Polymorphic forms of human apolipoprotein[a]: inheritance and relationship of their molecular weights to plasma levels of lipoprotein[a]

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Abstract The plasma concentration of human lipoprotein[a], Lp[a], is highly correlated with coronary artery disease. The protein moiety of Lp[a], apoLp[a], consists of two apoproteins, apo[a] and apoB-100, linked by one or more disulfide bonds(s). Apo[a], the protein unique to Lp[a], exists in polymorphic forms that exhibit different apparent molecular weights (M_r). Polyacrylamide gel electrophoresis in sodium dodecyl sulfate followed by immunoblotting was used to separate and visualize these different forms and to determine the polymorphic pattern of apo[a] in the plasma samples of 692 individuals. A total of 11 different polymorph bands ranging in M_r from 419 kD to 838 kD could be resolved, but only 1 or 2 bands were present per individual. The polymorphic band pattern for an individual was assigned to 1 of the 66 different phenotype designations representing the total number of possible single- and double-band combinations of the 11 detectable bands. All 11 of the possible single-band phenotypes but only 32 of the 55 possible double-band phenotypes were represented. There were 412 plasma samples (59.5%) that contained a single band, 274 (39.6%) contained two bands, and only 6 (0.9%) had no detectable apo[a] band. A highly significant inverse correlation was found between the M_r of the band(s) present and the plasma apoLp[a] concentration ($r = -0.461$; $\varrho = 0.0001$). The correlation was better between apoLp[a] and single-band ($r = -0.495$; $\varrho = 0.0001$) than double-band ($r = -0.382$; $\varrho = 0.0001$) phenotypes. Of the 274 individuals exhibiting double-band phenotypes, the lower M_r band was more intense in 141 (51.4%), the two bands were equally intense in 85 (31.0%), while the higher M_r band was more intense in 48 (17.5%). Based upon the hypothesis that apo[a] polymorphism is controlled by different alleles at a single locus, the frequency of the 11 alleles determined from the observe phenotypes (low M_r→high M_r) was: band 1) 419 kD, 0.00875; band 2) 489 kD, 0.00510; band 3) 536 kD, 0.0555; band 4) 553 kD, 0.0758; band 5) 613 kD, 0.135; band 6) 680 kD, 0.0824; band 7) 705 kD, 0.104; band 8) 742 kD, 0.151; band 9) 760 kD, 0.246; band 10) 796 kD, 0.128; band 11) 838 kD, 0.00802. The observed distribution of phenotypes in the population was compared by chi-square analysis to that predicted on the basis of simple Mendelian inheritance, and the hypothesis was rejected ($\chi^2 = 921.7$; $\varrho < 0.001$). Significantly, the single-band phenotypes are over-represented in the population compared to that predicted. The study of a single family pedigree demonstrated high heritability of apo[a] polymorph size, but several deviations from a simple Mendelian pattern of inheritance were observed. These results suggest that the apparent sizes of apo[a] polymorphs in the plasma compartment are not completely determined by the gene that codes for apo[a], but that post-translational processes may also be involved.—**Gaubatz, J. W., K. I. Ghanem, J. Guevara, Jr., M. L. Nava, W. Patsch, and J. D. Morrisett.** Polymorphic forms of human apolipoprotein[a]: inheritance and relationship of their molecular weights to plasma levels of lipoprotein[a]. *J. Lipid Res.* 1990. **31:** 603–613.

Supplementary key words lipoprotein[a] • size polymorphism • apoB • immunoblotting • phenotype • heritability

In 1963, Berg (1) and Berg and Mohr (2) discovered the Lp[a] antigen in human plasma and proposed it as a qualitative genetic marker whose inheritance pattern appeared to fit an autosomal dominant model. A great deal of clinical interest was generated by early qualitative tests that demonstrated a positive correlation between the presence of Lp[a] and certain parameters associated with coronary heart disease (3–4). Following the development of quantitative measurements (5–7), it was shown that the Lp[a] lipoprotein represented a quantitative trait with evidence for a single major gene as well as polygenic influence (8–10). Additionally, quantitative assays for Lp[a] have provided the basis for numerous further studies linking plasma Lp[a] levels to atherosclerosis (11–14).

Although this important correlation of Lp[a] with heart disease was demonstrated more than 27 years ago, only recently have major advances in the structural properties

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; EDTA, ethylene diamine tetraacetic acid; Tris, tris-(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Lp[a], lipoprotein[a] normally isolated from the d 1.060– 1.120 g/ml fraction of human plasma; apoLp[a], the total moiety of Lp[a], including apo[a] and apoB; apo[a], an apolipoprotein unique to Lp[a]; apoB, an apolipoprotein present in Lp[a], LDL, VLDL, and chylomicrons; Ig-G, immunoglobulin G; χ^2, the computed value of chi-square; M_r, apparent molecular weight.

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of Lp[a] been made. In 1983, it was first established that Lp[a] contained apoB disulfide-linked to apo[a] (15, 16), both of which are very high M_r proteins. The apoB was found to be of uniform size (B-100), but the apo[a] exhibited size polymorphism (15). The density heterogeneity of Lp[a] has been attributed to apo[a] species of differing molecular weights (17). This size polymorphism of apo[a] may originate from polypeptides of differing lengths and/or differing extents of glycosylation. The polymorphic pattern of apo[a] is characteristic and constant for each individual (18). Several recent reports by Utermann et al. (19–21), have described the resolution of six M_r polymorphs of apo[a]; no individual possessed more than two M_r species. However, apo[a] polymorphs were totally absent from 44% to 49% of the population tested. Their data suggested that apo[a] polymorphs are genetically controlled and are associated with Lp[a] levels. In this report we describe the observation of 11 different polymorphs of apo[a] in 686 subjects; at least one polymorph is detectable in >99% of the population. The molecular weights of apo[a] polymorphs are inversely correlated with plasma apoLp[a] levels ($r = -0.461$; $\varrho = 0.0001$).

METHODS

Plasma collection and storage

Blood was collected into 10-ml tubes containing 15 mg EDTA and immediately centrifuged at 4°C. The plasma was then decanted and aliquoted into screw-cap vials and stored frozen at either −70°C or −20°C. The stability of Lp[a] immunoreactivity as measured by ELISA (22) was evaluated. Essentially no significant change in immunoreactivity was observed for plasma samples stored at −20°C for up to 6 months.

Immunochemical analyses

ApoLp[a] (i.e., Lp[a] protein consisting of apo[a] and apoB) was measured in each plasma sample by an enzyme-linked immunosorbent assay (ELISA) described elsewhere (22). Polyclonal antibodies to apo[a] and apoB were raised in goat and rabbit and used as a purified immunoglobulin G fraction. Purified Lp[a] was used as a primary standard and its protein content (apoLp[a]) was determined by the method of Lowry et al. (23) using bovine serum albumin as a standard. Hence, the immunoreactivity of apo[a] within Lp[a] was related to the mass of the protein (apo[a] + apoB) in the same sample.

