Apolipoprotein E isoform phenotyping methodology and population frequency with identification of apoE1 and apoE5 isoforms[1]

Jose M. Ordovas,[2] Lois Litwack-Klein, Peter W. F. Wilson, Mary M. Schaefer, and Ernst J. Schaefer

Lipid Metabolism Laboratory, USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA 02111, and Framingham Heart Study, National Heart, Lung, and Blood Institute, 118 Lincoln Street, Framingham, MA 01801

Abstract Minigel methodology has been utilized for apolipoprotein (apo) E isoform phenotyping and criteria for distinguishing the apoE4/4 phenotype (mean apoE4/apoE3 ratio: 5.81) from the apoE4/3 phenotype (mean ratio: 1.01) and the apoE3/3 phenotype (mean apoE3/apoE2 ratio: 2.67) from the apoE3/2 phenotype (mean ratio: 0.76) based on gel scanning were developed. ApoE allele frequencies in 1209 subjects were: apoE3, 0.786; apoE4, 0.135; apoE2, 0.075; apoE5, 0.002; and apoE1, 0.002. Subjects with the apoE2 allele tended to have higher plasma very low density lipoprotein (VLDL) cholesterol and lower low density lipoprotein (LDL) cholesterol concentrations than subjects with the apoE3 allele, while the converse was true for subjects with the apoE4 allele. Subjects with the rare apoE1 allele had values similar to those with the apoE2 allele, while subjects with the rare apoE5 allele had values similar to those with the apoE4 allele.—**Ordovas, J. M., L. Litwack-Klein, P. W. F. Wilson, M. M. Schaefer, and E. J. Schaefer.** Apolipoprotein E isoform phenotyping methodology and population frequency with identification of apoE1 and apoE5 isoforms. *J. Lipid Res.* 1987. **28:** 371–380.

Supplementary key words very low density lipoproteins • low density lipoproteins • high density lipoproteins • hyperlipoproteinemia

Apolipoprotein (apo) E is a glycoprotein present in all mammalian species studied (1). In human plasma, apoE is associated with triglyceride-rich lipoproteins and high density lipoproteins (HDL) (2-4). Human apoE is synthesized in a variety of tissues as a preprotein 317 amino acids in length (5, 6). The preprotein undergoes intracellular proteolysis, glycosylation, and extracellular desialylation, resulting in a single polypeptide chain of 299 amino acids with a calculated molecular weight of 34, 145 (7, 8). The amino acid sequence as well as the mRNA nucleotide sequence of apoE are known for several species (8-10). The human apoE gene has been isolated, characterized, and mapped to chromosome 19, as have the genes for apoC-I, apoC-II, and the LDL receptor (10-14). ApoE can bind to the LDL (apoB, E) receptor, as well as the apoE receptor, and is therefore important in determining triglyceride-rich lipoprotein catabolism (1, 2, 15-27). ApoB, E receptor activity is regulated by dietary factors and decreases with age, while apoE receptor activity does not appear to have such regulation (2, 15, 21).

In plasma, three major isoforms of apoE (E4, E3, and E2), differing in their isoelectric points, have been detected when delipidated very low density lipoproteins (VLDL) are subjected to isoelectric focusing at a pH range of 4.0 to 6.5 (16-19, 24). These isoforms are determined by three alleles at a single genetic locus (17). Other minor isoforms can be detected when apoVLDL is subjected to two-dimensional gel electrophoresis and these forms are associated with varying degrees of sialylation. The most common apoE allele is E3, while E4 and E2 are less common (17, 18, 28-32). ApoE4 differs from apoE3 due to an arginine for cysteine substitution at residue 112, while apoE2 differs from apoE3 due to a cysteine for arginine substitution at residue 158 (33, 34). As a result of these three different alleles, six apoE phenotypes are observed in the normal population. Other rare isoforms (E1, E5, and E7) have been recently reported (36-39).

A number of studies have demonstrated the association of different apoE phenotypes with lipid disorders (1, 16, 17, 28, 31, 40-43). Type III hyperlipoproteinemia (HLP)

Abbreviations: IEF, isoelectric focusing; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; HLP, hyperlipoproteinemia.

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[2]Address correspondence to Dr. Jose M. Ordovas, Lipid Metabolism Laboratory, USDA Human Nutrition Research Center on Aging at Tufts University, 711 Washington Street, Boston, MA 02111.

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or dysbetalipoproteinemia is characterized by palmar and tubo-eruptive xanthomas, premature peripheral vascular and coronary artery disease, and accumulation of abnormal cholesterol-rich VLDL and intermediate density lipoproteins (23). Type III HLP has been associated with the apoE2/2 phenotype, as well as with other various rare mutations of apoE, including apoE1, and familial apolipoprotein E deficiency (16, 26-28, 36). ApoE1 has also recently been associated with severe hypertriglyceridemia (37). ApoE1 in this latter case has been reported to contain cysteine instead of arginine at residue 158, similar to apoE2, as well as an additional amino acid substitution which is probably of no functional significance. It has been shown that apoE2 has a decreased fractional catabolic rate and markedly impaired binding to the apoB, E receptor as compared to apoE3 (25, 34, 35).

The apoE4 allele is more frequent in type V HLP, characterized by marked hypertriglyceridemia due to accumulations of both chylomicrons and VLDL as well as an increased risk of recurrent pancreatitis (31, 40). More recently the apoE4 allele has also been associated with elevations in plasma low density lipoprotein (LDL) cholesterol levels (41-43). The etiology of these associations remains unclear, since apoE4 and apoE3 have similar binding properties to the apoB, E receptor (34). However, apoE4 is catabolized more rapidly than apoE3, possibly resulting in down-regulation of the liver LDL receptor and increased levels of plasma LDL constituents (44). In addition, two kindreds from Japan have recently been described as having an apoE5 allele (38, 39). These subjects had type II HLP (increased LDL cholesterol), and some affected kindred members had premature atherosclerosis.

