown on the left side of the gel are in numbers of K4 repeats. In the pedigrees the numbers denote the ages on the family members in years at the time of sampling. The squares and circles represent males and females, respectively, while open symbols and shaded symbols represent normal and FHB, respectively. The characteristics of the family members and the full pedigree are shown in appendix IV.



Isoform 1	n	22	22	22	22	24	22
Isoform 2	28	34	28	34	34	34	28
Lp(a) mg/dl	1.0	2.5	11.2	3.4	1.8	3.5	2.0
			†				†

Figure 6.2

Example 2 of apo(a) immunoblot showing the inheritance of apo(a) isoforms in family 174-1. The gel was run, blotted and developed exactly as described in chapter 2 section 2.5. The exposure time was 5 min. One informative sibling pairs with identical apo(a) phenotypes and *APO(a)* alleles ibd is shown in lanes 3 and 7 (†). Size markers shown on the left side of the gel are in numbers of K4 repeats. In the pedigrees the numbers denote the ages on the family members in years at the time of sampling. The squares and circles represent males and females, respectively, while open symbols and shaded symbols represent normal and FHB, respectively. The characteristics of the family members and the full pedigree are shown in appendix IV.

ApoB occupies a central role in lipid metabolism and normally exists as two isoforms in plasma, apoB-100 and apoB-48. ApoB-100 is the principal protein component of LDL. FHB is an autosomal codominant disorder characterized by low plasma concentrations of apoB and LDL cholesterol. Most cases of FHB have been shown to be due to mutations in the *APOB* gene resulting in the production of a truncated form of apoB-100 (Linton et al., 1993).

The results presented in chapter 5 suggested that the LDL receptor plays playing an important role in the control of plasma Lp(a) concentrations. The level of apoB, the other main apolipoprotein apart from apo(a) in Lp(a), also appears to be a limiting factor in the control of plasma Lp(a) levels. Those siblings who had lower circulating apoB levels due to a defect in one of their *APOB* alleles had significantly lower levels of plasma Lp(a). This may be due to a defect in Lp(a) production. Diminished availability of apoB at the hepatocyte surface, where Lp(a) assembly has been hypothesized to occur after secretion of free apo(a) (White et al., 1994), may limit the assembly of the apo(a):apoB dipeptide. These findings are therefore entirely consistent with the previously stated hypothesis that plasma Lp(a) levels are controlled by production rather than degradation rates of the particle.

As described above in section 5.4 the size of the circulating Lp(a) pool will be a function of both the rate of Lp(a) production and Lp(a) catabolism. In FHB there is a clearly defined defect in apoB production but no defect in the clearance rate of apoB-containing lipoproteins. We may therefore hypothesise that the diminished plasma concentration of Lp(a) observed in those individuals with FHB was due to a decreased production rate of Lp(a). This finding is consistent with the findings presented in chapter 5 and adds further weight to the hypothesis describing a general control mechanism for the plasma Lp(a) concentration dependent on substrate availability. If we re-examine the equilibrium equation describing Lp(a) assembly:

$$\text{Apo(a)} + \text{LDL} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Lp(a)}$$

it is immediately obvious that if the concentration of LDL is decreased then there will be a shift towards the left, i.e. not favouring Lp(a) production. This

again assumes the apo(a) production rate to be invariant, which will be the case in those sibling pairs where the powerful effects of the *APO(a)* gene polymorphisms have been normalised by studying siblings with identical *APO(a)* alleles. This is the exact opposite of the situation observed in FH, as described in chapter 5, where the LDL concentration was increased and Lp(a) assembly was favoured by a shift to the right.

Study of these FHB kindreds has therefore provided further evidence to support the hypothesis that the plasma concentration of Lp(a) while predominately controlled by polymorphisms at the *APO(a)* locus is also influenced by other genes that code for key proteins that control the plasma concentrations of LDL, viz. the LDL receptor and apoB-100.

Table 6.1

Comparison of plasma Lp(a) levels in normal sibling pairs with the same apo(a) phenotype.

Sibling pair ID numbers	Apo(a) phenotype	*APO(a)* alleles ibd	Plasma Lp(a) mg. dL^{-1}		WSR
			Normal	Normal	
174-1-29/36	22/34	+	3.4	2.5	
174-1-28/29	22/34	+	4.6	3.4	
174-2-20/22	17/29	+	22.8	19.6	
174-2-3/5	14/17	+	68.2	76.2	
174-2-6/11	14/17	+	79.8	78.0	
174-2-5/6	14/17	+	76.2	79.8	
174-2-5/11	14/17	+	76.2	78.0	
174-2-3/6	14/17	+	68.2	79.8	
174-2-3/11	14/17	+	68.2	78.0	
174-1-28/36	22/34	+	4.6	2.5	
174-2-2/16	13/22	+	15.6	19.0	
174-1-26/32	23/34		1.9	1.0	
174-1-36/39	22/34	+	2.5	1.8	
Median			22.8	19.6	P=0.15 (NS)
Median (only ibd pairs)			45.5	47.9	P=0.11 (NS)

ibd: identical by descent
WSR: Wilcoxon Signed Rank Test

Table 6.2

Comparison of plasma Lp(a) levels in hypobetalipoproteinaemic sibling pairs with the same apo(a) phenotype.

Sibling pair ID numbers	Apo(a) phenotype	*APO(a)* alleles ibd	Plasma Lp(a) mg. dL^{-1}		WSR
			FHB	FHB	
174-2-4/10	17/27	+	20.7	35.2	
174-2-4/7	17/27	+	20.7	18.6	
174-2-7/10	17/27	+	18.6	35.2	
174-2-17/41	27/29	+	1.2	2.5	
153-4/5	34/34	+	4.0	1.0	
144-4/5	23/34		3.0	3.0	
Median			11.3	10.8	P=0.25 (NS)
Median (only ibd pairs)			18.6	18.6	Insufficient data for WSR

ibd: identical by descent
WSR: Wilcoxon Signed Rank Test

Table 6.3

Comparison of plasma Lp(a) levels in normal and hypobetalipoproteinaemic sibling pairs (i.e. one sibling with hypobetalipoproteinaemia and one normal) with the same apo(a) phenotype.

Sibling pair ID numbers	Apo(a) phenotype	*APO(a)* alleles ibd	Plasma Lp(a) mg. dL^{-1}		WSR
			Normal	FHB	
174-1-39/37	22/34	+	1.8	1.8	
174-2-21/17	27/29	+	2.0	1.2	
174-2-21/41	27/29	+	2.0	2.5	
174-1-31/19	22/28	+	11.2	2.0	
174-1-29/37	22/34	+	3.4	1.8	
174-1-36/37	22/34	+	2.5	1.8	
174-1-28/37	22/34	+	4.6	1.8	
174-2-20/44	17/29	+	22.8	20.0	
174-2-22/44	17/29	+	19.2	20.0	
153-3/4	34/34	+	5.0	4.0	
153-3/5	34/34	+	5.0	1.0	
145-6/7	19/26	+	37.7	24.6	
145-8/4	25/34	+	3.0	1.0	
144-3/6	23/30		18.0	1.0	
Median			4.8	1.8	P=0.002
Median (only ibd pairs)			4.6	1.8	P=0.003

ibd: identical by descent
WSR: Wilcoxon Signed Rank Test

Chapter 7

Conclusions

Though our challenges are fearsome, so are our strengths. Americans have ever been a restless, questing, hopeful people. And we must bring to our task today the vision and will of those who came before us. ... Our democracy must be not only the envy of the world but the engine of our own renewal. There is nothing wrong with America that cannot be cured by what is right with America.

President William J.Clinton, 1993.
Inaugural Address

7.1 Introduction

It was while performing one of the many apo(a) phenotyping Western blots in Dallas that I listened to the new President's words on the lab. radio as they were being spoken in Washington DC. The rhetroic, so essential to any inaugural speech, was punctutated with an uncommon wisdom and there truly was a sense of a new beginning in America that day.

Words of a new beginning are appropriate for the final chapter of a thesis for all that has gone before in science is merely the prelude to that which will follow. In this chapter I shall review the findings presented in this thesis and offer some direction for the next stage of the still extensive work to be done

on Lp(a). This chapter is then the starting point for my work and hopefully the work of others in the months and years ahead.

Most theses will present some interesting data, some conclusions, but many more interesting questions. This work has been no different. In chapter 1 the aims of this thesis were set forth. It is the purpose of this final chapter to assess the extent to which these aims have been satisfied and, in turn, the overall contribution made to this field.

7.2 The *APO(a)* "null" allele may be any size

The inverse relationship between the length of the apo(a) glycoprotein and the plasma concentration of Lp(a) with which it is associated has been thoroughly documented (Utermann et al., 1987). With the knowledge that many *APO(a)* alleles were apparently associated with no detectable plasma protein product it was hypothesised that these non-expressing alleles would be clustered at the large end of the apo(a) size spectrum. With the analyses presented in chapter 4 this hypothesis was found to be incorrect. *APO(a)* alleles that are non-expressing or "null" may be any size. This conclusion was only made possible by simultaneous investigation of the *APO(a)* gene and its glycoprotein product apo(a), using apo(a) genotyping by pulsed-field gel electrophoresis and apo(a) phenotyping by high resolution immunoblotting. These findings have stimulated interest in the search for controlling sequences in and around the *APO(a)* locus that may be responsible for the transcriptional regulation of this gene.

7.3 The *APO(a)* allele size polymorphism does not explain inter-racial differences in plasma Lp(a)

A major finding of the studies presented in chapter 4 is that the inter-ethnic differences in plasma concentrations of Lp(a) are not due to in differences in the *APO(a)* allele size distributions. This conclusion is evident from the following observations:

1) The Caucasians and Chinese groups studied had very similar distributions of plasma Lp(a) levels and yet had significantly different *APO(a)* allele distributions.

2) The African-Americans had a distinctly different plasma Lp(a) distribution from the other two groups, yet their *APO(a)* allele size distribution was not significantly different from the Caucasians.

It was also a striking finding that the relationship between *APO(a)* allele size and plasma Lp(a) level was so similar irrespective of ethnic origin. As described in chapter 4, the uniformity of this relationship suggests a fundamental underlying biological mechanism controlling the plasma Lp(a) concentration.

7.4 Plasma Lp(a) levels are affected by defects in the LDL receptor and *APOB* genes

The importance of the *APO(a)* gene in controlling plasma Lp(a) levels is without dispute. In Caucasians, Boerwinkle and his colleagues (1992) have quantified this contribution as >90% of the inter-individual variability in plasma Lp(a) concentration. However, the subjects used to define that figure were normolipidaemic and there has been considerable controversy in the literature on the contribution on other candidate genes that may control plasma Lp(a) levels in abnormal situations. Most notably the role of the LDL receptor has received considerable attention.

FH heterozygotes, who have a defective LDL receptor allele, have variably been described as having either normal or elevated plasma Lp(a) levels. Because the contribution of the enormous inter-individual varaiblity at the *APO(a)* locus may override any controlling factors this has to be removed from the analysis. In order to normalise the contribution of the *APO(a)* gene it is necessary to study and compare individuals with identical *APO(a)* alleles. It is quite insufficient merely to rely on equalizing the size variability between individuals for we know that there are further levels of heterogeneity at the *APO(a)* locus beyond the length of the gene. The only way to ensure that *APO(a)* alleles are identical, and their effect normalised, is to study sibling pairs who have inherited the same *APO(a)* alleles from their parents. These studies are difficult to perform for many subjects and families have to be examined before sufficiently large numbers of appropriate sibling pairs are identified.

In the studies presented in chapters 5 and 6 the LDL receptor and the *APOB* genes were studied respectively. The results were consistent and

allowed the formulation of the following hypothesis: Lp(a) plasma concentrations are in part determined by the availability of the LDL moiety. When LDL is present in the ECF in significantly higher concentrations than normal (as in FH) the assembly of Lp(a) is favoured and the plasma level rises. On the other hand, if the ECF LDL level is significantly lower than normal (as in FHB) the assembly of Lp(a) is not favoured and plasma levels fall. This is shown schematically in figure 7.1.

If this hypothesis is true then plasma Lp(a) levels should be lowered by pharmacological means that lower plasma LDL concentrations. To date most lipid lowering drugs have failed to lower plasma Lp(a) levels consistently. However, as argued in chapter 6, it may be that we have not yet used sufficiently powerful drugs to shift the equilibrium equation. Studies using new agents, which are already underway in different centres may provide useful new data in support of this hypothesis.