Electrophoretic procedures

To 50 μl of lyophilized plasma was added 500 μl SDS-PAGE sample buffer (10 mM Tris, 1 mM EDTA, 1% SDS, 10% glycerol, 2% 2-mercaptoethanol, pH 8.8). All sample loads for SDS-PAGE were normalized to 25 ng apoLp[a] based on the ELISA measurements. A reference plasma mixture containing four different M_r polymorphs of apo[a] from two individuals whose Lp[a] had previously been completely characterized (18) was included twice in each gel. Homogeneous slab gels containing 3.75% acrylamide, 0.10% bisacrylamide were used for SDS-PAGE as previously described (15, 18) except for the inclusion of 0.75% agarose (type C, Behring Diagnostics, La Jolla, CA). Electrophoresis was performed at 10°C for 18 h at 25 mA/gel constant current. Electrophoretic transfer of protein from gel to nitrocellulose was performed for 18 h at 50 volts as previously described (15, 18).

Apo[a] was localized on the nitrocellulose using a highly purified rabbit Ig-G fraction to apo[a], followed by ^{125}I protein A (ICN Biomedicals, Inc., Irvine, CA), with specific activity >30 μCi/mg. Radioactive labeled bands were visualized after overnight exposure at −70°C to X-omat XAR5 film (Eastman Kodak Co., Rochester, NY) using Lightning Plus intensifying screen (Dupont, Wilmington, DE). Apo[a] polymorph molecular weights were estimated using apoB-100 (M_r 512 kD) and cross-linked phosphorylase B (97.4 kD) oligomers (Sigma, St. Louis, MO) in the SDS-PAGE system; for M_r estimation the gel was stained with Coomassie Blue rather than blotted.

Statistical methods

Comparisons of single- to double-band phenotypes were performed by the Mann-Whitney test when the distribution was non-normal, and by the *t*-test when the distribution was normal (24). Comparison of observed frequencies of phenotypes to expected frequencies was performed by a modified Chi-Square analyis (25). Correlations were determined by simple linear regression analysis. All of these tests were performed on a SUN 3/60 workstation operating as part of the PROPHET National Biomedical Computer Resource (26).

Study subjects

Of the blood samples used in this study, 631 were collected from participants in the Atherosclerosis Risk in Communities (ARIC) study. ARIC selected representative samples of 4000 individuals aged 45–64 years from each of four defined U.S. communities in Maryland, Minnesota, Mississippi, and North Carolina (27) Their age, gender, and race distribution was intended to match that of the target communities, except that only black participants were selected in Mississippi. The present study used a consecutive series of blood samples received in ARIC's Central Lipid Laboratory between January 1 and September 1, 1988. Since the ARIC study attempts to recruit random subsamples at monthly intervals, such a consecutive series is approximately representative of ARIC's target populations. Thirty-one of the blood samples used in the present study were obtained from patients undergoing aorto-coronary re-bypass surgery as described by Cush-

ing et al. (28). The remaining 30 samples were drawn from laboratory and hospital personnel. The objective of the present study was not to draw correlations of apo[a] phenotype frequencies with unique characteristics of the patient population; that issue will be addressed in a subsequent publication. Rather, it was to demonstrate the existence of at least 11 different M_r polymorphs and to determine their distribution in a mixed population.

RESULTS

Distribution of apoLp[a] levels

All 692 subjects studied had plasma apoLp[a] concentration followed the same highly skewed non-Gaussian distribution reported previously (29), with a mean of 89 μg/ml and median of 52 μg/ml (**Fig. 1**).

Apo[a] size polymorphs and their phenotypic designations

Each sample load for SDS-PAGE was normalized to 25 ng of apoLp[a] based on its ELISA measurement. The detection limit of any one band was about 2.5 ng. Although all 692 samples were positive for apoLp[a] by ELISA, 6 (0.9%) had no detectable band by immunoblotting. With the SDS-PAGE system we have developed, 11 different size polymorphs of apo[a] were resolvable (**Fig. 2**) ranging in apparent molecular mass from 419 kD to 838 kD. These were assigned a number from 1 for the lowest to 11 for the highest apparent M_r. In individual plasmas, one of these 11 bands was generally present either alone, or in combination with one other band; only rarely were more than two predominant bands present. A species was observed that migrated to a position similar to (but not always the same as) that of apoB-100 and reacted weakly with anti-apo[a]. This species was present in all plasma samples, even those few in which no Lp[a] was detectable. It appeared with varying band intensity, depending upon the plasma volume required for the standard load of 25 μg apoLp[a]. Generally, the band was quite faint (see Figs. 2, 3, 6) and was usually distinguishable from the more intensely staining apo[a] polymorphs 3 and 4 that migrate to a similar position. This anomalous band has also been observed by other workers (19).

There were also some other faint, broader, lower molecular weight apo[a] positive bands (e.g., Fig. 6) that did not appear on gels too porous to retain these species. We suspect that these are very small amounts of apo[a] fragments resulting from specific proteolytic clips. Similar bands have been observed by Kratzin et al. (30).

A total of 66 different combinations of single- and double-band patterns was possible from the principal 11 bands. Therefore, a phenotype designation from 1 to 66 was assigned to each sample (**Table 1**). The numbers for the phenotype designation were ordered so as to indicate the progression of apoLp[a] polymorphs from lowest to highest M_r. This numbering system provides a convenient way of cataloging the phenotypes and making them amenable to statistical analysis.

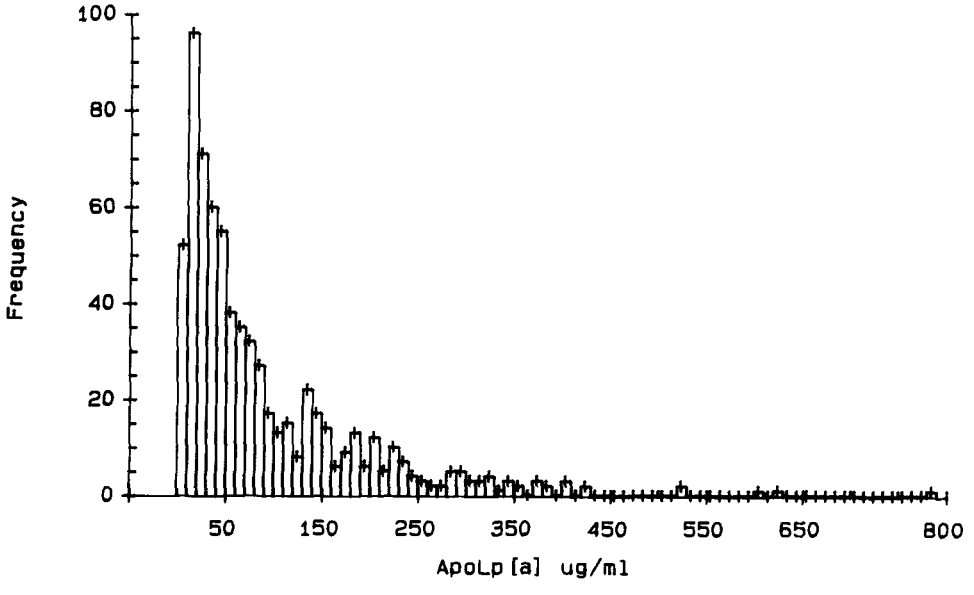

Fig. 1. The distribution (in intervals of 10 μg/ml) of plasma apoLp[a] concentrations measured by ELISA in 692 human plasma samples.