The purpose of the present study was to develop a rapid, accurate, and inexpensive method for apoE isoform determination, and to utilize this method to assess apoE phenotypes in a large population participating in an ongoing community study attempting to precisely define the importance of various coronary artery disease risk factors (The Framingham Offspring Study).

METHODS

Lipoprotein isolation and quantitation

Blood was collected in 0.1% Na_2EDTA, and plasma was separated at 4°C by centrifugation at 3000 rpm for 30 min. Plasma was subjected to ultracentrifugation at density 1.006 g/ml for 18 hr at 4°C. VLDL was separated by tube slicing, and brought to its original 5-ml volume with 0.85% NaCl, 0.01% Na_2EDTA solution. The cholesterol concentrations in whole plasma, the 1.006 g/ml infranate, and the HDL fraction were quantitated by an enzymatic method on an Abbott 200 bichromatic analyzer (Abbott Laboratories, Dallas, TX) utilizing Abbott single vial reagent (AGENT) (45). Plasma triglyceride concentrations were determined in similar fashion (45). VLDL and low density lipoproteins (LDL) were precipitated from plasma by the dextran magnesium sulfate procedure as previously described (45). Our laboratories participate in the Centers for Disease Control-National Heart, Lung, and Blood Institute Lipid Research Clinics Standardization program, and values obtained for total cholesterol, triglycerides, and HDL cholesterol have consistently been within the established reference ranges.

Sample preparation

Isolated VLDL were dialyzed versus 10 mM ammonium bicarbonate for at least 3 hr (a minimum 100-fold excess in volume) with two changes of the dialysis fluid (dialysis tubing 12,000-14,000 molecular weight cut off, 0.4 cm dry cylinder diameter). VLDL were then frozen at − 70°C for at least 1 hr, and lyophilized overnight. VLDL samples were delipidated with 3 ml of chloroform-methanol 2:1 (v/v), vortexed for 1 min, and placed on a rocker for at least 1 hr. Two ml of ethyl ether was added, the tubes were vortexed and placed on a rocker for at least 30 min, and then they were centrifuged at 3,000 rpm for 15 min at 4°C. The supernatant was aspirated off without disturbing the protein pellet, and the delipidation process was repeated in identical fashion except that 5 ml of ethyl ether was utilized. The samples were dried in an oven at 37°C or under a nitrogen stream. The dried protein pellet was resolubilized in 100 μl of 0.01 M Tris, 0.01 M dithiothreitol, 8 M urea solution, pH 8.2 (24.024 g of urea was dissolved in 50 ml of distilled water and passed through an Amberlite MB-1A column, then 8 mg of Tris-HCl and 8 mg of dithiothreitol were added; the solution was titrated with 0.1 M NaOH in 8 M urea; the Amberlite column was made with a 10-ml syringe with the tip filled with glass wool and contained 5 ml of Amberlite MB-1A obtained from Sigma Co., St. Louis, MO). The resolubilized protein pellet was allowed to sit for approximately 10 min, revortexed, and placed in an oven for 1 hr at 37°C. The protein content was determined by the Bio-Rad protein assay method (46).

Isoelectric focusing

Either the Bio-Rad model 360 mini vertical slab gel apparatus or the Hoefer SE 250 Mighty Small II gel apparatus were utilized for the minigel methodology. Gel solutions were made up as follows: 1.5 g of acrylamide and 40 mg of bisacrylamide were dissolved in 19 ml of 8 M urea and filtered through a 0.45-μm filter (Millex-GS. Millipore Corp. Bedford, MA). Thereafter 1 ml of ampholines (LKB, pH 4-6.5) was added, and 10-ml aliquots were separated off and degassed. Twenty five microliters of 10% ammonium persulfate and 3 μl of TEMED were added to each 10 ml of aliquot, and mixed well. Slab gels

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were poured with each 10-ml aliquot; a 10-well template was utilized. Once the gels were polymerized (1-2 hr) the templates were removed, and the wells were rinsed with upper buffer (5 mM NaOH, 5 ml of 1 M NaOH in 1 liter of distilled water). Gels were stored for up to 1 week prior to use. The lower electrophoresis buffer was 10 mM phosphoric acid (5.5 ml of 1 M phosphoric acid in 1 liter of distilled water).

Twenty micrograms of protein was loaded with a Hamilton syringe below a layer of upper buffer into each well. Gels were run for 2 hr at constant current with initial voltage set at 400 volts. When one gel was run, a current of 11.1 amperes was applied; for two slabs, 22.2 amperes of current was applied. Following the run, gels were removed from the glass plates and placed in fixing solution (30 g of trichloroacetic acid, and 13 g of sulfosalicylic acid in 500 ml of distilled water) for 30 min. The gels were then equilibrated in destaining solution (200 ml of glacial acetic acid and 600 ml of methanol in 1200 ml of distilled water) for 10 min, and stained with Coomassie blue (1 g of Coomassie blue, 450 ml of methanol, 100 ml of glacial acetic acid, and 450 ml of distilled water) for about 3 hr. Gels were destained in destaining solution until the background was clean (usually overnight), and then they were scanned with an LKB Ultroscan laser scanning densitometer, utilizing a scan speed of 6, an integration factor of 1, and output setting T. The absorption setting was varied depending upon the intensity of the bands. The integrator was set at peak width 0.04, chart speed at 2, attenuation at 10, and the threshold setting at 10. The gel background was set by scanning a blank portion of the gel. Gels were dried for permanent storage on a Bio-Rad model 224 gel drier for 30 min.