7.5 The case for high resolution apo(a) phenotyping

Lp(a) is without doubt one of the most fascinating of the human lipoproteins. The locus structure of the gene encoding the complex glycoprotein, apo(a), presents us with two major intellectual challenges. Its highly repetitive structure and close similarity to adjacent loci in the human genome pose difficult technical problems for those wishing to study polymorphisms and mutations in this stretch of DNA. Elegant solutions have been found by a number of key workers in this field, most notably Lackner and her colleagues (1991, 1993), whose methods provided a foundation for the work presented here. In addition, nature presents us with a gene of such startling inter-individual variation that it is clear that the range of apo(a) protein sizes begs the question: can all these *APO(a)* gene products be regarded as the same protein, at least in functional terms.

The high degree of apo(a) heterogeneity may be regarded as going beyond mere polymorphism. The largest isoform thus far described has 51 K4 repeats while the smallest has 12. These two proteins, although sharing all the key motifs in common, differ in size by approximately 480 kDa.

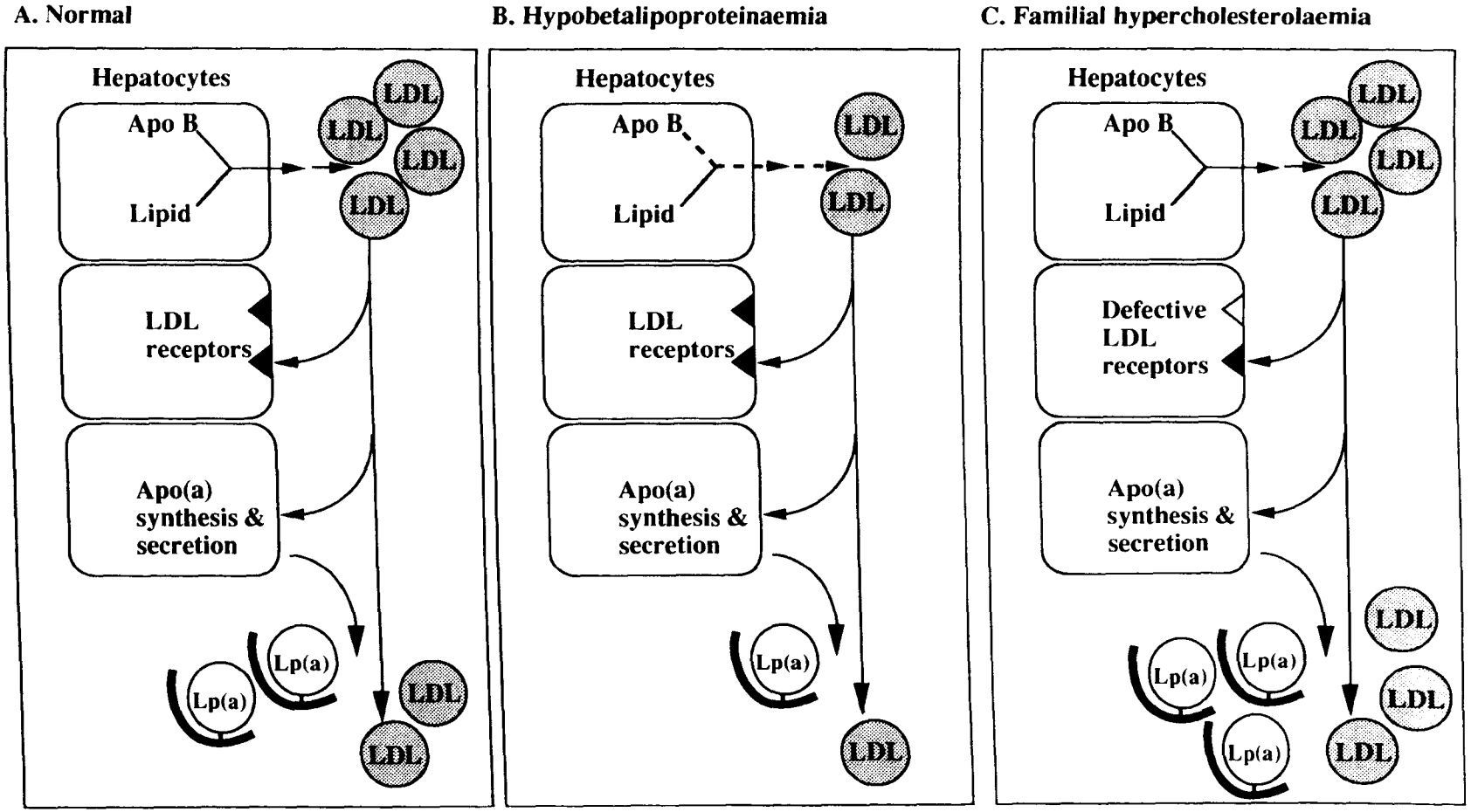


FIGURE 7.1.

Summary of the effects of normolipidaemia (Panel A.), hypobetalipoproteinaemia (Panel B.) and familial hypercholesterolaemia (Panel C.) on the assembly of Lp(a). In all three circumstances the apo(a) secretion rate is assumed to be constant. In the normal situation apoB is synthesised and associated with lipid in the hepatocyte before being secreted as a lipoprotein particle. These lipoproteins undergo extrahepatic delipidation and yield LDL particles. LDL may then be removed from the ECF by the action of the LDL receptor or may be come associated with apo(a) secreted and potentially immobilised on the hepatocyte cell surface. In FHB there is reduced apoB synthesis and therefore reduced circulating LDL. The LDL receptors function normally but there is only a limited amount of LDL available as a substrate for Lp(a) assembly and lower plasma Lp(a) levels result. In FH, there is normal apoB secretion but the defective LDL receptor function results in an excess of plasma LDL. This, in turn, results in increased plasma Lp(a) levels.

The extension of this argument is that the resulting Lp(a) particles containing such differently sized apo(a) isoforms must be viewed as equally diverse in their potential function(s) and atherogenicity. Thus, it is quite simply insufficient to define an individual's Lp(a) status or phenotype on the basis of a plasma estimation of the circulating mass of Lp(a) particles. Some measure of the type and potentially the quality of these particles must be taken into account.

Early attempts at characterising the apo(a) constituent of Lp(a), based on SDS-PAGE gave useful but limited information. This form of low resolution apo(a) phenotyping has been used by many groups and features as a an analytical tool throughout the literature. However, the originator of this method, Utermann (1989), has even now adopted the higher resolution methods developed by others (Lackner et al., 1991). The clear advantage of a high resolution system is that it provides complete definition of the apo(a) size range, which ascends in discrete steps of single K4 units. This system therefore provides the investigator with a tool to define the Lp(a) / apo(a) phenotype of an individual with much improved accuracy. As this system separates the apo(a) isoforms solely on the basis of size it tells us nothing of the potential compositional heterogeneity that may exist in Lp(a). The sugar content of the apo(a) and the lipid complement of the LDL moiety are not defined by this method and this may be seen as a potential drawback. Apart from these obvious manifestations of Lp(a) heterogeneity that are unresolved by this method there is also a more subtle characteristic of the apo(a) protein that is not touched on by this method.

As we know that *APO(a)* alleles of the same length have differences in sequence, albeit silent polymorphisms, we may hypothesise that there may also be differences in DNA sequence that lead to amino acid changes and potentially to changes in the tertiary structure and perhaps glycosylation pattern of the apo(a) protein, which may in turn lead to differences in function. This would result in the presence of apo(a) isoforms, which have the same length and therefore the same migration on the SDS-agarose gel used in high resolution apo(a) phenotyping, but which may have differences in composition and therefore, potentially, differences in function.

The apo(a) phenotyping system presented is therefore a major advance over simple plasma Lp(a) concentration measurements but it does not provide absolute differentiation of all individual apo(a) isoform types. Collectively, the different levels of polymorphism in the *APO(a)* gene are known to contribute

>90% of the inter-individual variability of plasma Lp(a) levels. The length polymorphism, which is controlled solely by the corresponding length polymorphism of the *APO(a)* gene is known to contribute approximately 42% of the variability in Lp(a) levels. As such it is probably the single most important controlling factor of plasma Lp(a) concentrations.

The research presented in this thesis was performed to contribute new information to our understanding of the mechanisms controlling plasma Lp(a) levels. I believe the results presented are useful additions to our existing knowledge base, especially the discovery that so-called "null" *APO(a)* alleles are independent of allele size as was previously thought, and that the functions of the *APOB* and LDL receptor genes do have an important impact on the plasma Lp(a) level.

7.6 Full circle

This thesis began at the very beginning of the Lp(a) story with the words Kåre Berg used to name his fascinating lipoprotein. It is fitting then that Prof. Berg should have the lasts words in this thesis, which has tried to extend our knowledge of his discovery. Over more than three decades Berg has published extensively on Lp(a) and, although by his own admission he would not claim to have dominated the field, he has always been present supporting and nudging research in the right direction like the proud father of a developing child. His most recent paper (Berg, 1994) has called investigators throughout the world to task over the most basic element of Lp(a) research: its measurement.

"Lp(a) lipoprotein determination should now be included in any clinical or scientific study of CHD risk. Lp(a) lipoprotein measurements must be based either on methods used in research laboratories or on techniques firmly validated and monitored against such methods and reagents."

Kåre Berg, 1994

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Appendix I

Suppliers of Reagents, Equipment and Software.

Amersham International plc
Arlington Heights, IL
ECL™ Western blot detection reagents
Hybond™-C extran nitrocellulose membrane
Sheep anti-mouse peroxidase linked antibody

Beckman Instruments,Inc
Fullerton, CA
Ultracentrifuges
Rotors
Bench-top low speed centrifuge
Ultra-Clear ™ centrifuge tubes
GeneLine II ™ pulsed-field gel electrophoresis apparatus

Becton Dickinson & Co
Lincoln Park, NJ
Vacutainer system

Leucoprep tubes

Bio-Rad
Hercules, CA
Ultra pure DNA grade agarose

Boehringer Mannheim
Indianapolis, IN
Proteinase K

BRL Life Technologies
Gaithersburg, MD
Horzontal gel electrophoresis apparatus

Claris™
440 Clyde Ave, Mountain View, CA, USA.
MacDraw II

Cricket Software Inc
40 Valley Stream, Malvern, PA, USA.
Cricket Graph Version 1.3.1
Statworks Version 1.2

Dupont
Wilmington, DE
Reflection™ autoradiography film
Intensifying screen

Dynatech
Chantilly, VA
Automated microtitre plate washer

Eastman Kodak
Eastman Kodak Co, Rochester, NY, USA.
X-OMAT™ autoradiography film

Hoefer Scientific Instruments
San Francisco, CA
Hoefer Transphor Cell (electroblotting appartaus)

ICN Biomedicals
Irvine, CA
Nylon membrane for Southern blotting

Intermountain Scientific
Bountiful, UT
96 well microtitre plates

Microsoft Corporation
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MS Word Version 5.1a

Molecular Devices
Menlo Park, CA
Automated microtitre plate reader

New Brunswick Scientific
Edison, NJ
Microtitre plate incubator/shaker

New England Biolabs
Beverly, MA
*Kpn*I
*Hpa*I
Low range PFG markers

Sigma
St. Louis, MO
General chemicals

Terumo Medical Corp.
Elkton, MD
Lipoprotein (a) ELISA kit

Appendix II

Individual plasma Lp(a) concentrations, *APO(a)* allele sizes and apo(a) isoform sizes of the subjects reported in chapter 4.