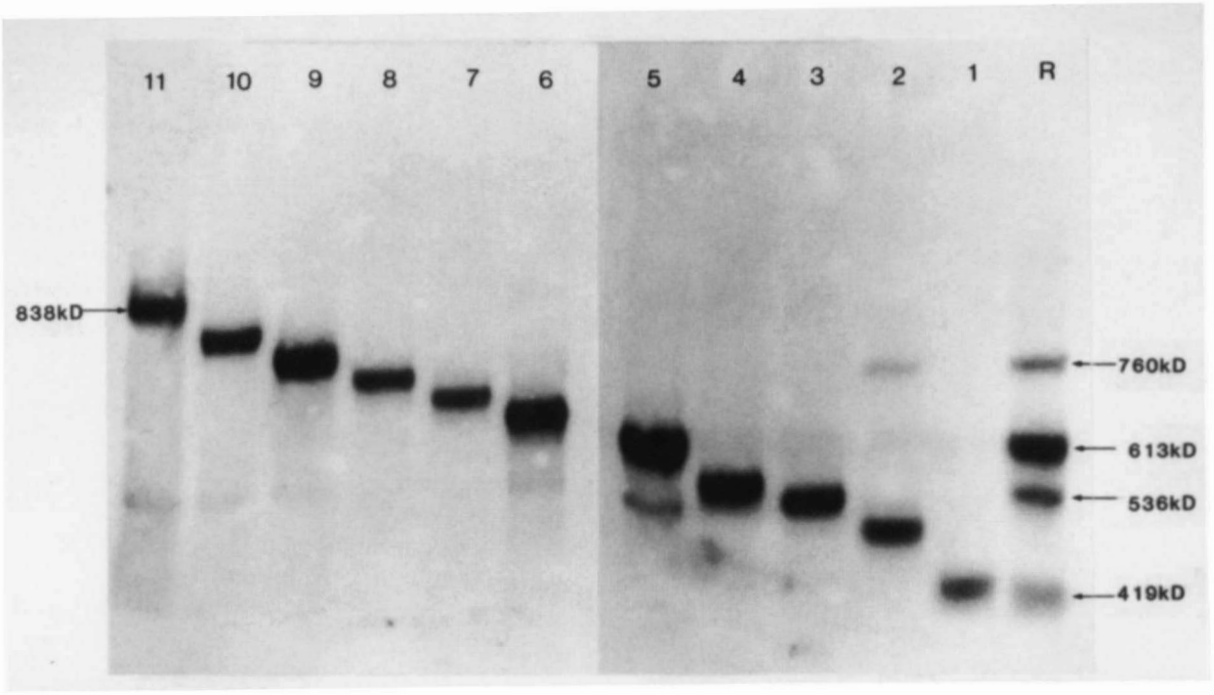

Fig. 2. Eleven polymorphs of apo[a] resolved by immunoblot analysis following 3.75% SDS-PAGE. The apparent molecular weights determined for the respective polymorphs were: 1) 419 kD; 2) 489 kD; 3) 536 kD; 4) 553 kD; 5) 613 kD; 6) 680 kD; 7) 705 kD; 8) 742 kD; 9) 760 kD; 10) 796 kD; and 11) 838 kD. The reference mixture R contains apo[a] polymorphs 1, 3, 5, and 9.

Apo[a] polymorphic patterns in the subject population

The apo[a] polymorphic band pattern for an individual was assigned to one of the 66 phenotypic designations. Some typical patterns are shown (**Fig. 3, Table 2**). Of the 692 samples analyzed, only 6 had no detectable band. All 11 of the possible single-band phenotypes but only 32 of the 55 possible double-band phenotypes were represented. There were 412 samples (59.5%) classified as single band and 274 samples (39.6%) as double band. The relative intensities of the two bands from individuals exhibiting the double-band pattern varied. For these double-band phenotypes, the lower M_r band was predominant in 141 individuals (51.4%), the two bands were equal in 85 (31.0%), while the higher M_r band was predominant in 48 (17.5%).

The distribution of apo[a] phenotypes in the study population was nonuniform (**Fig. 4**). Of the eight highest frequency phenotypes, seven were single band with the designations 61 (band 9, n = 107); 57 (band 8, n = 69); 39 (band 5, n = 52); 64 (band 10, n = 46); 52 (band 7, n = 45); 31 (band 4, n = 34); and 46 (band 6, n = 30). The highest frequency double-band phenotype was designation 43 (bands 5, 9; n = 30). Based upon the hypothesis that apo[a] polymorphism is controlled by different alleles at a single locus, the frequencies of the 11 alleles (1 band = 1 allele) determined from the observed phenotypes were: band 1) 0.00875; band 2) 0.0051; band 3) 0.0554; band 4) 0.0758; band 5) 0.135; band 6) 0.0824;

TABLE 1. Phenotype designation for each of 66 apolipoprotein[a] polymorphs

Phenotype Designation	Band Pattern		Phenotype Designation	Band Pattern	
1	1	--	34	4	7
2	1	2	35	4	8
3	1	3	36	4	9
4	1	4	37	4	10
5	1	5	38	4	11
6	1	6	39	5	--
7	1	7	40	5	6
8	1	8	41	5	7
9	1	9	42	5	8
10	1	10	43	5	9
11	1	11	44	5	10
12	2	--	45	5	11
13	2	3	46	6	--
14	2	4	47	6	7
15	2	5	48	6	8
16	2	6	49	6	9
17	2	7	50	6	10
18	2	8	51	6	11
19	2	9	52	7	--
20	2	10	53	7	8
21	2	11	54	7	9
22	3	--	55	7	10
23	3	4	56	7	11
24	3	5	57	8	--
25	3	6	58	8	9
26	3	7	59	8	10
27	3	8	60	8	11
28	3	9	61	9	--
29	3	10	62	9	10
30	3	11	63	9	11
31	4	--	64	10	--
32	4	5	65	10	11
33	4	6	66	11	--