Determination of isoelectric points

The isoelectric points of the rare apoE5 and apoE1 isoforms were calculated using standards (the Sigma wide range marker System) previously reported values for the other apoE and apoC isoforms (17, 47).

Two-dimensional gel electrophoresis

For two-dimensional gel electrophoresis, individual stained lanes were cut from the monodimensional IEF slab gel and run immediately or kept stored frozen at −70°C. The second dimension was run in an SDS polyacrylamide gradient gel (4 to 22.5%) as previously described (48).

Modification of VLDL

To characterize the number of cysteines and charge differences in apoE, VLDL were treated with cysteamine (33). Briefly, after dialysis the VLDL sample (150 μg) was treated with 1.0 mg of cysteamine, dissolved in 0.1 M ammonium bicarbonate for 4 hr at 37°C, and the sample was then lyophilized and IEF was performed as described above.

To assess the effect of desialylation on the IEF pattern, the VLDL sample (1 mg of protein in 500 μl of 0.15 M NaCl and 0.02 M sodium acetate, pH 5.2) was incubated with 0.01 U of neuraminidase (*Clostridium perfringens* 0.6 U/ml, Boehringer Mannheim Biochemicals, Indianapolis, IN) for 30 min at 37°C. Thereafter the VLDL sample was subjected to IEF as described above.

Statistical analysis

Results were tabulated and analyzed utilizing the RS1 software package (Bolt, Benarek, Newman, Cambridge, MA) on a VAX 11/780 computer (Digital Equipment Corp., Maynard, MA). Statistical differences were assessed by *t*-test analysis. For those parameters that did not have a normal distribution (plasma triglyceride and VLDL cholesterol), logarithmic transformation was performed prior to the analysis.

RESULTS

The complex pattern of apoE isoforms observed after subjecting delipidated VLDL to isoelectric focusing results from both charge and size differences, and makes the assessment of different phenotypes difficult without the use of two-dimensional gel electrophoresis or neuraminidase treatment. The method presented in this report permits rapid characterization of apoE isoform phenotypes, with a limited amount of VLDL protein utilizing a combination of minigel electrophoresis (**Fig. 1**) and laser densitometric scanning. The integration of the different apoE bands (resulting from the different genetic variants and/or their multiple sialylated forms) allows the calculation of ratios between bands at specific isoelectric points. These ratios were used to identify specific phenotypes (**Table 1**). The mean apoE4/apoE3 ratio in individuals with the apoE4/3 phenotype was 1.01, (range 0.31-1.28). The same mean ratio for individuals with the apoE4/4 phenotype was 5.81, (range 1.21-66.00). The mean apoE3/apoE2 ratio in subjects with the apoE3/2 phenotype was 0.76, (range 0.21-1.19), while in subjects with the apoE3/3 phenotype the ratio was 2.67 (range 1.03-17.50). The phenotypes were verified by two-dimensional gel electrophoresis in those subjects in whom the ratio precluded definitive diagnosis as well as in randomly selected subjects with varying apoE phenotypes (n = 68) (Table 1). If one uses a cut-point of 1.20 for the apoE4/apoE3 ratio, only one subject (0.57%) was misclassified by monodimensional isoelectric focusing in distinguishing between apoE4/4 and apoE4/3 pheno-

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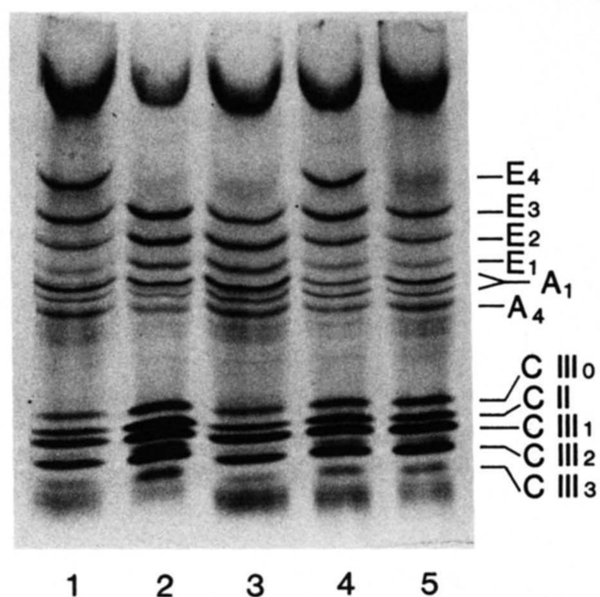

Fig. 1. Minigel isoelectric focusing patterns of VLDL apolipoproteins from subjects with various apoE phenotypes; lane 1, apoE4/3; lane 2, apoE3/3; lane 3, apoE3/2; lane 4, apoE4/3; and lane 5, apoE3/3.

types. For the apoE3/apoE2 ratio, the cut-point chosen was 1.10 to distinguish between apoE3/3 and apoE3/2 phenotypes, which resulted in a 1% misclassification. **Fig. 2** shows typical densitometric scans for subjects with the apoE4/4 and apoE4/3 phenotypes. The reproducibility of these ratios was tested by running samples of two individuals in different gels (four runs for each one of the samples); the average coefficient of variation was 8.1%. The same methodology can be used to determine the ratios of the different sialylated forms of apoC-III and the ratio of apoC-III to apoC-II, since these proteins are also detected in this gel system.