Caucasians

Subject No.	Subject's initials	Plasma Lp(a) ($mg.dl^{-1}$)	*APO(a)* allele 1	*APO(a)* allele 2	Apo(a) isoform 1	Apo(a) isoform 2
1	KS	44	21	25	21	25
3	NR	5	24	28	-	-
4	CL	32	18	33	18	33
6	RK	<1	24	30	24	n
7	LE	30	26	32	-	-
9	GB	83	19	27	-	-
12	LF	6	27	28	-	-
15	MB	29	18	24	18	24
18	HH	20	24	31	24	31
21	GM	19	26	33	-	-
22	MD	7	24	29	n	29
24	TO	7	25	27	25	n
26	RB	9	28	30	-	-
27	KN	8	20	24	-	-
29	DJ	7	27	32	-	-
30	JM	20	24	30	24	30
31	DN	7	32	34	32	34
32	FP	33	23	28	23	28
33	KP	1	25	33	25	33
35	BB	16	28	28	28	28
36	JL	1	28	31	n	31
37	KL	3	27	29	27	n
38	DS	16	31	33	31	33
39	ST	8	20	22	-	-
40	PD	2	26	38	n	38
41	DR	47	18	29	-	-
42	SH	5	22	28	22	n
43	TO	9	22	31	22	31
44	WC	5	22	33	-	-
45	RM	5	22	30	-	-
46	PM	<1	25	34	-	-
47	VR	19	20	29	-	-
48	DS	<1	29	30	n	n
49	DS	61	19	34	19	34
50	SS	66	19	26	19	26
51	ET	1	26	26	26	26
52	AT	47	19	21	19	21
53	MD	17	26	31	26	n
54	LO	7	20	33	20	33
55	ML	18	25	51	25	51
56	MM	1	27	39	27	39
57	PC	10	26	39	26	n
58	JG	29	31	32	31	32
59	RL	2	19	24	19	n
60	CD	29	20	21	n	21
61	MP	62	20	24	20	n

Caucasians contd

Subject No.	Subject's initials	Plasma Lp(a) ($mg.dl^{-1}$)	*APO(a)* allele 1	*APO(a)* allele 2	Apo(a) isoform 1	Apo(a) isoform 2
64	SG	9	28	33	28	n
65	RT	36	15	23	15	23
66	ER	4	33	34	33	n
67	JC	<1	25	34	n	34
68	MB	8	23	30	23	n
69	KN	13	28	30	28	30
70	LA	38	18	26	18	26
71	ES	4	28	29	n	29
72	RW	34	19	23	19	n
73	KS	17	26	27	26	n
74	LW	14	26	37	26	n
75	KM	33	23	23	23	23
76	HP	2	19	28	19	n
77	MT	4	24	26	24	n
78	VT	22	26	27	-	-
79	DH	7	25	25	25	25
80	BJ	1	24	36	n	36
81	BC	15	27	30	27	30
82	ML	3	22	32	22	32
83	JH	7	29	33	29	n
84	KH	25	24	24	24	24
85	CM	27	22	24	22	24
86	MF	<1	26	30	26	30
87	MF	32	21	33	21	n
88	MP	54	19	33	19	33
89	MP	27	18	23	18	23
90	KH	7	23	25	23	25
91	SS	39	16	32	16	n
92	RC	6	28	31	28	31
93	DS	5	15	28	n	28
94	DS	9	26	30	n	30
97	TC	28	22	27	22	27
98	LS	74	29	35	29	n
99	LC	24	28	29	23	26
101	HW	3	29	30	29	n
102	JB	36	20	31	20	n
103	SS	4	20	21	n	n
104	GT	42	16	26	16	n
105	JT	18	23	26	-	-
106	EP	88	14	37	14	n
107	MS	3	26	32	26	32
108	ES	<1	23	37	n	n
109	BH	10	22	29	22	29
110	MH	7	26	26	26	26
111	RF	9	27	32	27	n
112	RW	4	20	30	n	30
113	KW	15	19	33	19	33
114	MC	3	26	28	n	28
115	VE	7	26	27	26	27
116	CN	<1	29	40	29	40
117	AN	<1	19	30	19	30
118	PW	8	30	36	30	36
119	CW	<1	30	32	30	n
120	EH	7	28	30	-	-

Caucasians contd

Subject No.	Subject's initials	Plasma Lp(a) ($mg.dl^{-1}$)	*APO(a)* allele 1	*APO(a)* allele 2	Apo(a) isoform 1	Apo(a) isoform 2
121	BH	10	27	27	27	27
122	JC	<1	-	-	-	-
123	JC	48	13	28	13	28
133	NC	15	23	32	23	32
134	HC	3	32	33	32	n
135	MR	4	32	33	32	n
136	BR	45	19	30	19	n
137	LW	1	28	36	n	n
138	LW	9	31	34	-	-
139	HG	<1	24	29	n	n
140	NG	2	28	30	28	n
141	TM	4	28	29	n	29
142	MM	2	26	27	n	27
143	XX	29	19	32	19	n
145	GH	36	16	33	16	n
146	SH	6	27	32	n	32
147	SP	71	19	33	19	33
148	TP	21	23	24	23	24
149	CL	28	21	32	21	n
150	CL	<1	26	30	n	n
151	KT	<1	30	33	n	33
152	CT	51	23	23	23	23
153	TB	47	19	33	19	33
154	JB	5	25	26	25	26
155	DS	22	21	23	21	n
156	JS	9	25	36	25	36
157	RL	14	27	28	27	28
158	SL	41	21	21	21	21
159	SH	12	22	30	22	30
160	JH	10	22	30	22	30
161	KM	6	21	31	20	31
162	PM	15	28	34	28	34
163	RT	5	32	33	32	33
164	IT	5	21	22	21	22
165	CE	1	29	32	n	32
166	SE	1	28	29	28	n
167	CS	1	26	28	n	n
168	CS	30	22	30	22	n
169	GD	52	21	33	21	n
170	DD	1	26	32	n	32
171	CR	32	26	32	26	32
172	DR	52	18	27	18	27
173	RC	4	30	32	30	32
174	LC	3	26	26	26	26
175	RM	4	30	32	n	32
176	MM	42	18	25	18	n
177	JF	4	32	35	32	n
178	EF	51	20	33	20	33
179	JT	48	18	22	18	22
180	CT	7	30	31	30	31
181	EM	<1	33	36	n	n
182	BM	6	23	32	n	32
183	CJ	27	24	26	24	26
184	JJ	10	30	31	30	n

Caucasians contd

Subject No.	Subject's initials	Plasma Lp(a) ($mg.dl^{-1}$)	*APO(a)* allele 1	*APO(a)* allele 2	Apo(a) isoform 1	Apo(a) isoform 2
185	JH	50	18	27	18	27
186	SH	9	28	29	28	29
187	PP	38	19	30	19	30
188	LP	19	18	29	18	n
189	MP	60	19	25	19	26
190	SP	41	19	20	19	n
191	SS	2	25	33	n	33
192	GS	4	22	32	22	32
193	JB	16	28	30	28	30
194	MB	<1	24	35	n	n
195	PQ	9	23	27	n	27
196	DQ	10	25	31	n	31
197	WM	12	28	40	28	40
198	SM	1	26	28	n	n
199	GW	40	20	35	20	n
200	KW	54	15	26	15	26
201	DW	3	22	26	22	n
202	DW	7	25	32	n	32

Chinese

Subject No.	Subject's initials	Plasma Lp(a) ($mg.dl^{-1}$)	*APO(a)* allele 1	*APO(a)* allele 2	Apo(a) isoform 1	Apo(a) isoform 2
C1	WC	5	33	33	33	33
C2	KL	15	33	33	33	33
C4	YH	26	23	34	23	34
C5	BZ	3	27	29	n	29
C6	HZ	8	23	27	23	n
C7	ST	10	28	35	28	35
C8	XX	14	26	33	26	33
C9	SP	2	34	36	34	36
C10	MY	31	24	36	24	36
C11	YY	8	34	36	34	36
C12	GS	33	15	34	15	34
C13	JC	13	31	32	n	32
C14	LH	33	22	33	22	33
C15	SW	8	33	35	33	35
C16	BG	45	21	33	21	33
C17	XZ	5	34	34	34	34
C18	XW	13	23	41	23	41
C19	JL	3	22	33	n	33
C21	QJ	6	32	35	32	35
C22	JC	63	15	32	15	32
C23	JC	4	33	35	33	n
C24	JL	4	33	34	n	34
C25	KL	11	28	34	28	34
C26	PL	2	33	37	33	37
C27	XL	30	25	33	25	33
C29	BZ	5	34	38	34	38
C30	XT	34	24	34	23	34
C31	CZ	3	32	33	n	33
C32	WT	10	32	33	32	33
C33	YS	26	29	34	29	34
C34	CM	7	30	30	30	30
C35	YX	3	32	33	n	33
C36	KL	32	19	33	19	33
C37	MZ	8	25	33	25	33
C38	CL	5	33	34	33	n
C39	YY	3	36	36	36	36
C40	LF	49	20	26	20	n
C41	MZ	6	34	35	34	35
C42	YZ	13	31	34	31	34
C44	ZG	17	24	33	24	33
C45	YH	14	31	35	31	35
C46	YW	1	33	33	33	33
C47	EG	3	35	35	35	35
C48	CY	13	28	32	28	32
C50	ST	9	30	31	n	31
C51	SN	7	32	37	32	37
C52	GT	5	32	33	32	33
C53	CH	11	32	34	32	34
C54	SH	5	31	37	31	37
C55	CS	27	25	35	25	35
C57	HT	6	32	40	32	40
C58	CY	4	32	36	32	n
C59	WF	11	31	43	31	n
C60	YY	13	30	32	30	32
C61	PT	32	30	32	30	32
C62	MD	68	19	40	19	40

Chinese contd

Subject No.	Subject's initials	Plasma Lp(a) ($mg.dl^{-1}$)	*APO(a)* allele 1	*APO(a)* allele 2	Apo(a) isoform 1	Apo(a) isoform 2
C63	HY	1	26	34	n	34
C64	YL	13	29	34	29	34
C65	HC	3	35	39	35	39
C66	PC	9	32	34	32	34
C67	FC	3	33	35	33	35
C68	YC	17	21	34	21	34
C69	CY	2	32	34	32	34
C71	HC	<1	35	36	n	n
C72	LW	26	26	31	26	31
C73	SW	68	22	33	22	33
C74	LY	9	30	33	30	33
C75	YY	<1	32	36	32	36
C76	TL	<1	31	31	31	31
C77	BY	1	28	35	28	n
C78	SL	9	29	30	29	30
C79	CL	7	28	35	28	35
C80	PL	14	28	36	28	36
C81	TC	10	23	32	23	32
C82	TC	2	32	44	32	44
C83	DS	4	18	33	n	33
C84	CH	<1	38	41	n	41
C85	MC	6	29	33	29	33
C86	JW	6	29	37	29	37
C87	SS	21	23	33	23	33
C88	SS	3	25	33	n	33
C89	MC	5	33	33	33	33
C90	FC	3	33	37	33	37
C93	GC	14	33	33	33	33
C94	HC	<1	28	35	n	n
C96	LK	2	21	32	n	32
C97	JK	3	31	31	31	31
C98	CY	14	25	29	25	29
C99	CC	54	28	33	28	33
C100	YW	3	33	33	33	33
C101	EL	3	32	34	32	34
C102	CF	16	28	35	28	35
C103	HH	156	18	27	18	27
C104	IC	73	19	33	19	33
C105	TC	10	28	38	28	38

African Americans

Subject No.	Subject's initials	Plasma Lp(a) ($mg.dl^{-1}$)	*APO(a)* allele 1	*APO(a)* allele 2	Apo(a) isoform 1	Apo(a) isoform 2
B1	JJ	3	24	27	n	n
B2	RG	71	20	23	20	23
B3	RM	50	19	31	19	31
B4	LB	34	25	32	25	32
B5	PM	10	30	36	30	36
B6	MJ	43	23	26	23	26
B7	DK	8	22	25	32	38
B8	JT	22	26	35	26	n
B9	AF	4	25	29	n	29
B10	BH	24	27	33	27	33
B11	MD	23	24	32	24	32
B12	RT	120	21	24	21	24
B13	NW	30	24	26	24	n
B14	BW	44	18	20	-	-
B15	AE	64	16	18	16	18
B18	RM	40	22	24	22	24
B19	BJ	68	21	32	21	32
B20	BH	12	26	29	26	29
B21	FE	31	29	31	29	31
B22	AT	72	20	24	-	-
B23	CH	32	25	27	25	27
B24	DB	41	23	28	23	28
B25	CJ	65	23	37	23	37
B26	CA	7	25	32	n	32
B27	CD	29	24	27	24	27
B28	MT	14	26	26	26	26
B29	JS	37	23	28	23	n
B30	MC	17	28	29	28	29
B31	LJ	36	20	30	20	n
B32	SJ	32	22	32	22	32
B33	PW	90	18	18	-	-
B34	JH	63	23	23	23	23
B35	JJ	24	29	30	29	30
B36	NB	33	21	24	21	n
B37	CB	58	27	28	27	28
B38	RW	37	23	31	23	31
B39	JH	25	27	33	27	33
B40	BN	52	25	26	n	26
B41	JG	13	30	33	30	33
B42	BG	86	18	23	18	23
B43	JW	51	21	28	21	28
B44	JR	49	20	24	-	-
B45	DL	118	19	19	19	19
B46	DT	29	18	27	18	27
B47	CL	53	24	27	24	27
B48	BB	<1	30	30	30	30
B49	KN	8	21	33	21	33
B50	SH	12	27	28	n	28
B51	TS	11	25	33	25	n
B52	SP	39	26	28	26	28
B54	CB	49	20	30	20	30
B55	GW	26	26	29	26	29
B56	SC	32	26	27	26	27
B57	AW	51	24	28	24	28
B58	BW	10	23	26	23	n
B59	AH	2	25	26	n	n