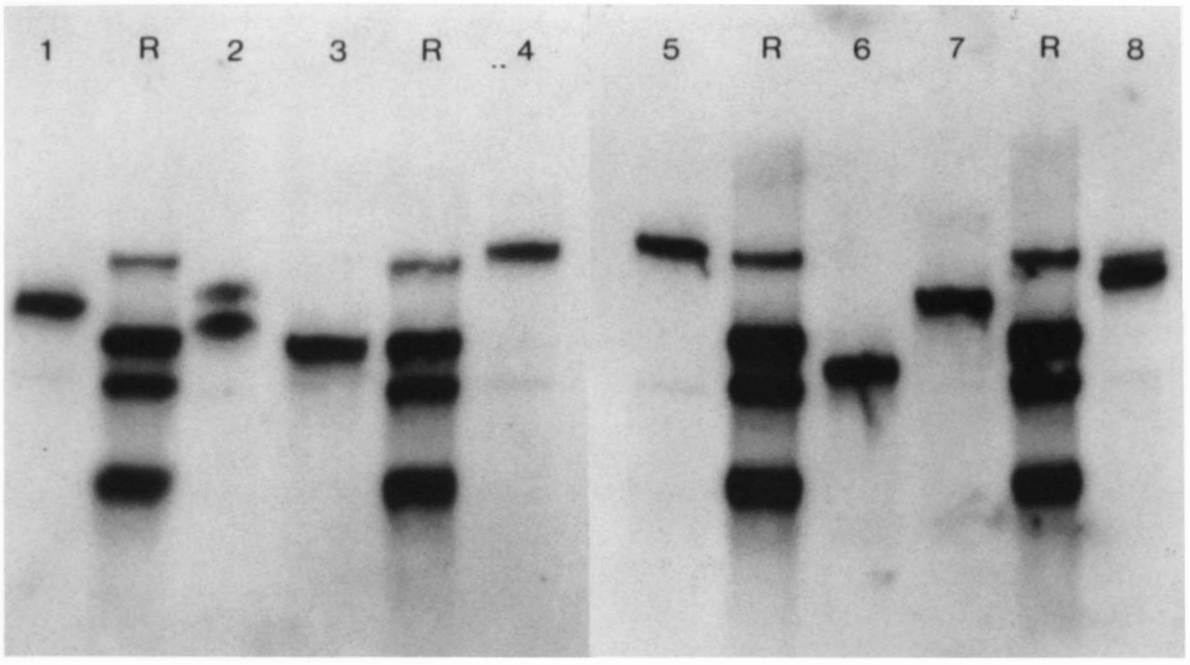

Fig. 3. Representative apo[a] phenotyping of individuals by immunoblot analysis after 3.75% SDS-PAGE. Eight different plasma samples were analyzed in lanes 1-8. The reference mixture R contains polymorphs 1, 3, 5, and 9. Table 2 presents a detailed classification.

band 7) 0.104; band 8) 0.151; band 9) 0.246; band 10) 0.128; band 11) 0.00802. The observed distribution of phenotypes in the population was compared by modified Chi-Square analysis (25) to that predicted on the basis of simple Mendelian inheritance, assuming Hardy-Weinberg equlibrium (**Table 3**).

A conservative limitation on use of the Chi-Square test requires the value of any expected cell frequency to be greater than 5. To meet this rigorous requirement, adjacent cells were sometimes combined. In the case where a cell could be combined with either of two neighboring cells to satisfy this condition, it was combined with the adjacent cell having the same low molecular weight band, if possible. The following phenotype designations were combined using this procedure: 1) 1-11; 2) 12-21; 3) 22-23; 4) 29-30; 5) 31-32; 6) 37-38; 7) 44-45; 8) 46-47; 9) 50-51; 10) 55-56; 11) 59-60; 12) 62-63; 13) 64-66. This provided 35 groupings yielding 34 degrees of freedom. Based on this analysis, $\chi^2 = 921.7$, $\varrho = 0.001$ and the hypothesis was rejected.

Correlation of apo[a] polymorphic patterns with plasma apoLp[a]

A highly significant inverse correlation was found between apoLp[a] concentration and phenotype designation (n = 686, $r = -0.461$, $\varrho = 0.0001$; **Fig. 5**). The correlation was somewhat higher for single-band (n = 412, $r = 0.495$, $\varrho = 0.0001$) than double-band (n = 274, $r = 0.382$, $\varrho = 0.0001$) phenotype. While there was a highly significant inverse correlation between apoLp[a] concentration and M_r of the polymorph present, an extremely high variation in concentrations was found for each of the 11 single-band patterns (**Table 4**).

A statistical comparison was performed between the single-band group (n = 412) and the double-band (n = 274) group with respect to plasma concentrations of apoLp[a]. Subjects with the single-band phenotypes had a significantly lower apoLp[a] concentration ($\varrho = 0.0001$) than the double-band group. The double-band phenotypes could be divided into three groups based upon the relative intensities of their bands: *1*) low M_r band most intense (n = 141), *2*) bands equally intense (n = 85), and *3*) high M_r bands most intense (n = 48). For each of these three double-band groups, the apoLp[a] level of each individual sample was compared to the mean apoLp[a] level of each of the two constituent bands, as determined for the single

TABLE 2. ApoLp[a] concentration and phenotypic classification of the eight individual plasma samples shown in Fig. 4

Sample	Number of Bands	Polymorphs Present	Predominant Band(s)	Phenotype Designation	ApoLp[a]
					μg/ml
1	1	7	--	52	43
2	2	6,7	0	47	76
3	1	5	--	39	113
4	1	10	--	64	18
5	1	9	--	61	39
6	1	4	--	31	226
7	1	7	--	52	137
8	2	8,9	8	58	168

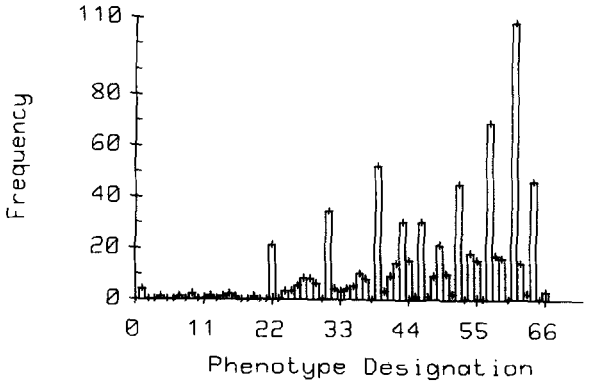

Fig. 4. The distribution of 66 apo[a] phenotypic classifications in 686 individual plasma samples. Table 1 lists the 66 total possible single- and double-band combinations of the 11 different apo[a] polymorphs.

band phenotypes (Table 4), then scored for the phenotype with the least difference. Of the 142 samples in which the low M_r band was most intense, 100 (70.4%) were scored for the apoLp[a] level closest to the level of the lower M_r single band. Of the 85 samples in which the bands were

TABLE 3. Observed frequencies and expected frequencies of the 66 phenotypes in 686 subjects