The frequencies of various apoE phenotypes as well as the lipid values in a normal representative United States community sample with a similar number of young and elderly males and females (age range 22–71 years) are given in Tables 2–5. The allele frequency in our entire sample was: apoE3, 0.786; apoE4, 0.135; apoE2, 0.075; apoE5, 0.002 and apoE1, 0.002. The apoE phenotype frequencies, plasma lipids and lipoprotein cholesterol concentrations for male and female subjects are presented in **Table 2** and **Table 3**. Males with the apoE3/2 phenotype had significantly lower plasma cholesterol ($P < 0.001$) and LDL cholesterol levels ($P < 0.001$) than did males with the apoE3/3 phenotype. Males with the uncommon apoE2/2 phenotype had significantly higher plasma triglyceride ($P < 0.01$) and VLDL cholesterol levels ($P < 0.001$), and significantly lower LDL cholesterol levels ($P < 0.01$) than did males with the apoE3/3 phenotype. Males with the uncommon apoE4/2 phenotype had significantly higher plasma triglyceride ($P < 0.01$) and VLDL cholesterol ($P < 0.05$) concentrations than did males with the apoE3/3 phenotype. Males with the apoE4/3 phenotype had significantly higher plasma triglyceride values ($P < 0.01$) and lower HDL cholesterol levels than did apoE3/3 males (see Table 2).

For females, fewer differences were observed. Females with the apoE3/2 phenotype had significantly lower total cholesterol ($P < 0.05$) and LDL cholesterol levels ($P < 0.01$) than did apoE3/3 females. Females with the apoE2/2 phenotype had significantly lower LDL cholesterol levels ($P < 0.01$) while females with the apoE4/3 phenotype had significantly higher LDL cholesterol values ($P < 0.01$) as compared to apoE3/3 females.

In order to assess whether the increase in total lipids that takes place with age was affected by the apoE phenotype, we divided both males and females into two age groups: under 45 years and 45 years and over (**Table 4** and **Table 5**). In general, there was a trend for older subjects to have higher lipid levels than younger subjects, but only in apoE3/3 males were statistically significant differences observed, with older males having significantly higher plasma total cholesterol ($P < 0.001$), total triglyceride ($P < 0.001$), VLDL cholesterol ($P < 0.001$), and LDL cholesterol concentrations ($P < 0.001$) than younger males. Sample size in other groups was significantly smaller (see Table 4). For females (see Table 5) older women with the apoE3/3, apoE4/3, and apoE3/2 phenotypes had significantly higher plasma total cholesterol, total triglyceride, VLDL cholesterol, and LDL cholesterol levels than did their younger counterparts. Sample size for other phenotypes was small.

The unusual apoE isoforms apoE5 and apoE1 were also detected in this population. Two subjects with the apoE5/3 phenotype were detected (one male, one female),

TABLE 1. Ratio of isoforms based on densitometric scanning[a]

ApoE Phenotype	4/3	3/2
E4/4		
(n = 27)[b]	5.81 (1.213–66.00)	
(n = 11)[c]	4.72 (1.213–12.12)	
E4/3		
(n = 161)[b]	1.01 (0.30–1.28)	
(n = 23)[c]	1.00 (0.54–1.28)	
E3/3		
(n = 514)[b]		2.66 (1.03–17.50)
(n = 14)[c]		1.88 (1.03–2.96)
E3/2		
(n = 99)[b]		0.76 (0.21–1.20)
(n = 20)[c]		0.90 (0.44–1.09)

[a] Ratios given as mean values. Values in parentheses indicate ranges.
[b] Based on total sample size of 801 subjects.
[c] Based on total sample size of 68 subjects whose apoE phenotypes were verified by two-dimensional gel electrophoresis.

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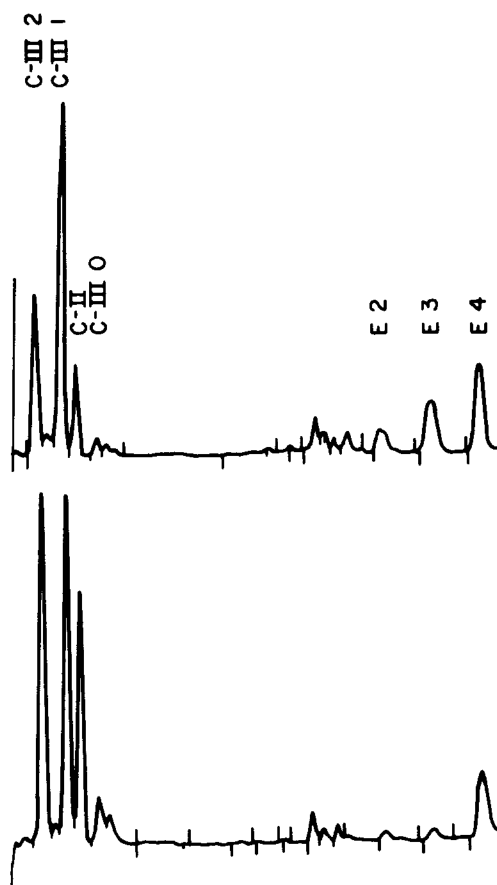

Fig. 2. Scanning densitometry of VLDL isoelectric focusing patterns of VLDL apolipoproteins derived from subjects with the apoE4/3 phenotype (top) and the apo4/4 phenotype (bottom), respectively.