African Americans contd

Subject No.	Subject's initials	Plasma Lp(a) ($mg.dl^{-1}$)	*APO(a)* allele 1	*APO(a)* allele 2	Apo(a) isoform 1	Apo(a) isoform 2
B60	NY	35	20	28	20	28
B61	CS	29	22	29	22	29
B62	JW	22	18	29	n	29
B63	GJ	9	29	42	29	42
B64	JM	39	21	33	21	33
B65	JS	24	26	26	26	26
B66	BP	18	18	29	18	29
B67	MT	27	19	28	n	28
B68	AT	54	27	29	27	29
B69	VJ	5	20	23	-	-
B70	RS	90	25	25	25	25
B71	EK	92	18	26	18	26
B72	SS	1	18	30	n	n
B73	MS	64	21	31	21	31
B74	KS	23	23	26	-	-
B75	DP	25	24	33	n	33
B76	JD	42	21	29	21	29
B77	EJ	41	18	22	18	22
B78	PR	4	29	37	29	n
B79	MK	32	27	33	27	33
B80	BD	91	22	25	22	25
B81	MC	45	27	28	27	28
B82	PR	76	22	30	22	30
B83	IR	51	21	28	21	28
B84	CW	17	27	27	27	27
B85	GJ	21	28	28	28	28
B86	RL	33	23	25	n	33
B87	LB	12	27	28	27	28
B88	NB	49	22	25	22	25
B89	JH	10	26	30	n	30
B90	AL	5	29	34	n	34
B91	TA	36	19	37	19	37
B92	AS	5	31	31	31	31
B93	LP	33	23	30	23	30
B94	WS	84	23	26	23	26
B95	MS	52	25	31	25	31
B96	IM	12	26	27	26	27
B97	DB	14	18	21	18	n
B98	EP	34	27	39	27	39
B99	JC	19	13	34	13	34
B100	VH	59	22	25	22	25
B101	AR	60	17	25	17	25
B102	WC	62	21	27	25	29
B104	CB	74	24	28	24	28
B105	IC	<1	35	38	35	38
B106	JW	81	21	23	21	23
B200-1	MK	5	26	40	26	n
B200-2	WK	45	22	24	22	24
B201-1	EC	97	21	27	21	n
B201-2	SC	37	13	24	n	24

Appendix III

Characteristics of the FH family members subjects reported in chapter 5.

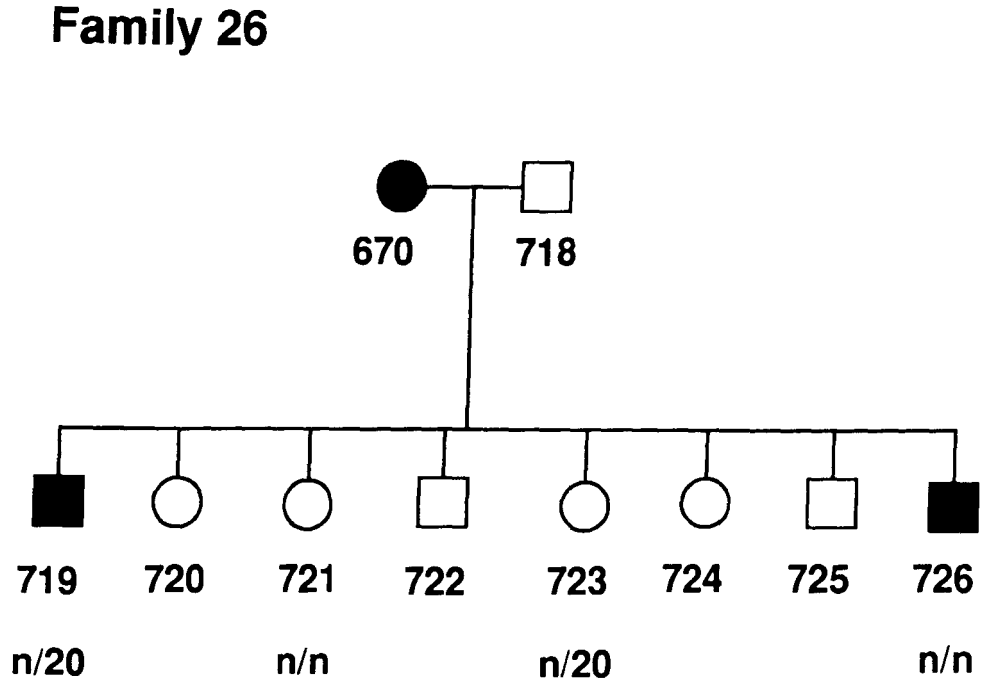
Subject No.	Age	Sex	TC	TG	LDL-C	Lp(a)	Apo(a) phenotype	FH
26-341	24	M	9.0	1.9	7.0	22.0	n/14	+
26-342	22	M	3.5	0.8	2.4	10.0	n/22	-
26-343	20	F	7.9	2.0	5.6	13.0	n/22	+
26-344	17	M	4.8	1.2	3.2	18.0	n/14	-
26-393	24	F	6.3	1.3	4.6	29.0	17/34	+
26-394	23	M	4.1	0.7	2.4	2.0	32/34	-
26-395	21	F	4.4	0.9	2.7	4.0	32/34	-
26-397	18	F	4.5	0.6	3.0	21.0	17/34	-
26-399	13	M	4.7	0.6	3.2	33.0	17/34	-
26-496	17	M	4.1*	1.2*	3.1*	57.0	22/30	+
26-497	8	M	4.0	0.6	†	64.0	22/30	-
26-527	28	M	6.4	1.8	5.3	64.0	22/30	+
26-719	24	M	6.4	1.5	4.7	35.0	n/20	+
26-721	20	F	4.9	1.9	2.2	1.0	n/n	-
26-723	16	F	4.0	0.7	2.3	56.0	n/20	-
26-726	8	M	7.7	1.0	6.4	1.0	n/n	+
625-30	44	M	6.7	1.9	4.0	8.0	28/39	-
625-42	35	M	4.0	1.2	2.5	12.0	28/39	-
625-52	32	M	4.8	2.1	3.3	17.0	26/28	-
625-59	29	F	4.7	1.2	2.4	22.0	26/28	-
625-64	28	F	6.5	0.6	5.1	20.0	26/28	+
625-69	24	F	4.1	0.7	1.8	20.0	26/28	-
625-70	22	F	4.0	0.6	2.5	16.0	26/28	-
653-1-1	43	M	10.7	1.5	5.8	31.0	20/26	+
653-1-5	37	F	7.8	1.1	5.5	28.0	20/26	+
653-1-12	68	M	9.4	4.6	5.0	37.0	20/26	+
653-1-24	79	M	5.0	2.4	3.0	60.0	20/26	-
653-1-28	17	M	6.6	1.2	5.1	28.0	n/20	+
653-1-29	16	F	8.3	1.8	†	24.0	n/20	+
653-1-30	14	F	3.9	1.0	2.1	2.0	n/19	-
653-1-31	10	M	6.9	1.1	5.2	3.0	n/19	+
653-1-41	42	M	11.7	0.9	4.8	10.0	20/26	+
653-1-81	18	F	7.2	0.9	5.6	10.0	21/28	+
653-1-83	11	M	8.6	0.7	7.2	17.5	21/28	+
653-1-268	66	F	5.9	1.3	3.7	13.6	n/28	-
653-1-272	52	F	7.5	1.2	2.8	14.4	n/28	-

Age: in years at time of sampling, Sex: F female, M male.
TC (total cholesterol), LDL-C and TG (triglyceride): in mmol. L^{-1}
Lp(a): in mg. dL^{-1}
FH (familial hypercholesterolaemia): + FH, - normal.
* lipid values on lipid lowering drug therapy
† sample for LDL-C measurement lost during analysis.

Characteristics of the FH family members reported in chapter 5 (continued).

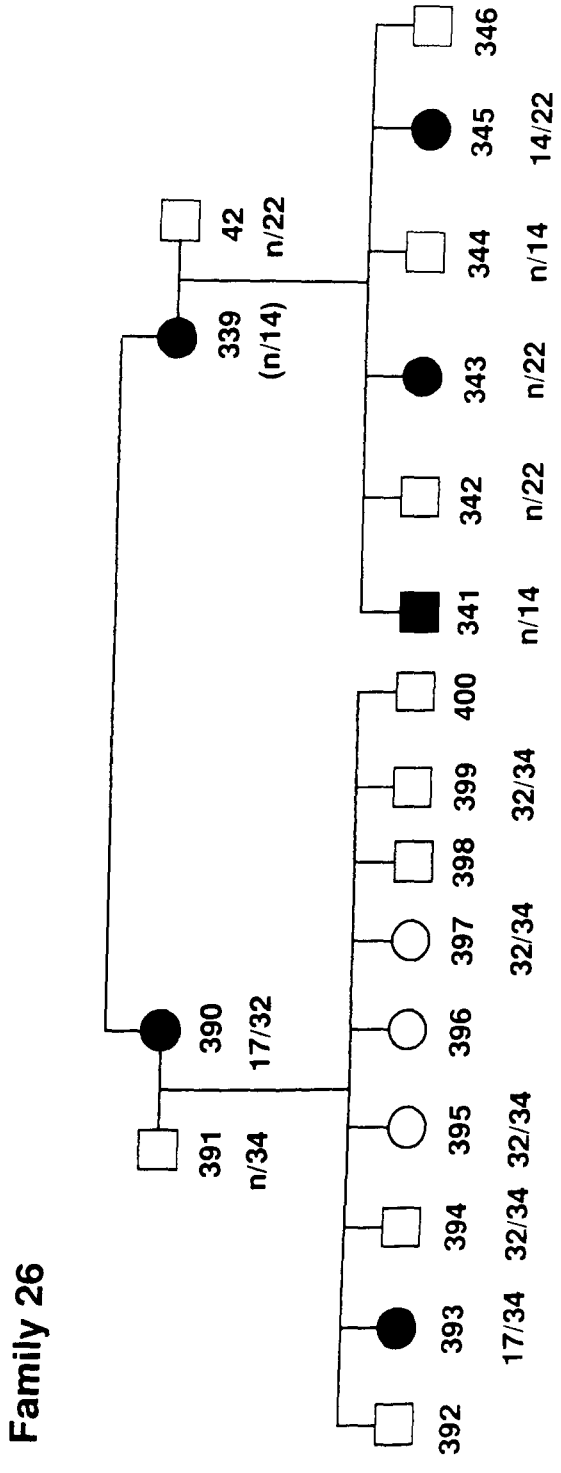
Subject No.	Age	Sex	TC	TG	LDL-C	Lp(a)	Apo(a) phenotype	FH
653-2-102	31	M	5.6	2.0	3.4	4.6	n/30	-
653-2-104	28	M	5.5	1.7	3.6	3.7	n/30	-
653-2-105	26	F	4.3	1.0	2.5	5.0	n/30	-
653-4-103	21	F	4.2	0.5	2.5	11.8	26/34	-
653-4-104	18	F	4.1	0.8	2.5	38.9	n/19	-
653-4-107	11	M	6.5	1.4	4.8	40.7	n/19	+
653-4-108	10	M	7.1	0.9	5.1	22.0	26/34	+
653-4-109	7	F	3.9	0.9	2.2	7.2	26/34	-
653-4-110	6	F	6.7	1.3	4.9	16.7	26/34	+
653-5-102	36	M	9.8	6.2	4.2	10.7	29/31	+
653-5-109	18	F	4.1	1.3	2.7	7.0	29/31	-
653-6-103	20	M	5.3	1.5	3.1	45.9	20/33	-
653-6-105	13	F	12.6	2.7	9.9	63.0	20/33	+
659-61	46	M	15.8	5.4	6.2	45.0	n/15	+
659-63	40	M	4.1	4.1	1.5	37.0	n/15	-
659-125	29	M	8.1	1.4	7.0	47.0	n/20	+
659-127	20	M	7.9	1.9	5.8	42.0	n/20	+
659-139	15	F	4.8	2.3	3.2	56.0	15/24	+
659-140	12	F	5.1	1.9	3.9	62.4	15/24	+
659-143	18	M	6.8	1.7	4.5	4.4	n/20	+
659-144	16	M	5.5	1.6	4.9	39.9	15/20	+
659-145	12	F	3.6	1.9	2.3	45.1	15/20	-
659-147	7	M	5.9	1.3	4.6	3.9	n/20	+
659-151	23	F	5.0	1.2	2.7	36.0	n/21	-
659-152	20	M	8.0	3.1	5.2	21.0	n/21	+
71002076-264	16	F	6.4	1.0	4.8	14.0	n/27	+
71002076-265	14	F	3.2	0.4	2.2	9.0	n/27	-
71002076-266	9	F	3.4	0.4	2.0	3.0	n/27	-

Age: in years at time of sampling, Sex: F female, M male.
TC (total cholesterol), LDL-C and TG (triglyceride): in mmol. L^{-1}
Lp(a): in mg. dL^{-1}
FH (familial hypercholesterolaemia): + FH, - normal.