Phenotype Designation	Observed	Expected	Phenotype Designation	Observed	Expected
1	4	0.05	34	4	10.82
2	0	0.06	35	5	15.7
3	0	0.66	36	10	25.69
4	1	0.91	37	8	13.31
5	*0*	*1.62*	*38*	*0*	*0.83*
6	0	0.99	39	52	12.5
7	1	1.25	40	3	15.26
8	0	1.81	41	9	19.26
9	2	2.96	42	14	27.97
10	0	1.54	43	30	45.75
11	0	0.1	44	15	23.71
12	1	0.02	45	1	1.48
13	0	0.38	46	30	4.66
14	1	0.53	47	1	11.66
15	2	0.94	48	9	17.07
16	1	0.58	49	21	27.92
17	0	0.73	50	10	14.47
18	0	1.06	51	2	0.91
19	1	1.73	52	45	7.42
20	0	0.9	53	0	21.54
21	0	0.06	54	18	35.24
22	21	2.05	55	15	18.26
23	0	5.69	56	0	1.14
24	3	10.13	57	69	15.6
25	3	6.18	58	17	51.17
26	5	7.8	59	16	26.52
27	8	11.33	60	0	1.66
28	8	18.54	61	108	41.85
29	6	9.61	62	14	43.38
30	0	0.6	63	2	2.72
31	34	3.94	64	46	11.24
32	4	14.04	65	0	1.41
33	3	8.57	66	3	0.04

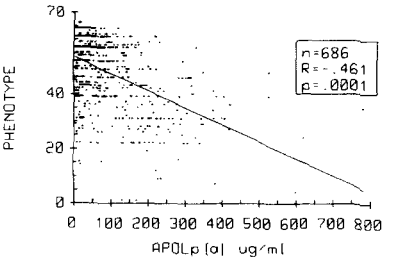

Fig. 5. Correlation between plasma apoLp[a] concentration and phenotype.

of equal intensity, 39 (45.9%) was scored for apoLp[a] concentration most resembling the levels of the low M_r band, and 46 (54.1%) most resembling the level of the high M_r band. Of the 47 samples in which the high M_r band was most intense, 36 (76.6%) had apoLp[a] levels closest to the mean level of the higher M_r single band. Hence, the pair-wise comparison of these groups revealed a significantly higher apoLp[a] concentration in those samples where the relative intenstity of the low M_r band was greater (ϱ, always <0.008).

Study of family pedigree

The apo[a] polymorphic pattern of a family with seven children was studied. Both parents were double-band phenotypes, the father having polymorphs 5 and 8 (with 8 predominant), and the mother having polymorphs 6 and 8 (with 6 predominant) (**Fig. 6, Table 5**). If a simple mode of inheritance were responsible for transmission of the polymorphs, one would expect the children to exhibit the single-band polymorph 8 or the double-band phenotypes consisting of polymorphs 5, 8, or 6, 8. When the usual normalized 25 ng sample load was used, the patterns of three of the children were exceptional. Child 1 had polymorph 5 as the most intense band and a much less intense polymorph 9, while child 3 expressed only polymorph 6, and child 4 only polymorph 5. When the

TABLE 4. Mean apoLp(a) concentration in plasma samples of individuals with single band phenotypes

Apo[a] Band	Phenotype Designation	n	Mean ApoLp[a]	SD
			μg/ml	
1	1	4	126	51.8
2	12	1	224	---
3	22	21	167.9	91.6
4	31	34	173.5	114.6
5	39	52	88.3	110
6	46	30	111	108.1
7	52	45	62.4	52.5
8	57	69	55.7	50.8
9	61	107	41.6	34.5
10	64	46	33.7	21.7
11	66	3	17	3

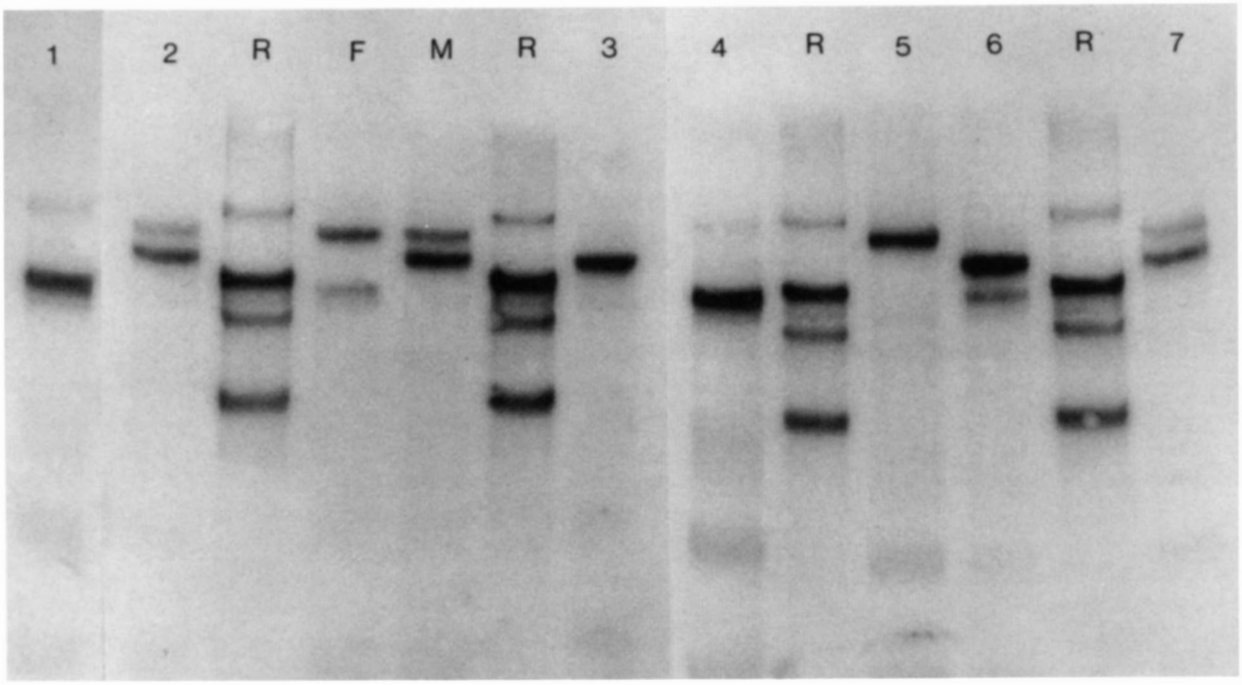

Fig. 6. Immunoblot analysis of apo[a] phenotypes in a single nine-member family demonstrated the inheritance/expression of the apo[a] polymorphs. F represents the father, M the mother, and 1-7 the children. The reference mixture R contains polymorphs 1, 3, 5, and 9. A detailed classification is presented in Table 5.

plasma of children 3, 4, and 5, classified as single-band phenotypes, and child 6 were re-run at a 100 ng apoLp[a] sample load (4 ×), only the classification of child 4 was affected due to the appearance of a low intensity band corresponding to polymorph 9. Polymorph 5, which was present only in the father and was the least intensely expressed band among all polymorphs in both parents, was the most intensely expressed polymorph in children 1 and 4. Additionally, these offspring expressed, albeit weakly, polymorph 9 which was not exhibited by either parent. Child 3 expressed only polymorph 6; presumably inherited from the mother. The polymorphic patterns for children 2, 5, 6, and 7 were strictly in accordance with a simple mode of inheritance.