and both had elevated LDL cholesterol values. This was not the case in one subject with the apoE5/2 phenotype. **Fig. 3** and **Fig. 4** show the isoelectric focusing and two-dimensional electrophoresis patterns of a subject with the apoE5/3 phenotype. Cysteamine treatment of VLDL from one of the subjects with the apoE5/3 phenotype resulted in a shift of the apoE3 band by one charge unit, but no change in charge of apoE5 band after isoelectric focusing. These data indicate lack of cysteine in the apoE5 band in this subject. Two-dimensional electrophoresis revealed no appreciable differences in the molecular weight of the apoE5 variant when compared to apoE3 or apoE2. The calculated isoelectric point of the apoE5 allele using appropriate standards was 6.52. After neuraminidase treatment of the same apoE5/3 sample, only two bands corresponding to the non-sialylated forms of apoE5 and apoE3 were observed in the IEF gel. Two subjects with the apoE2/1 phenotype were also detected (one male, 1 female), and both subjects had markedly decreased LDL cholesterol levels. Cysteamine treatment of the VLDL isolated from a subject with the apoE2/1 phenotype caused a shift of two units in each of the bands, resulting in bands in the E3 and E4 positions, respectively. Neuraminidase treatment removed all the sialylated bands of both apoE2 and apoE1. Molecular weight determination after SDS polyacrylamide gel electrophoresis did not show any difference between the apoE2 and the apoE1 alleles. The calculated isoelectric point of the apoE1 isoform was 5.76. All the subjects with the apoE5 variant as well as those with the apoE1 variant were Caucasian.

DISCUSSION

ApoE plays an important role in the receptor-mediated uptake of triglyceride-rich lipoproteins (2, 15–18). It has recently been demonstrated that more than 15% of the total variation in total plasma and LDL cholesterol levels can be attributed to this single gene locus (41–43). For this reason a rapid and accurate method of apoE isoform determination, allowing for the study of large numbers of individuals, is of interest. The method presented here offers a number of advantages over those previously reported. Only 20 μg of apoVLDL solubilized in urea is necessary. The electrophoretic run time is reduced from

TABLE 2. Male subjects (n = 602)

Phenotype	Frequency	Cholesterol	Triglyceride	Cholesterol VLDL	Cholesterol LDL	Cholesterol HDL	Ratio VLDL C/TG
				mg/dl ± SD			
E 3/3 (n = 392)	0.651	212 ± 39	138 ± 107	28 ± 20	138 ± 35	45 ± 11	0.213 ± 0.08
E 4/3 (n = 108)	0.179	217 ± 4	171 ± 143[a]	34 ± 26	141 ± 36	42 ± 10[a]	0.205 ± 0.08
E 3/2 (n = 61)	0.101	192 ± 37[b]	158 ± 149	34 ± 30	116 ± 28[b]	42 ± 11	0.216 ± 0.08
E 4/4 (n = 20)	0.033	213 ± 38	127 ± 118	26 ± 24	140 ± 37	47 ± 15	0.205 ± 0.07
E 4/2 (n = 14)	0.023	223 ± 33	234 ± 199[a]	48 ± 43[c]	132 ± 35	43 ± 12	0.201 ± 0.04
E 2/2 (n = 4)	0.007	192 ± 38	273 ± 72[a]	71 ± 12[b]	84 ± 33	38 ± 4	0.269 ± 0.07
E5 Variant (n = 2)	0.003	207 ± 57	175 ± 165	24 ± 24	139 ± 46	45 ± 13	0.131 ± 0.01
E1 Variant (n = 1)	0.002	117	100	21	55	41	0.2100

Statistically significant difference from phenotype 3/3: [a], $P < 0.01$; [b], $P < 0.001$; [c], $P < 0.05$.

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TABLE 3. Female subjects (n = 607)

Phenotype	Frequency	Cholesterol	Triglyceride	Cholesterol VLDL	Cholesterol LDL	Cholesterol HDL	Ratio VLDL C/TG
				mg/dl ± SD			
E 3/3 (n = 373)	0.614	209 ± 47	122 ± 230	24 ± 33	130 ± 38	56 ± 16	0.2069 ± 0.11
E 4/3 (n = 119)	0.196	217 ± 40	113 ± 74	21 ± 14	140 ± 39[a]	56 ± 15	0.1915 ± 0.08
E 3/2 (n = 82)	0.135	198 ± 41[b]	120 ± 84	25 ± 19	117 ± 34[a]	56 ± 16	0.2073 ± 0.10
E 4/4 (n = 18)	0.030	216 ± 42	92 ± 41	18 ± 11	139 ± 39	60 ± 10	0.1915 ± 0.09
E 4/2 (n = 10)	0.016	201 ± 42	90 ± 34	22 ± 13	123 ± 32	52 ± 19	0.1928 ± 0.08
E 2/2 (n = 3)	0.005	172 ± 53	106 ± 58	36 ± 22	76 ± 24[a]	60 ± 11	0.3207 ± 0.07
E5 Variant (n = 1)	0.002	266	147	32	177	57	0.2176
E1 Variant (n = 1)	0.002	166	113	32	70	64	0.2831

Statistically significant difference from phenotype 3/3: [a], $P < 0.01$; [b], $P < 0.05$.

16 hr to 2 hr. The small volumes of solutions required to prepare the gel represent a considerable savings in reagent costs. The scanning of the gels permits an easier identification of the different apoE phenotypes as well as the possibility of studying the association of differing apoC-III/apoC-II ratios with dyslipidemic states (49–52).