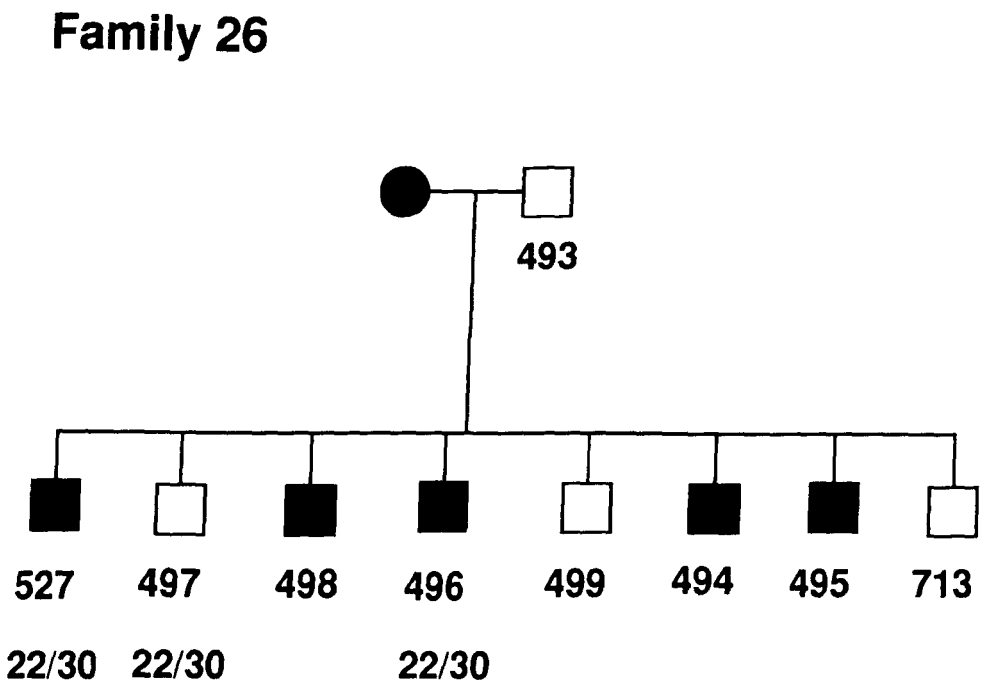
FH pedigree 1.

The squares and circles represent males and females, respectively, while open symbols and shaded symbols represent normal and FH, respectively. The number immediately below the subject symbol denotes that subject's family identification (ID) number. The pairs of numbers below the ID numbers denote the apo(a) phenotye of the subject as assessed by high resolution immunoblotting. Where an 'n' is recorded this denotes a null or non-expressing *APO(a)* allele.



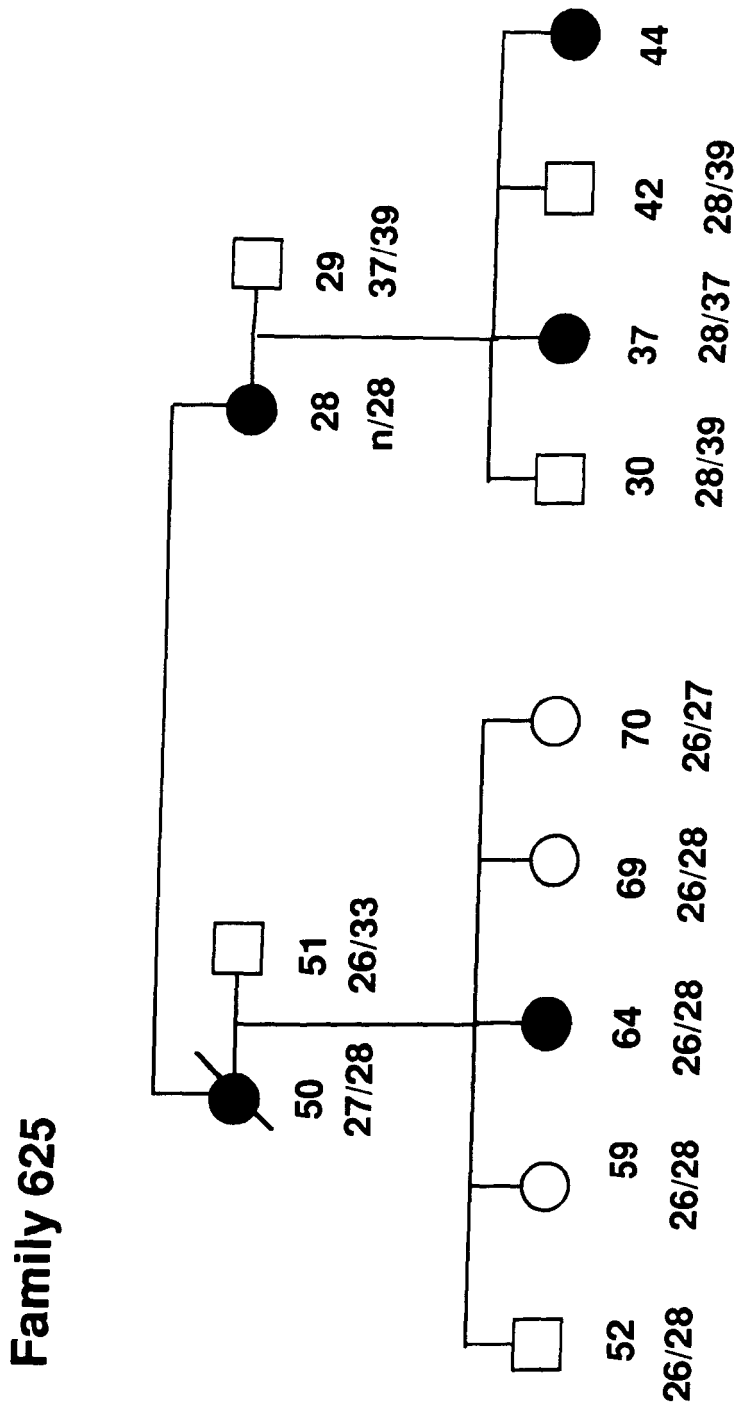
FH pedigree 2.

The squares and circles represent males and females, respectively, while open symbols and shaded symbols represent normal and FH, respectively. The number immediately below the subject symbol denotes that subject's family identification (ID) number. The pairs of numbers below the ID numbers denote the apo(a) phenotye of the subject as assessed by high resolution immunoblotting. Where an 'n' is recorded this denotes a null or non-expressing *APO(a)* allele.



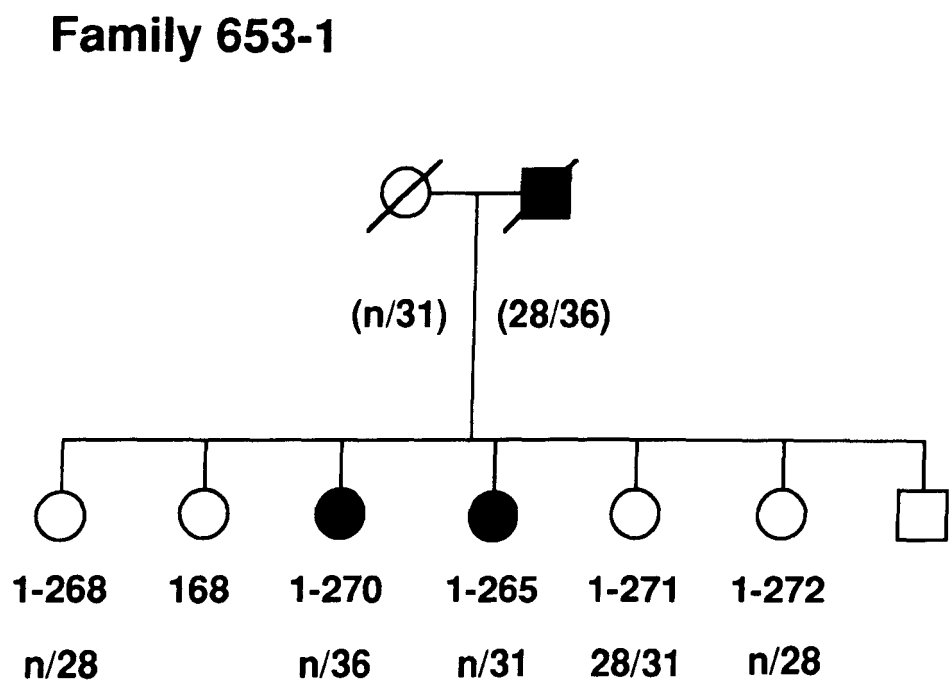
FH pedigree 3.

The squares and circles represent males and females, respectively, while open symbols and shaded symbols represent normal and FH, respectively. The number immediately below the subject symbol denotes that subject's family identification (ID) number. The pairs of numbers below the ID numbers denote the apo(a) phenotye of the subject as assessed by high resolution immunoblotting. Where an 'n' is recorded this denotes a null or non-expressing *APO(a)* allele.



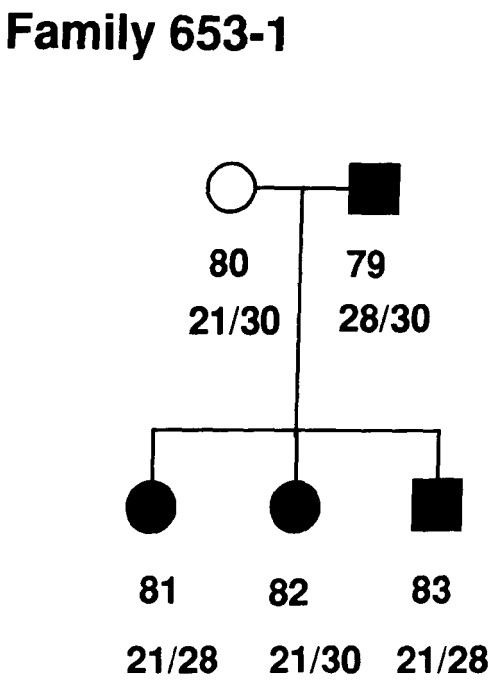
FH pedigree 4.

The squares and circles represent males and females, respectively, while open symbols and shaded symbols represent normal and FH, respectively. The number immediately below the subject symbol denotes that subject's family identification (ID) number. The pairs of numbers below the ID numbers denote the apo(a) phenotye of the subject as assessed by high resolution immunoblotting. Where an 'n' is recorded this denotes a null or non-expressing *APO(a)* allele.



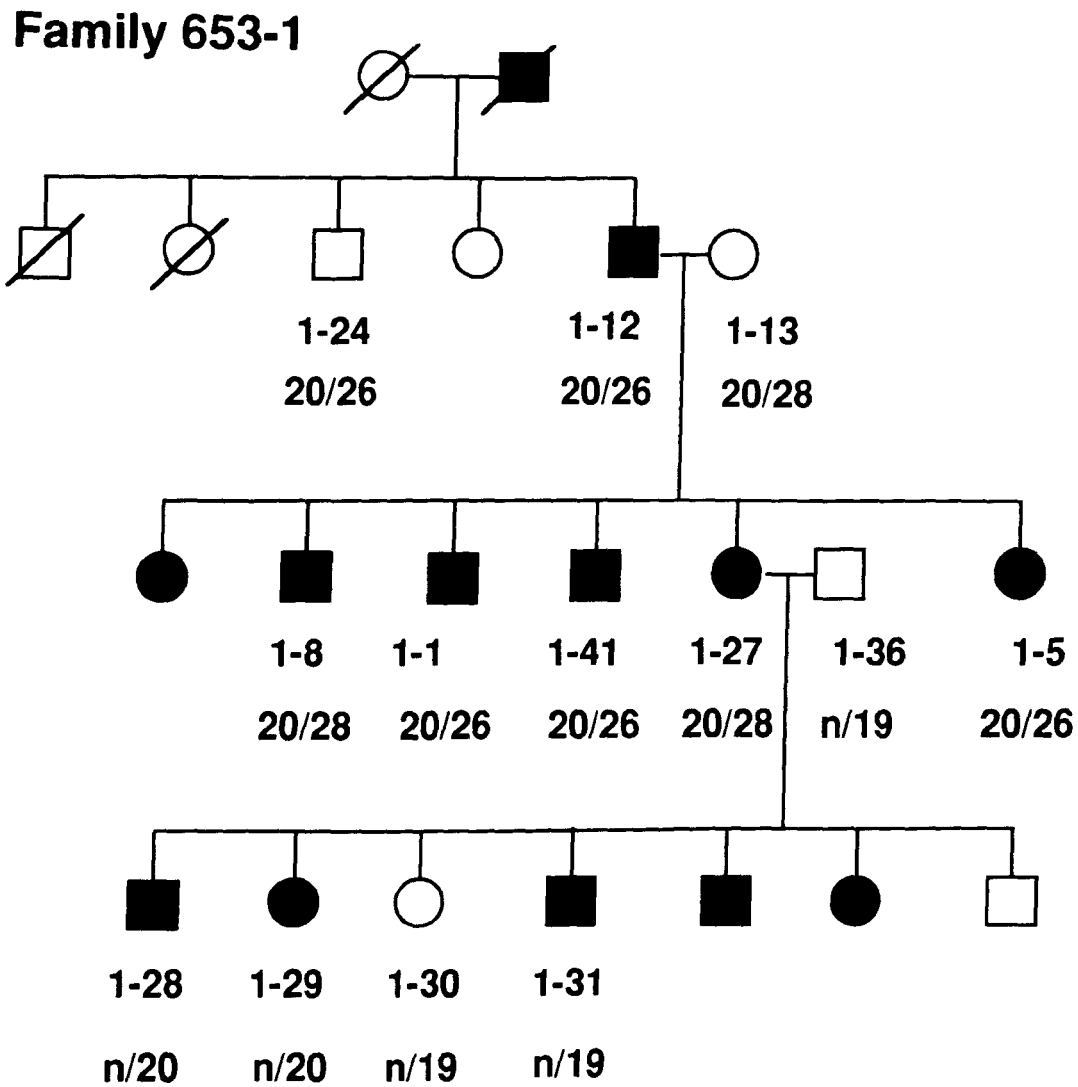
FH pedigree 5.