DISCUSSION

We have developed an extremely sensitive, high resolution method for determining the size of apo[a] polymorphs in individual plasma samples. This method is capable of detecting one or more polymorphs in >99% of a population, and resolving at least 11 polymorphs of differing apparent M_r. Utermann et al. (19) have reported studies in which 49% of the population measured had no detectable bands by immunoblotting although 77% were positive by electroimmunoassay. Among individuals with detectable band(s), they observed that 90% display only a single band. A null phenotype/allele was postulated to account for this finding, and was the most frequently ob-

TABLE 5. Plasma apoLp[a] concentrations and phenotypic classifications of a nine-member family (Fig. 6)

Sample	Number of Bands	Polymorphs Present	Predominant Band	Phenotype Designation	ApoLp[a]
					μg/ml
F	*2*	*5,8*	*8*	*42*	*89*
M	*2*	*6,8*	*6*	*48*	*70*
1[a]	2	5,9	5	43	23
2	2	6,8	6	48	88
3[a]	1	6	--	46	71
4[a]	1	5	--	39	22
5	1	8	--	57	32
6	2	5,6	6	43	109
7	2	6,8	6	48	70

[a]Children that exhibited apo[a] polymorphic patterns different from that expected on the basis of a simple mode of inheritance/expression.

served allele in the population (>0.5). This null allele was used in phenotype classification and allele frequency calculations. Albers, Adolphson, and Hazzard (29) have concluded from their immunoassay data that there are no true Lp[a]-negative subjects. In light of recent studies indicating high sequence similarity between one apo[a] polymorph and plasminogen (31, 32), and because of the significant levels (mean = 476 μg/ml) of plasminogen ($M_r \approx 90$ kD) in human plasma (33), the possibility existed that plasminogen made a significant contribution to the apparent apoLp[a] immunoreactivity. In turn, this would artifactually increase the number of individuals who were falsely reactive for apo[a], but for whom no apo[a] band was detectable by immunoblotting. Finally, this would lead to a discrepancy between the percentage of individuals positive for Lp[a] by immunoassay, but negative by immunoblotting for a size polymorph in the apparent M_r range of apo[a] (>300 kD). Alternatively, if the null phenotype were due simply to assay insensitivity, the null allele would lead to inaccuracies in classification of single versus double band phenotypes, and perhaps to an over-simplification that could result in erroneous conclusions or significant omissions. Initially, Utermann et al. (20), reported that the apo[a] phenotype frequencies they observed were not significantly different from those expected at Hardy-Weinberg equilibrium. More recently, however, they (34) have indicated that the relative apo[a] phenotype frequencies they observe with their gel system are significantly different from those expected, assuming Hardy-Weinberg equilibrium and utilizing their estimated apo[a] allele frequencies. These authors feel that the most likely explanation for the observed inconsistencies are misclassification of the apo[a] phenotypes, especially with regard to the operational null allele. In our immunoblotting assay, each sample load was normalized to 25 ng apoLp[a] based on its ELISA. Of all the samples measured, >99% had apo[a] polymorphs detectable by immunoblotting. Generally, there was good correspondence in the total intensity of bands (as judged by visual inspection) between samples normalized in this manner, indicating that possible elevations in the apoLp[a] measurement due to plasminogen, even among low Lp[a] positive individuals, was minimal.

We have resolved a total of 11 size polymorphs of apo[a] ranging in M_r from 419 kD to 838 kD. Two of these (polymorphs 1 and 2) were smaller than B-100. Utermann et al. (20) reported 6 polymorphs of apo[a] among 441 subjects. In separate studies, 6 bands were also found in screening 20 human subjects (35) and in a population of 70 subjects (30). Nine bands were reported among a population of 165 baboons (36). It is not easy to determine which of our apo[a] polymorph designations correspond to those of Utermann et al. (20) who designate theirs as F, B, S1, S2, S3, S4. Their B polymorph migrates with an electrophoretic mobility identical to that of apoB-100, which has an M_r of 513 kD and is similar to that of our polymorph 3 (Fig. 2). Their faster migrating polymorph F could correspond to our polymorphs 1 or 2, while their slower migrating species S1–S4 probably corresponds to one or more of our polymorphs 4–11.

Because apo[a] contains a high proportion of carbohydrate, especially sialic acid (18, 35, 37), glycosylation has been suggested as a possible factor contributing to differences in the apparent M_r of apo[a] polymorphs (38, 39). Utermann et al. (19) found that while neuraminidase treatment led to an absolute decrease in apparent M_r of about 50 kD for the 6 polymorphs they resolve, the relative M_r differences were unchanged. In the present study we also found that the 11 polymorphs maintained their same relative electrophoretic positions after neuraminidase treatment. But these results should be interpreted with caution: sialic acid represents only abut 30–50% of the total carbohydrate present on apo[a] (37); the carobohydrate content of apo[a] seems to vary among individuals (18); and carbohydrate can cause glycoproteins to migrate anomalously in SDS-containing buffer (40). It would be instructive to compare the apparent M_r values of all polymorphs after they have been fully deglycosylated. Recent cDNA sequence analysis has shown that one of the kringles in the 503 kD polymorph is repeated 27 times (32). This kringle has an M_r of about 13 kD. The primary molecular basis for the polymorphism in apo[a] could be variations in polypeptide chain length due to differing numbers of these kringle units. Recently, Hixson et al. (41), in a survey of hepatic RNA from 22 baboons, detected a variety of sizes of apo[a] transcripts that corresponded with the relative mobilities and number of serum apo[a] isoforms. These results provide evidence that apo[a] glycoprotein isoforms can be due to structural differences in apo[a] transcripts. Observed exceptions to correspondence between apo[a] transcripts and isoforms may be due to post-translational glycosylation. As better SDS-PAGE systems are developed, additional polymorphs will probably be discovered.

ApoLp[a] levels in the present study population had the same highly left-skewed non-normal distribution reported by several other investigators (6, 14). When the actual phenotype patterns were examined, only a highly significant inverse correlation between the phenotype designations (and hence the M_r of the apo[a] polymorph present), and apoLp[a] concentrations were found ($r = -0.461$; $\varrho = 0.0001$). These results are in agreement with the findings of Utermann et al. (19–21) who suggested that the same genes are involved in determining both the apparent size of the apo[a] protein and the concentration of the Lp[a] complex. This proposal is compatible with genetic models that assume a major gene effect. However, other superimposed effects such as variations in promoter strength, Lp[a] assembly, secretion, plasma

compartment processing, and cellular catabolism as well as polygenic influence may be reflected in the high standard deviations of the mean apoLp[a] for single-band phenotypes (Table 4). Differences in apo[a] polymorph frequencies in the population do reflect somewhat the skewed non-normal distribution of apoLp[a] levels in the population. However, the polymorph with the greatest frequency (0.246) is band 9 with a M_r of 760 kD. The frequency declines to 0.128 and 0.00802 for bands 10 (769 kD) and 11 (838 kD), respectively. When the distribution of apoLp[a] was examined over intervals of 10 μg/ml compared to 20 μg/ml, a decrease in the frequency of individuals with <10 μg/ml apoLp[a] was revealed (Fig. 1). A similar finding was also obtained when a highly sensitive radioimmunoassay was used to measure Lp[a] (29). If the molecular basis of differences in M_r for the polymorphs is due to duplication of a gene region that codes for a particular kringle, the decline in frequency of polymorphs 10 and 11 after peaking with polymorph 9 could reflect the more recent introduction of these alleles into the gene pool.