Monodimensional isoelectric focusing was inadequate for precise apoE phenotyping in only 7 of 1207 (0.9%) samples. Based on our data, the optimal cut-point for the apoE4/apoE3 ratio to distinguish between the apoE4/4 and the apoE4/3 phenotypes was 1.20, with those subjects having higher ratios having the former phenotype and those subjects having lower ratios having the latter phenotype. The mean apoE4/apoE3 ratio for subjects with the apoE4/4 phenotype at 5.81 was over fivefold higher than that observed for subjects with the apoE4/3 phenotype (1.01). In our experience, finding a ratio above 1.5 was always associated with the apoE4/4 phenotype, and having a ratio below 1.1 was always associated with the apoE4/3 phenotype. Similarly, the optimal cut-point for the apoE3/apoE2 ratio to distinguish betweeen the apoE3/3 and apoE3/2 phenotypes was 1.10, with subjects having higher ratios having the former phenotype and subjects with lower ratios having the latter phenotype. The mean apoE3/apoE2 ratio for subjects with the apoE3/3 phenotype at 2.67 was over threefold higher than that observed for subjects with the apoE3/2 phenotype (0.76). In our experience, finding an apoE3/apoE2 ratio

TABLE 4. Young (under 45 years) and old (45 years and over) males

Phenotype	Age Category	N	Cholesterol	Triglyceride	Cholesterol VLDL	Cholesterol LDL	Cholesterol HDL	Ratio VLDL C/TG
					mg/dl ± SD			
E 3/3	young	164	200 ± 36	124 ± 110	26 ± 17	129 ± 35	45 ± 12	0.22 ± 0.10
	old	193	221 ± 34[a]	148 ± 103[a]	30 ± 20[a]	145 ± 31[a]	45 ± 11	0.212 ± 0.07
E 4/3	young	38	207 ± 45	147 ± 117	29 ± 23	135 ± 41	43 ± 9	0.21 ± 0.08
	old	61	223 ± 40	180 ± 154	36 ± 27	144 ± 35	42 ± 11	0.206 ± 0.07
E 3/2	young	24	188 ± 39	164 ± 172	34 ± 33	110 ± 29	44 ± 13	0.22 ± 0.09
	old	31	197 ± 38	164 ± 144	35 ± 31	122 ± 29	40 ± 9	0.215 ± 0.07
E 4/4	young	7	225 ± 40	100 ± 60	22 ± 13	157 ± 30	45 ± 9	0.22 ± 0.04
	old	9	212 ± 34	153 ± 168	29 ± 34	133 ± 42	50 ± 19	0.182 ± 0.08
E 4/2	young	7	220 ± 31	176 ± 111	36 ± 24	144 ± 26	40 ± 9	0.20 ± 0.06
	old	6	231 ± 26	306 ± 277	64 ± 60	125 ± 42	43 ± 14	0.202 ± 0.02
E 2/2	young	3	174 ± 172	78 ± 87	66 ± 10	69 ± 19	39 ± 4	0.25 ± 0.07
	old	1	245	257	83	128	34	0.323
E5 Variant	young	none						
	old	2	207 ± 57	175 ± 165	24 ± 24	139 ± 46	45 ± 13	0.131 ± 0.01
E1 Variant	young	1	117	100	21	55	41	0.210
	old	0						

Statistically significant difference between young and old: [a], $P < 0.001$.

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above 1.4 was always associated with the apoE3/3 phenotype, while a ratio below 1.0 was always associated with the apoE3/2 phenotype.

The broad range of ratios found in the population studied could represent physiological variation, as well as differences in preparation of samples. Previous reports indicate that variation in the ratio of apoE3/apoE2 may be due to carbamylation of the apoE3 isoform by urea subproducts present in the solubilization buffer, as well as sialylation (38). Based on our own experience, the use of freshly deionized urea in the preparation of the solubilization buffer is essential to minimize carbamylation.

The method described in this report is being utilized in a systematic study of cardiovascular risk factors in the Framingham Offspring Study. ApoE phenotype will be correlated with plasma apoE and apoB concentrations, lipoprotein levels, and cardiovascular disease in a case-controlled as well as a prospective fashion.

The data presented are consistent with previous reports, indicating that the allele frequency of the different apoE isoforms is similar in a variety of Caucasian populations (41–43). Moreover, our data indicate that apoE phenotype plays a significant role in modulating plasma lipid and lipoprotein cholesterol concentrations in a normal population. With regard to plasma triglyceride levels, a significant elevation was observed in males with the apoE4/3 phenotype, the apoE4/2, and the apoE2/2 phenotype, as compared to subjects with the apoE3/3 phenotype. No such differences were observed in females. Similar differences were observed for VLDL cholesterol

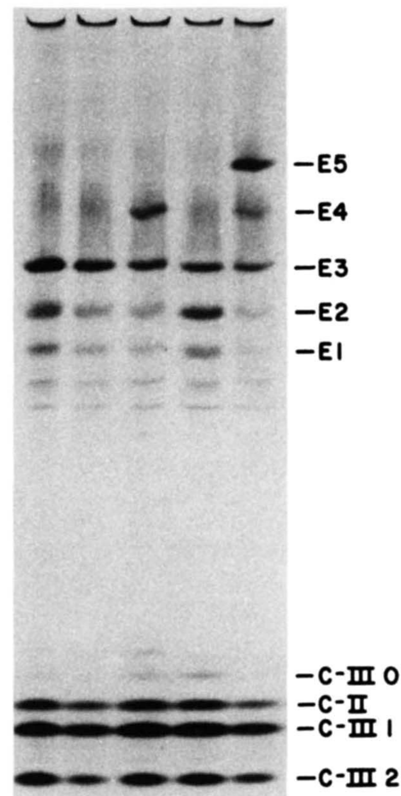

Fig. 3. Monodimensional isoelectric focusing gel of VLDL apolipoproteins derived from a subject with the apoE5/3 phenotype (far right) as compared to gels from subjects with apoE3/3, apoE3/3, apoE4/3, and apoE3/2 phenotypes (from left to right).