The squares and circles represent males and females, respectively, while open symbols and shaded symbols represent normal and FH, respectively. The number immediately below the subject symbol denotes that subject's family identification (ID) number. The pairs of numbers below the ID numbers denote the apo(a) phenotye of the subject as assessed by high resolution immunoblotting. Where an 'n' is recorded this denotes a null or non-expressing *APO(a)* allele.



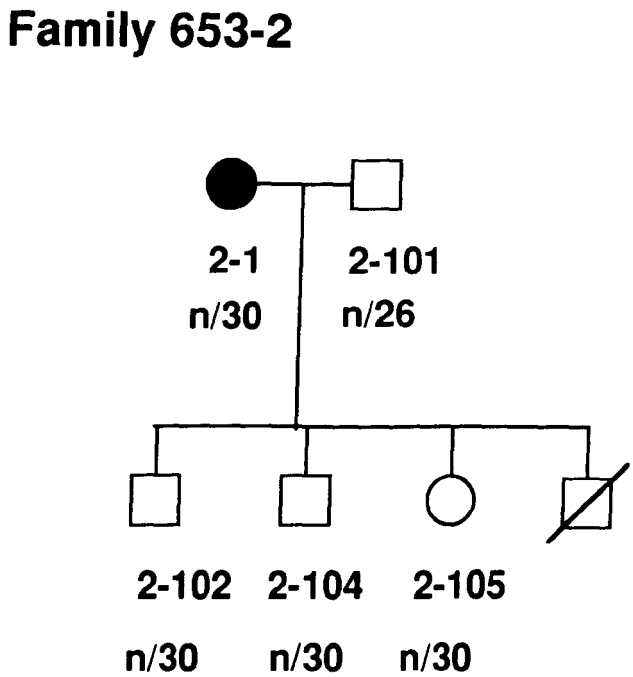
FH pedigree 6.

The squares and circles represent males and females, respectively, while open symbols and shaded symbols represent normal and FH, respectively. The number immediately below the subject symbol denotes that subject's family identification (ID) number. The pairs of numbers below the ID numbers denote the apo(a) phenotye of the subject as assessed by high resolution immunoblotting. Where an 'n' is recorded this denotes a null or non-expressing *APO(a)* allele.



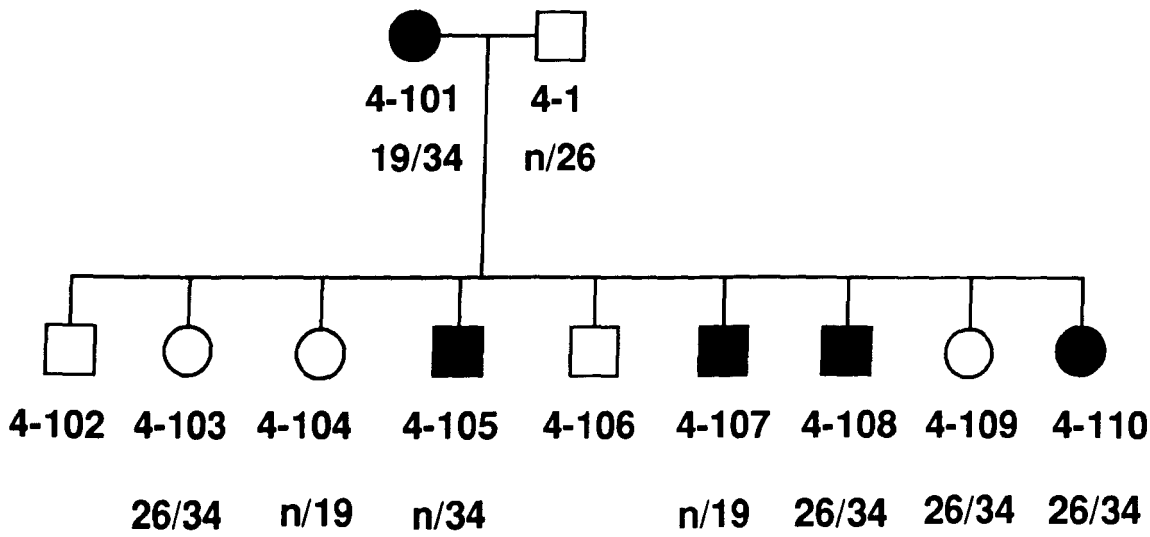
FH pedigree 7.

The squares and circles represent males and females, respectively, while open symbols and shaded symbols represent normal and FH, respectively. The number immediately below the subject symbol denotes that subject's family identification (ID) number. The pairs of numbers below the ID numbers denote the apo(a) phenotye of the subject as assessed by high resolution immunoblotting. Where an 'n' is recorded this denotes a null or non-expressing *APO(a)* allele.



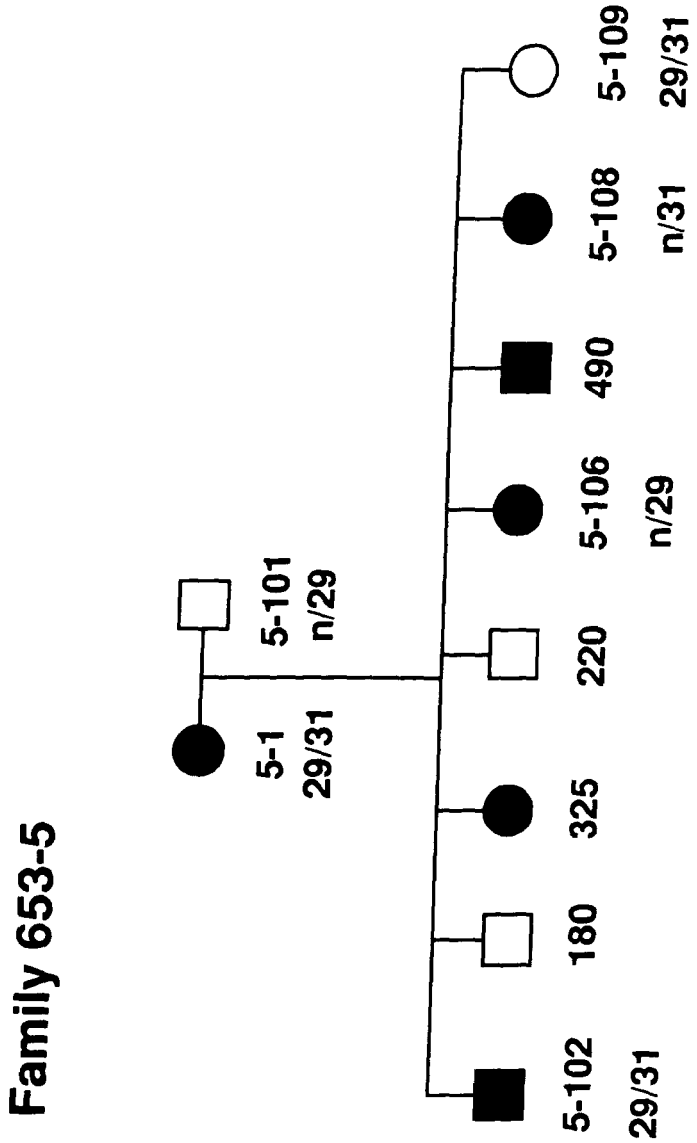
FH pedigree 8.

The squares and circles represent males and females, respectively, while open symbols and shaded symbols represent normal and FH, respectively. The number immediately below the subject symbol denotes that subject's family identification (ID) number. The pairs of numbers below the ID numbers denote the apo(a) phenotye of the subject as assessed by high resolution immunoblotting. Where an 'n' is recorded this denotes a null or non-expressing *APO(a)* allele.



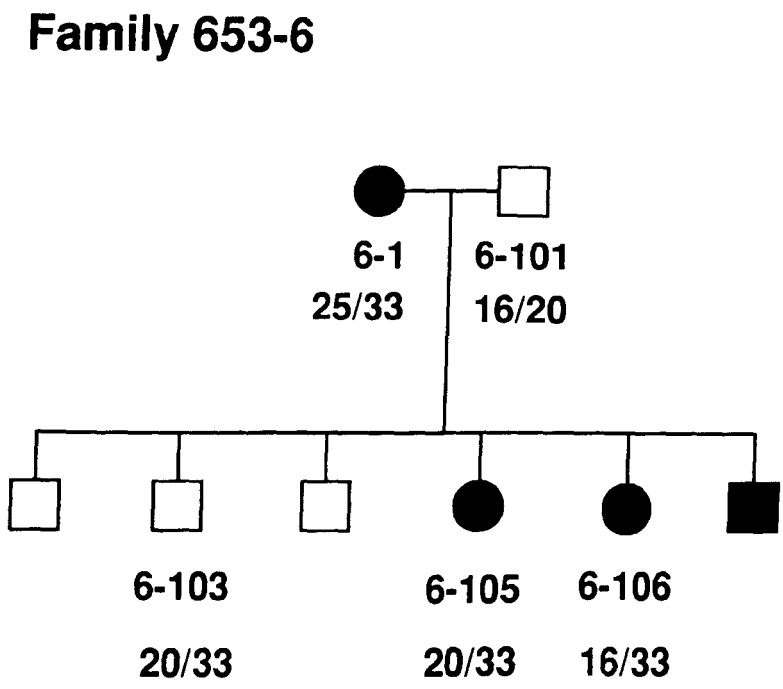
FH pedigree 9.

The squares and circles represent males and females, respectively, while open symbols and shaded symbols represent normal and FH, respectively. The number immediately below the subject symbol denotes that subject's family identification (ID) number. The pairs of numbers below the ID numbers denote the apo(a) phenotye of the subject as assessed by high resolution immunoblotting. Where an 'n' is recorded this denotes a null or non-expressing *APO(a)* allele.