A comparison of single-band to double-band phenotypes revealed that the former group had a significantly lower apoLp[a] level ($p = 0.0001$). All sample loads were normalized to 25 ng apoLp[a] so there was no selection based on sample differences in apoLp[a] concentration. Since our immunoblotting assay detected levels down to 2.5–5.0 ng apoLp[a], only when one polymorph constituted more than 80–90% of the total individual's apoLp[a] would a second polymorph go undetected. The samples falsely classified as single-band phenotypes should occur most often when a low M_r polymorph associated with high apoLp[a] concentration and a high M_r polymorph associated with low apoLp[a] concentration are genotypically present. Such an individual would then be falsely classified as a single-band phenotype containing only the low M_r polymorph. Such a systematic misclassification would give rise to a single-band group with a significantly higher apoLp[a] concentration than that of the double-band group, just the opposite of our experimental observations. Calculation of the expected number of phenotypes based on the allele frequencies (Table 3) also revealed a striking difference between the observed and the expected findings. If the polymorphs are inherited by a simple Mendelian mechanism, there is a great over-representation in the population of the single-band compared to the double-band phenotypes. One possible reason for this disparity is that additional modifying factors, perhaps inherited independently of the structural alleles, suppress the expression of an apo[a] polymorph genotypically present. Heritable differences in the contribution of specific apoB alleles to plasma apoB concentrations have recently been demonstrated which result in an unequal expression of the two gene products (42). Such an influence upon gene expression is also compatible with the large standard deviation in the mean apoLp[a] levels for the individual polymorphs (Table 4). The relative differences in the intensities of the two bands observed among the double-band phenotypes presumably also reflect this variability in expression not directly related to the structural allele. When two bands with widely different M_r were present, the more intense one was generally the more strongly associated with apoLp[a] concentration. A second possible reason for the observed disparity may be related to the different ethnic and other groups included in this study. The observed allele frequencies are actually averages of subgroup frequencies; hence there may be fewer heterozygotes than expected in the study population.

A study of the apo[a] polymorphs expressed among a nine-member family produced instructive results (Fig. 6, Table 5). Both parents were double-band phenotypes with similar levels of apoLp[a]. Only one polymorph was shared, hence this polymorph would be expected to give rise to the only single-band phenotype among the offspring. Among the seven children, three exceptions to simple Mendelian inheritance were noted. Children 1 and 4 expressed almost solely polymorph 5, which was found only in the father and was the least intensely expressed polymorph among those present in both parents. When higher sample loads were used, an additional very weak band classified as polymorph 9 could be demonstrated, a polymorph found in neither parent. One explanation for this apparent anomaly, yet consistent with Mendelian inheritance, is that the allele corresponding to polymorph 8 in the mother was inherited, but secondary modification of the polypeptide through increased glycosylation resulted in an increased M_r and a decreased expression/detection of this polymorph. Since the sample load was normalized to 25 ng apoLp[a], the intensity of band 5 (inherited from the father) does not represent an increased synthesis of this polymorph, as reflected by their apoLp[a] concentration, but rather a suppression of the expression of the polymorph inherited from the mother in these children. Child 3 expressed only polymorph 6, apparently inherited from the mother. A fourfold increased sample load failed to reveal other polymorphs, but this exception probably also reflects a suppressed expression of the polymorph that was inherited from the father.

A careful analysis of apo[a] polymorphs in additional family studies, coupled with polypeptide and carbohydrate structural analysis, will be needed in order to more fully understand the molecular basis of apo[a] polymorphism and the modifying factors involved. The increased detectability and resolution of apo[a] polymorphs achieved in the present study provide an improved method of analysis for pursuing this objective.