TABLE 5. Young (under 45 years) and old (45 years and over) females

Phenotype	Age Category	N	Cholesterol	Triglyceride	Cholesterol			Ratio VLDL C/TG
					VLDL	LDL	HDL	
					mg/dl ± SD			
E 3/3	young	144	188 ± 32	81 ± 50	17 ± 11	115 ± 30	56 ± 17	0.216 ± 0.11
	old	203	224 ± 49[a]	151 ± 305[a]	28 ± 43[a]	139 ± 37[a]	57 ± 15	0.202 ± 0.12
E 4/3	young	48	202 ± 37	92 ± 49	18 ± 11	128 ± 34	57 ± 12	0.193 ± 0.07
	old	64	230 ± 37[b]	124 ± 84[b]	23 ± 16[c]	150 ± 38[b]	57 ± 17	0.192 ± 0.08
E 3/2	young	30	179 ± 33	87 ± 46	18 ± 13	107 ± 30	54 ± 14	0.204 ± 0.14
	old	47	212 ± 42[b]	143 ± 96[b]	20 ± 21[b]	125 ± 36[c]	57 ± 16	0.212 ± 0.07
E 4/4	young	8	197 ± 40	84 ± 29	19 ± 11	120 ± 33	59 ± 10	0.224 ± 0.11
	old	9	230 ± 41	84 ± 91	14 ± 7	152 ± 40	63 ± 9	0.163 ± 0.07
E 4/2	young	2	169 ± 1	116 ± 50	21 ± 7	106 ± 3.5	43 ± 9	0.186 ± 0.02
	old	7	210 ± 47	87 ± 31	19 ± 12	129 ± 38	52 ± 21	0.204 ± 0.093
E 2/2	young	none						
	old	2	202 ± 1.4	133 ± 50	47 ± 14	90 ± 3	65 ± 10	0.358 ± 0.027
E5 Variant	young	none						
	old	1	266	147	32	177	57	0.2176
E1 Variant	young	1	166	113	32	70	64	0.2831
	old	none						

Statistically significant difference between young and old: [a], $P < 0.001$; [b], $P < 0.01$; [c], $P < 0.05$.

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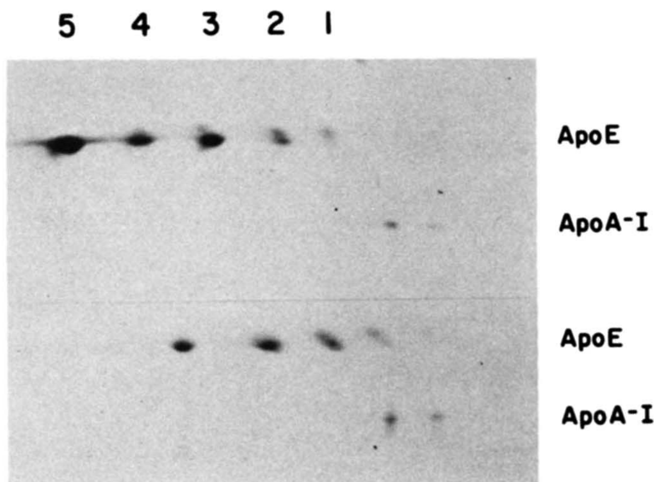

Fig. 4. Two-dimensional gel electrophoresis of VLDL apolipoproteins derived from a subject with the apoE5/3 phenotype (top) and a subject with the apoE3/2 phenotype.

levels. Males with the apoE4/2 and apoE2/2 phenotypes had significantly higher levels of VLDL cholesterol in plasma than did males with the apoE3/3 phenotype. Again no such differences were observed for females. In addition, there was a tendency for apoE phenotype to affect the VLDL cholesterol: plasma triglyceride ratio. Subjects with the apoE2 allele had the highest ratio and subjects with the apoE4 allele had the lowest ratio, but their differences were not statistically significant. These data suggest that both apoE phenotype and hormonal effects interact in modulating triglyceride metabolism.

With regard to LDL cholesterol levels, females with the apoE4/3 had significant elevations while females with the apoE3/2 and apoE2/2 phenotype had significantly lower levels than did females with the apoE3/3 phenotype. Only males with the apoE3/2 phenotype had significantly lower LDL cholesterol levels than did males with the apoE3/3 phenotype. Therefore, apoE phenotype appears to affect LDL metabolism as well, and differences observed in females are at least as great as those observed in males.

How different genetic forms of apoE modulate VLDL and LDL levels remains to be clearly defined. It has been shown that apoE2 has impaired binding to the LDL receptor (34, 35). It has also been documented that apoE2 residence time on normal lipoproteins in normolipidemic subjects and in subjects with type III HLP is significantly greater than apoE3 residence time (25). In contrast, apoE4 is catabolized more rapidly than apoE3 (44). It is possible that the impaired apoE-modulated uptake of chylomicron remnants in apoE2 homozygotes may upregulate the LDL receptor and lead to enhanced LDL degradation. Such a scheme would explain the decreased LDL levels observed in these subjects. The converse may be true in apoE4 homozygotes where enhanced uptake of chylomicron remnants may result in a down-regulation of the LDL receptor, increased VLDL synthesis, and decreased LDL uptake. The clear importance of apoE in chylomicron remnant uptake has been confirmed by lipoprotein abnormalities observed in familial apolipoprotein E deficiency (26, 27, 53).