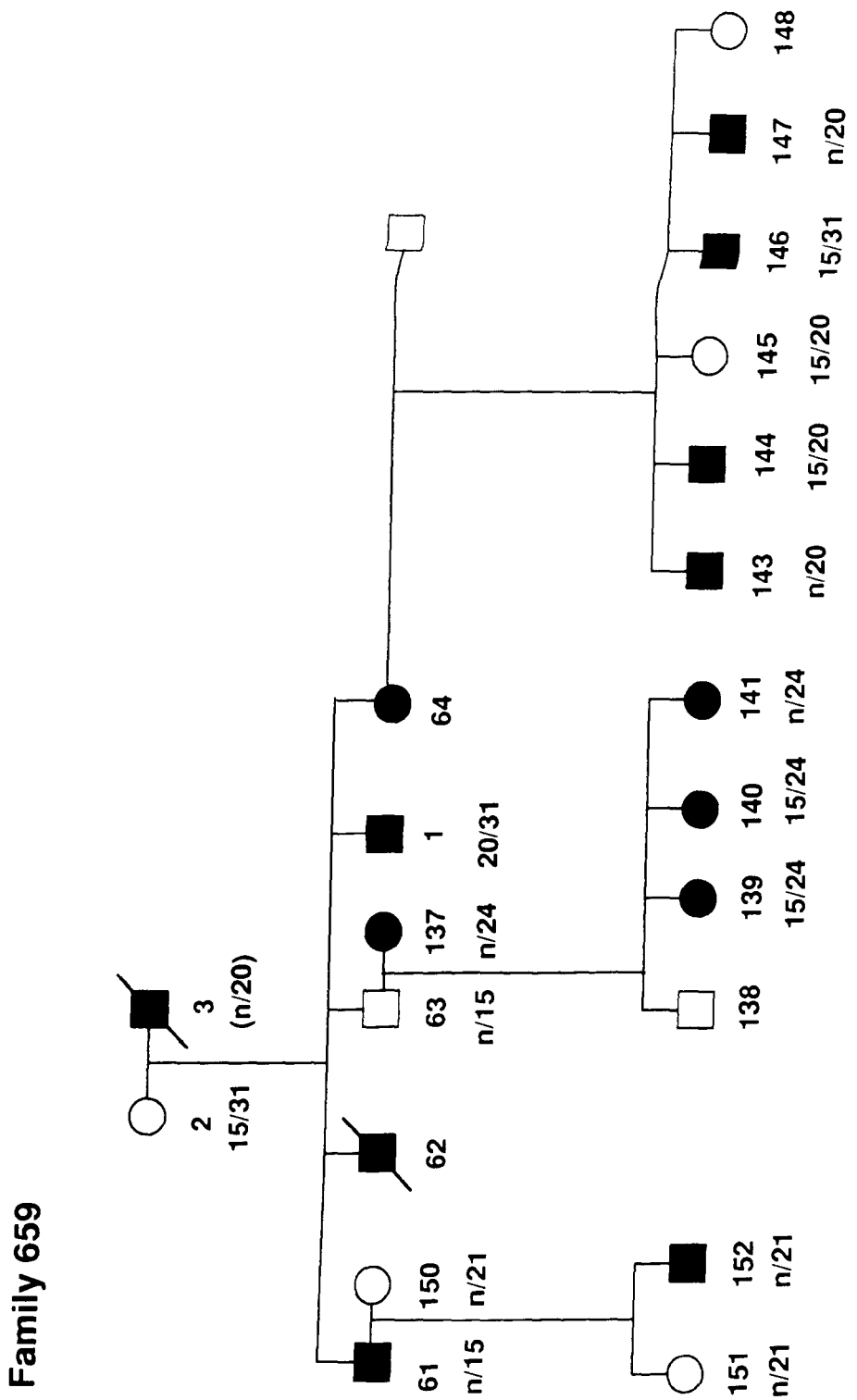
FH pedigree 10.

The squares and circles represent males and females, respectively, while open symbols and shaded symbols represent normal and FH, respectively. The number immediately below the subject symbol denotes that subject's family identification (ID) number. The pairs of numbers below the ID numbers denote the apo(a) phenotye of the subject as assessed by high resolution immunoblotting. Where an 'n' is recorded this denotes a null or non-expressing *APO(a)* allele.



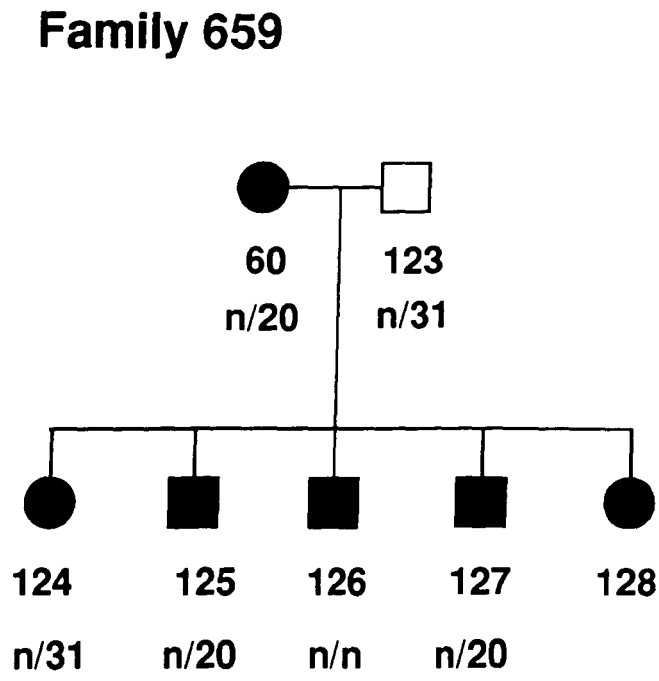
FH pedigree 11.

The squares and circles represent males and females, respectively, while open symbols and shaded symbols represent normal and FH, respectively. The number immediately below the subject symbol denotes that subject's family identification (ID) number. The pairs of numbers below the ID numbers denote the apo(a) phenotye of the subject as assessed by high resolution immunoblotting. Where an 'n' is recorded this denotes a null or non-expressing *APO(a)* allele.



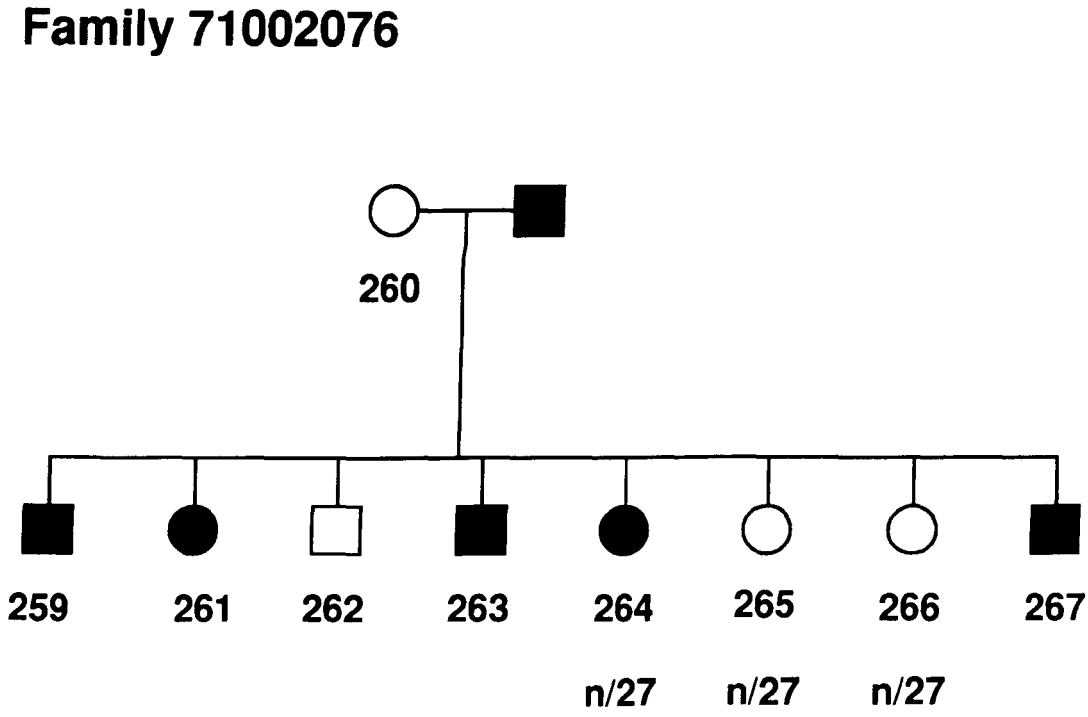
FH pedigree 12.

The squares and circles represent males and females, respectively, while open symbols and shaded symbols represent normal and FH, respectively. The number immediately below the subject symbol denotes that subject's family identification (ID) number. The pairs of numbers below the ID numbers denote the apo(a) phenotye of the subject as assessed by high resolution immunoblotting. Where an 'n' is recorded this denotes a null or non-expressing *APO(a)* allele.



FH pedigree 13.

The squares and circles represent males and females, respectively, while open symbols and shaded symbols represent normal and FH, respectively. The number immediately below the subject symbol denotes that subject's family identification (ID) number. The pairs of numbers below the ID numbers denote the apo(a) phenotye of the subject as assessed by high resolution immunoblotting. Where an 'n' is recorded this denotes a null or non-expressing *APO(a)* allele.



FH pedigree 14.

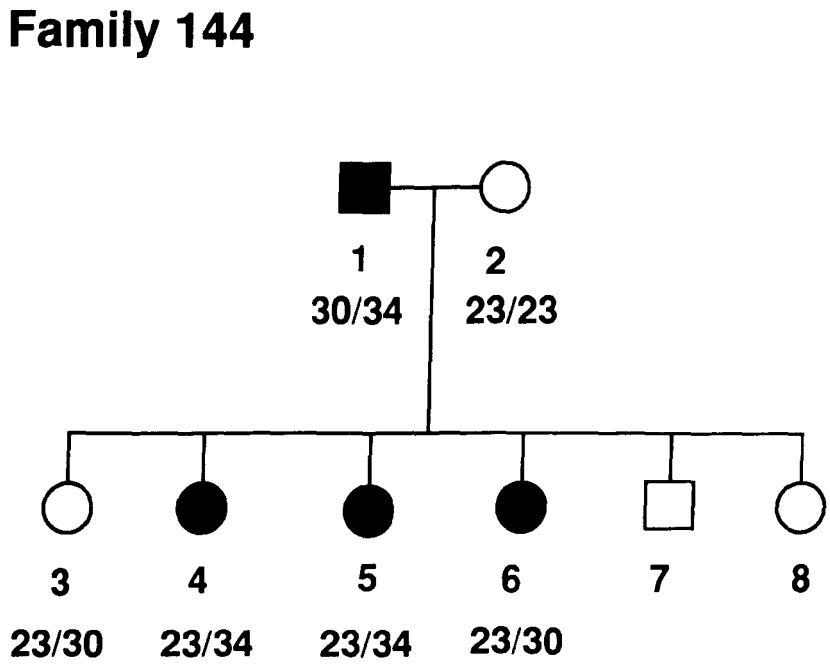
The squares and circles represent males and females, respectively, while open symbols and shaded symbols represent normal and FH, respectively. The number immediately below the subject symbol denotes that subject's family identification (ID) number. The pairs of numbers below the ID numbers denote the apo(a) phenotye of the subject as assessed by high resolution immunoblotting. Where an 'n' is recorded this denotes a null or non-expressing *APO(a)* allele.

Appendix IV

Characteristics of the FHB family members reported in chapter 6.

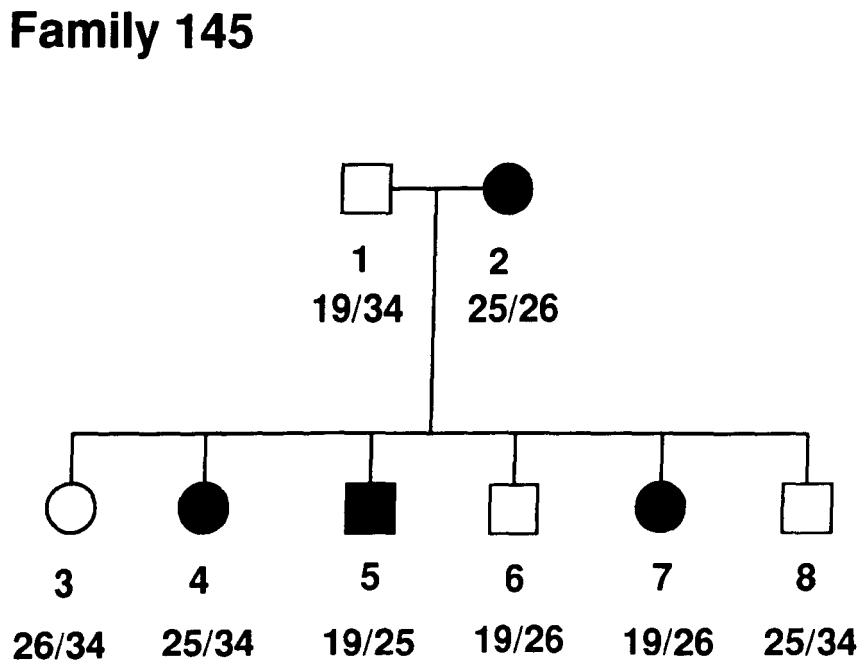
Subject No.	Age	Sex	TC	TG	LDL-C	Lp(a)	Apo(a) phenotype	FHB
144-3	10	F	3.9	0.5	2.1	18.0	23/30	-
144-4	9	F	3.9	0.7	1.7	3.0	23/34	+
144-5	7	F	3.3	0.9	1.2	3.0	23/34	+
144-6	5	F	2.6	1.1	1.1	1.0	23/30	+
145-4	14	F	3.3	2.0	1.3	1.0	25/34	+
145-6	10	M	4.9	1.8	2.7	37.7	19/26	-
145-7	8	F	3.5	2.1	1.4	24.6	19/26	+
145-8	4	M	3.4	0.8	1.8	3.0	25/34	-
153-3	14	F	3.5	0.9	2.1	5.0	34/34	-
153-4	12	F	2.1	0.4	0.6	4.0	34/34	+
153-5	11	F	1.9	0.3	0.4	1.0	34/34	+
174-1-19	24	M	2.2	0.1	0.6	2.0	22/28	+
174-1-26	17	F	4.6	0.9	3.0	1.9	23/34	-
174-1-28	27	M	4.8	2.7	2.7	4.6	22/34	-
174-1-29	30	F	4.5	0.4	2.5	3.4	22/34	-
174-1-31	31	F	7.1	1.6	5.2	11.2	22/28	-
174-1-32	20	M	4.1	2.6	1.5	1.0	23/34	-
174-1-36	34	M	4.6	0.1	3.1	2.5	22/34	-
174-1-37	22	F	3.8	2.1	1.2	1.8	22/34	+
174-1-39	28	M	8.3	1.3	6.5	1.8	22/34	-
174-2-2	58	F	5.6	1.8	3.4	15.6	13/22	-
174-2-3	38	F	5.6	0.1	2.4	68.2	14/17	-
174-2-4	70	F	2.5	1.0	0.8	20.7	17/27	+
174-2-5	36	F	6.0	1.8	4.0	76.2	14/17	-
174-2-6	42	M	7.4	15	5.0	79.8	14/17	-
174-2-7	65	F	4.0	1.3	1.4	18.6	17/27	+
174-2-10	66	M	3.4	1.8	0.9	35.2	17/27	+
174-2-11	35	F	4.9	0.9	3.2	78.0	14/17	-
174-2-16	69	M	*	*	*	19.0	13/22	-
174-2-17	40	F	3.5	1.1	0.9	1.2	27/29	+
174-2-20	20	F	4.1	0.6	2.4	22.8	17/29	-
174-2-21	41	M	4.7	1.3	2.7	2.0	27/29	-
174-2-22	36	M	5.2	4.0	2.2	19.6	17/29	-
174-2-41	39	F	3.3	1.0	0.8	2.5	27/29	+
174-2-44	24	F	3.0	0.6	1.0	20.0	17/29	+