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REFERENCES

1. Berg, K. 1963. A new serum type system in man—Lp system. *Acta Pathol. Microbiol. Scand.* **59:** 369–382.
2. Berg, K., and J. Mohr. 1963. Genetics of the Lp system. *Acta Genet. (Basel).* **13:** 349–360.
3. Berg, K., G. Dahlen, and M. H. Frick. 1974. Lp[a] lipoprotein and pre-β-$_1$ lipoprotein in patients with coronary heart disease. *Clin. Genet.* **6:** 230–235.
4. Dahlen, G., K. Berg, and M. H. Frick. 1976. Lp[a] lipoprotein/pre-β_1-lipoprotein, serum lipids and atherosclerotic disease. *Clin. Genet.* **9:** 558–566.
5. Harvie, N. R., and J. S. Schultz. 1970. Studies of Lp-lipoprotein as a quantitative genetic trait. *Proc. Natl. Acad. Sci. USA.* **66:** 99–103.
6. Albers, J. J., and W. R. Hazzard. 1974. Immunochemical quantification of human plasma Lp[a] lipoprotein. *Lipids.* **9:** 15–26.
7. Krempler, F., G. Kostner, and K. Bolzano. 1980. Turnover of lipoprotein[a] in man. *J. Clin. Invest.* **65:** 1483–1490.
8. Sing, C. F., J. S. Schultz, and D. C. Schreffler. 1974. The genetics of the Lp antigen. II. A family study and proposed models of genetic control. *Ann. Hum. Genet. (London).* **38:** 47–56.
9. Albers, J. J., P. Wahl, and W. R. Hazzard. 1974. Quantitative genetic studies of the human plasma Lp[a] lipoprotein. *Biochem. Genet.* **11:** 475–486.
10. Hasstedt, S. J., D. E. Wilson, C. Q. Edwards, W. N. Cannon, D. Carmelli, and R. R. Williams. 1983. The genetics of quantitative plasma Lp[a]: analysis of a large pedigree. *Am. J. Med. Genet.* **16:** 179–188.
11. Kostner, G. M., P. Avogaro, G. Cazzolato, E. Marth, G. Bittolo-Bon, and G. B. Quinici. 1981. Lipoprotein Lp[a] and the risk for myocardial infarction. *Atherosclerosis.* **38:** 51–61.
12. Jurgens, G., and P. Koltringer. 1985. A dominant role of lipoprotein [a] in the investigation and evaluation of parameters indicating the development of cerebral atherosclerosis. *Atherosclerosis.* **58:** 187–198.
13. Zenker, G., P. Koltringer, G. Bore, K. Niederkorn, K. Pfeiffer, and G. Jurgens. 1986. Lipoprotein[a] as a strong indicator for cerebrovascular disease. *Stroke.* **17:** 942–945.
14. Dahlen, G. H., J. R. Guyton, M. Attar, J. A. Farmer, J. A. Kautz, and A. M. Gotto, Jr. 1986. Association of levels of lipoprotein Lp[a], plasma lipids, and other lipoproteins with coronary artery disease documented by angiography. *Circulation.* **74:** 758–765.
15. Gaubatz, J. W., C. Heideman, A. M. Gotto, Jr., J. D. Morrisett, and G. H. Dahlen. 1983. Human plasma lipoprotein[a]. Structural properties. *J. Biol. Chem.* **258:** 4582–4589.
16. Utermann, G., and W. Weber. 1983. Protein composition of Lp[a] lipoprotein from human plasma. *FEBS Lett.* **154:** 357–361.
17. Fless, G. M., C. A. Rolih, and A. M. Scanu. 1984. Heterogeneity of human plasma lipoprotein[a]. Isolation and characterization of the lipoprotein subspecies and their apoproteins. *J. Biol. Chem.* **259:** 11470–11478.
18. Gaubatz, J. W., M. V. Chari, M. L. Nava, J. R. Guyton, and J. D. Morrisett. 1987. Isolation and characterization of the two major apoproteins in human lipoprotein[a]. *J. Lipid Res.* **28:** 69–79.
19. Utermann, G., H. J. Menzel, H. G. Kraft, H. C. Duba, H.G. Kemmler, and C. Seitz. 1987. Lp[a] glycoprotein phenotypes. Inheritance and relation to Lp[a]—lipoprotein concentrations in plasma. *J. Clin. Invest.* **80:** 458–465.
20. Utermann, G., H. G. Kraft, H. J. Menzel, T. Hopferwieser, and C. Seitz. 1988. Genetics of the quantitative Lp[a] lipoprotein trait. I. Relation of Lp[a] glycoprotein phenotypes to Lp[a] lipoprotein concentrations in plasma. *Hum. Genet.* **78:** 41–46.
21. Utermann, G., C. Duba, and H. J. Menzel. 1988. Genetics of the quantitative Lp[a] lipoprotein trait. II. Inheritance of Lp[a] glycoprotein phenotypes. *Hum. Genet.* **78:** 47–50.
22. Gaubatz, J. W., G. L. Cushing, and J. D. Morrisett. 1986. Quantitation, isolation, and characterization of human lipoprotein [a]. *Methods Enzymol.* **129:** 167–186.
23. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
24. Daniel, W. W. 1983. Biostatistics: A Foundation for Analysis in the Health Sciences. John Wiley and Sons, Inc., New York. Chap. 10, 352–387.
25. Roscoe, J. T., and J. A. Byars. 1971. An investigation of the restraints with respect to sample size commonly imposed on use of the Chi-square statistic. *J. Am. Stat. Assoc.* **66:** 755–759.
26. Raub, W. F. 1974. The PROPHET Computer System and Resource Sharing. *Fed. Proc.* **33:** 2390–2399.
27. The ARIC Investigators. 1989. The Atherosclerosis Risk in Communities (ARIC) Study: design and objectives. *Am. J. Epidemiol.* **129:** 687–702.
28. Cushing, G. L., J. W. Gaubatz, and B. J. Burdick, M. L. Nava, T. M. A. Bocan, D. Weilbaecher, J. R. Guyton, M. E. DeBakey, G. M. Lawrie, and J. D. Morrisett. 1989. Localization and quantitation of apolipoproteins [a] and B in coronary artery bypass vein grafts resected at reoperation. *Arteriosclerosis.* **9:** 593–603.
29. Albers, J. J., J. L. Adolphson, and W. R. Hazzard. 1977. Radioimmunoassay of human plasma Lp[a] lipoprotein. *J. Lipid Res.* **18:** 331–338.
30. Kratzin, H., V. W. Armstrong, M. Niehaus, N. Hilschmann, and D. Seidel. 1987. Structural relationship of an apolipoprotein[a] phenotype (570 kDa) to plasminogen: homologous kringle domains are linked by carbohydrate-rich regions. *Biol. Chem. Hoppe-Seyler.* **368:** 1533–1544.
31. Eaton, D. L., G. M. Fless, W. J. Kohr, J. E. McLean, Q-T. Xu, C. G. Miller, R. M. Lawn, and A. M. Scanu. 1987. Partial amino acid sequence of apolipoprotein[a] shows that it is homologous to plasminogen. *Proc. Natl. Acad. Sci. USA.* **84:** 3224–3228.
32. McLean, J. W., J. E. Tomlinson, W-J. Kaung, D. L. Eaton, E. Y. Chen, G. M. Fless, A. M. Scanu, and R. M. Lawn. 1987. cDNA sequence of human apolipoprotein[a] is homologous to plasminogen. *Nature.* **330:** 132–137.
33. Magoon, E. H., K. F. Austen, and J. Spragg. 1974. Immunoelectrophoretic analysis and radial immunodiffusion assay using plasminogen purified from fresh human plasma. *Clin. Exp. Immunol.* **17:** 345–359.
34. Boerwinkle, E., H. J. Menzel, H. G. Kraft, and G. Utermann. 1989. Genetics of the quantitative Lp[a] lipoprotein trait. *Hum. Genet.* **82:** 73–78.
35. Seman, L. J., and W. C. Breckenridge. 1986. Isolation and partial characterization of apolipoprotein[a] from human lipoprotein[a]. *Biochem. Cell Biol.* **64:** 999–1009.

36. Rainwater, D. L., G. S. Manis, and J. L. VandeBerg. 1989. Hereditary and dietary effects on apolipoprotein[a] isoforms and Lp[a] in baboons. *J. Lipid Res.* **30:** 549–558.
37. Fless, G. M., M. E. ZumMallen, and A. M. Scanu. 1986. Physico-chemical properties of apo[a] and Lp[a-] derived from the dissociation of human plasma Lp[a]. *J. Biol. Chem.* **261:** 8712–8718.
38. Morrisett, J. D., J. R. Guyton, J. W. Gaubatz, and A. M. Gotto, Jr. 1987. Lipoprotein[a]: structure, metabolism and epidemiology. *In* New Comprehensive Biochemistry. A. M. Gotto, Jr., editor. Elsevier Science Publishers, B. V. (Biomedical Division), The Netherlands. 129–152.
39. Morrisett, J. D., J. W. Gaubatz, R. D. Knapp, and J. G. Guevera. 1989. Structural properties of apo[a], a major apoprotein of human lipoprotein[a]. *In* Lipoprotein [a]: 25 Years of Progress. A. M. Scanu, editor. Academic Press, San Diego, CA. Chapter 4.
40. Segrest, J. P., R. L. Jackson, E. P. Andrews, and V. T. Marchesi. 1971. Human erythrocyte membrane glycoproteins: a re-evaluation of the molecular weight as determined by SDS polyacrylamide gel electrophoresis. *Biochem. Biophys. Res. Commun.* **44:** 390–395.
41. Hixson, J. E., M. L. Britton, G. S. Manis, and D. L. Rainwater. 1989. Lipoprotein[a] glycoprotein isoforms result from size differences in apo[a] mRNA in baboons. *J. Biol. Chem.* **264:** 6013–6016.
42. Gavish, D., E. A. Brinton, and J. L. Breslow. 1989. Heritable allele-specific differences in amounts of apoB and low-density lipoproteins in plasma. *Science.* **244:** 72–76.