Age: in years at time of sampling, Sex: F female, M male.
TC (total cholesterol), LDL-C and TG (triglyceride): in mmol. L^{-1}
Lp(a): in mg. dL^{-1}
FHB (familial hypobetalipoproteinaemia): + hypobetalipoproteinaemic, - normal.
* sample for lipid measurement lost during analysis, but subject 174-2-16 previously documented as normolipidaemic.



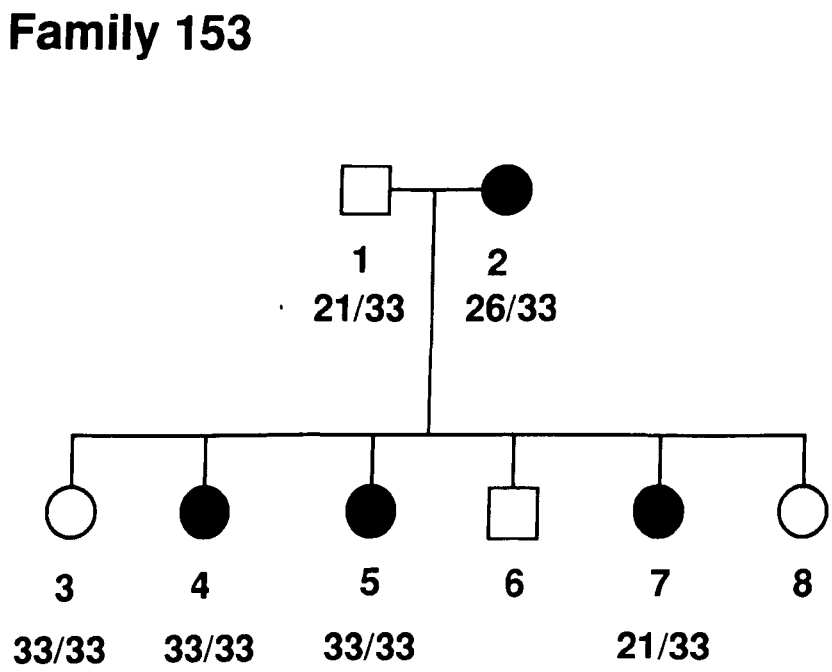
FHB pedigree 1.

The squares and circles represent males and females, respectively, while open symbols and shaded symbols represent normal and FHB, respectively. The number immediately below the subject symbol denotes that subject's family identification (ID) number. The pairs of numbers below the ID numbers denote the apo(a) phenotye of the subject as assessed by high resolution immunoblotting.



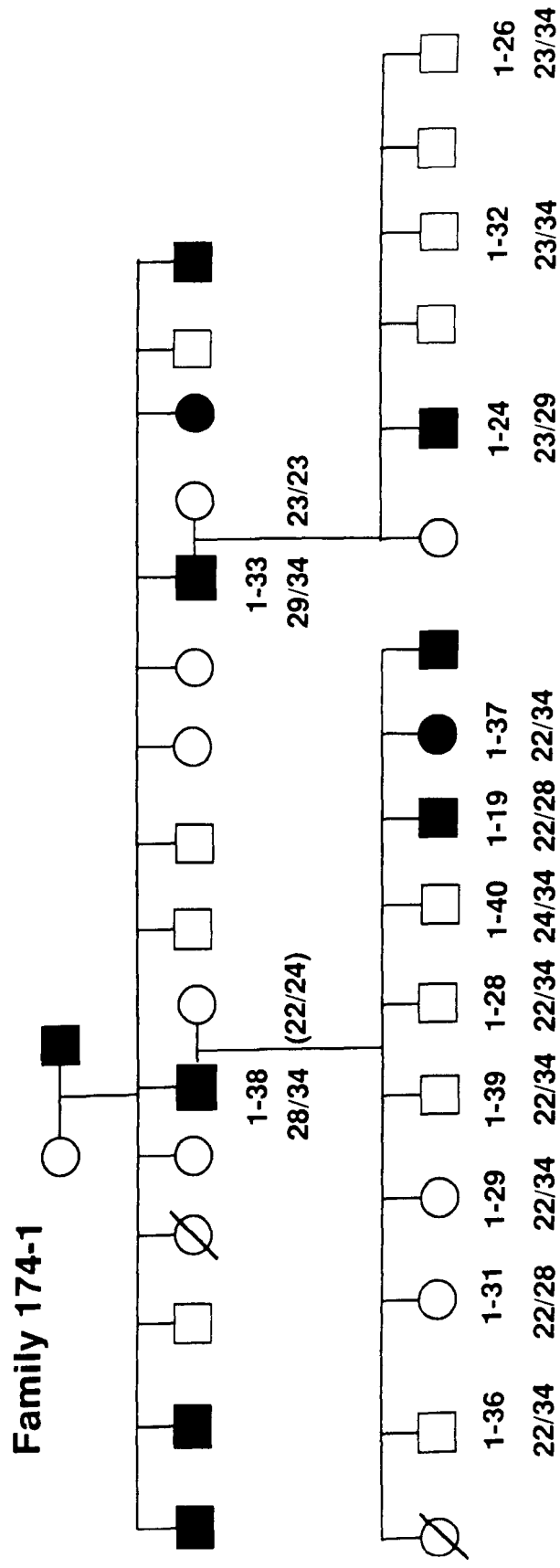
FHB pedigree 2.

The squares and circles represent males and females, respectively, while open symbols and shaded symbols represent normal and FHB, respectively. The number immediately below the subject symbol denotes that subject's family identification (ID) number. The pairs of numbers below the ID numbers denote the apo(a) phenotye of the subject as assessed by high resolution immunoblotting.



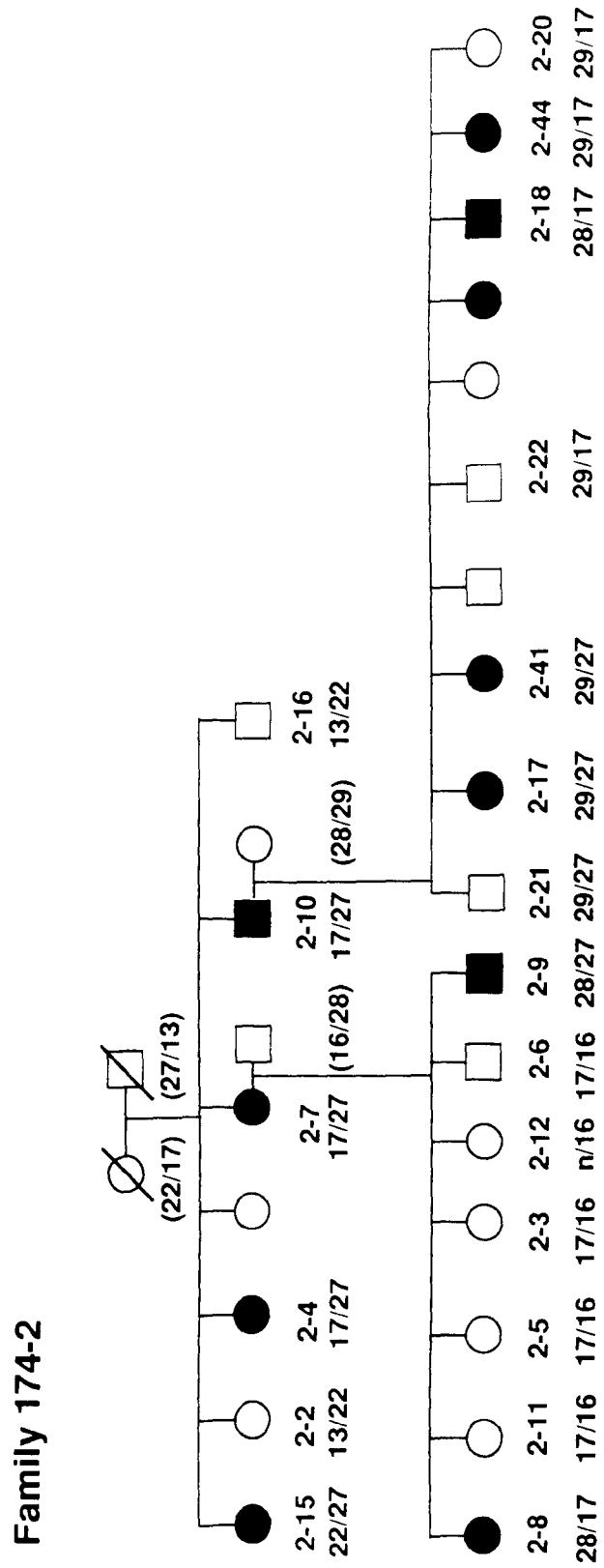
FHB pedigree 3.

The squares and circles represent males and females, respectively, while open symbols and shaded symbols represent normal and FHB, respectively. The number immediately below the subject symbol denotes that subject's family identification (ID) number. The pairs of numbers below the ID numbers denote the apo(a) phenotye of the subject as assessed by high resolution immunoblotting.



FHB pedigree 4.

The squares and circles represent males and females, respectively, while open symbols and shaded symbols represent normal and FHB, respectively. The number immediately below the subject symbol denotes that subject's family identification (ID) number. The pairs of numbers below the ID numbers denote the apo(a) phenotye of the subject as assessed by high resolution immunoblotting.



FHB pedigree 5.

The squares and circles represent males and females, respectively, while open symbols and shaded symbols represent normal and FHB, respectively. The number immediately below the subject symbol denotes that subject's family identification (ID) number. The pairs of numbers below the ID numbers denote the apo(a) phenotye of the subject as assessed by high resolution immunoblotting.

Glossary

Apo	apolipoprotein
Apo(a)	apolipoprotein(a) protein
APO(a)	apolipoprotein(a) allele
BSA	bovine serum albumin
cDNA	complimentary DNA
CHD	coronary heart disease
CV	coefficient of variation
d	density
Da	Dalton
EDTA	ethylene diamine tetra-acetate
EIA	electroimmunoassay
EID	electroimmunodiffusion
ELISA	enzyme linked immunosorbent assay
FH	familial hypercholesterolaemia
FHB	familial hypobetalipoproteinaemia
GRIPS	Göttingen risk incidence and prevalence study
HDL	high density lipoprotein
HMG CoA	hydroxy methyl glutaryl coenzyme A
HNF 1α	hepatocyte nuclear factor 1α
HRPO	horseradish peroxidase
IRMA	immunoradiometric assay
K	kringle
kb	kilobase
LDL	low density lipoprotein
LMP	low melting point
Lp(a)	lipoprotein (a)
MAb	monoclonal antibody
mRNA	messenger RNA
n	null or non-expressing
NP-40	nonidet P-40
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulphonyl fluoride
PTCA	percutaneous transluminal coronary angioplasty
RFLP	restriction fragment length polymorphism
RIA	radioimmunoassay
SDS	sodium dodecyl sulphate
SS	signal sequence
T	tail region
TBS	tris buffered saline
TC	total cholesterol
TG	triglyceride
TGF-ß	transforming growth factor-ß
tPA	tissue type plasminogen activator
VLDL	very low density lipoprotein
YAC	yeast artificial chromosome