The apoE1 mutation has been shown in at least one case to be due to the standard apoE2 alteration at residue 158 plus an additional substitution that may or may not be of functional significance (37). The apoE1 allele in our study was associated with decreased LDL values similar to the apoE2 allele. The analysis of the apoE1 of one of our subjects indicates that the mutation is similar to the one previously described. Whether apoE5 is identical to apoE4 plus an additional mutation that is not of functional importance remains to be determined. The apoE5 allele has been associated with elevated LDL levels similar to apoE4 (38), and this association is also observed in our population study. Our results indicate that the apoE5 variant described in this report is different from those previously described (38). The molecular weight determination indicates that our E5 has the same molecular weight as E3, while in the Japanese kindred the molecular weight was slightly lower. Also, cysteamine treatment caused a one-unit change shift in the apoE5 variant previously described, while the same treatment did not have any effect on our E5 protein.

Further research on apoE metabolism, genetics, and receptor interactions with the apoE receptor as well as the apoB, E receptor are clearly warranted. ■

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REFERENCES

1. Zannis, V. I., and J. L. Breslow. 1985. Genetic mutations affecting human lipoprotein metabolism. *Adv. Hum. Genet.* **14:** 125–215.
2. Mahley, R. W., and T. L. Innerarity. 1983. Lipoprotein receptors and cholesterol homeostasis. *Biochim. Biophys. Acta.* **737:** 197–222.
3. Shore, B., and V. Shore. 1973. Heterogeneity of human plasma very low density lipoproteins. Separation of species differing in protein components. *Biochemistry.* **12:** 502–507.
4. Weisgraber, K. H., and R. W. Mahley. 1978. Apoprotein (E-A-II) complex of human plasma lipoproteins. I. Characterization of this mixed disulfide and its identification in a high density lipoprotein subfraction. *J. Biol. Chem.* **253:** 6281–6288.

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5. Blue, M. L., D. L. Williams, S. Zucker, S. A. Khan, and C. Blum. 1983. Apolipoprotein E synthesis in human kidney, adrenal gland and liver. *Proc. Natl. Acad. Sci. USA.* **80:** 283-287.
6. Zannis, V. I., F. S. Cole, C. L. Jackson, D. M. Kurnit, and S. Karathanasis. 1985. Distribution of apolipoprotein A-I, C-II, C-III and E mRNA in fetal human tissues. Tissue-dependent induction of apolipoprotein E mRNA by cultures of human monocyte-macrophages. *Biochemistry.* **24:** 4450-4455.
7. Zannis, V. I., J. McPherson, G. Goldberger, S. K. Karathanasis, and J. L. Breslow. 1984. Synthesis, intracellular processing and signal peptide of human apoE. *J. Biol. Chem.* **259:** 5495-5499.
8. Rall, S. C., Jr., K. H. Weisgraber, and R. W. Mahley. 1982. Human apolipoprotein E. The complete aminoacid sequence. *J. Biol. Chem.* **257:** 4171-4178.
9. McLean, J. W., C. Fukazawa, and J. M. Taylor. 1983. Rat apolipoprotein E mRNA, cloning and sequencing of double stranded cDNA. *J. Biol. Chem.* **258:** 8993-9000.
10. Paik, Y. K., D. J. Chang, C. A. Reardon, G. E. Davies, R. W. Mahley, and J. M. Taylor. 1985. Nucleotide sequence and structure of the human apolipoprotein E gene. *Proc. Natl. Acad. Sci. USA.* **82:** 3445-3449.
11. Das, H. K., J. McPherson, G. A. P. Bruns, S. K. Karathanasis, and J. L. Breslow. 1985. Isolation, characterization and mapping to chromosome 19 of the human apolipoprotein E gene. *J. Biol. Chem.* **260:** 6240-6247.
12. Tata, F., I. Henry, A. F. Markham, S. C. Wallis, D. Weil, K. H. Grzeschik, C. Junien, R. Williamson, and S. E. Humphries. 1985. Isolation and characterization of a cDNA clone for human apolipoprotein CI and assignment of the gene to chromosome 19. *Hum. Genet.* **69:** 345-349.
13. Jackson, C. L., G. A. P. Bruns, and J. L. Breslow. 1984. Isolation and sequence of a human apolipoprotein CII cDNA clone and its use to isolate and map to human chromosome 19 the gene for apolipoprotein CII. *Proc. Natl. Acad. Sci. USA.* **81:** 2945-2949.
14. Francke, U., M. S. Brown, and J. L. Goldstein. 1984. Assignment of the human gene for the low density lipoprotein receptor to chromosome 19: synteny of a receptor, a ligand and a genetic disease. *Proc. Natl. Acad. Sci. USA.* **81:** 2826-2830.
15. Mahley, R. W., D. Y. Hui, T. L. Innerarity, and K. H. Weisgraber. 1981. Two independent lipoprotein receptors on hepatic membranes of dog, swine and man. Apo B, E and apo E receptors. J. Clin. Invest. **68:** 1197-1206.
16. Utermann, G., M. Jaeschke, and J. Menzel. 1975. Familial hyperlipoproteinemia type III: deficiency of a specific apolipoprotein (apoE-III) in the very low density lipoprotein. *FEBS Lett.* **56:** 352-355.
17. Zannis, V. I., and J. L. Breslow. 1981. Human very low density lipoprotein apolipoprotein E isoprotein polymorphism is explained by genetic variation and post-translational modification. *Biochemistry.* **20:** 1033-1041.
18. Zannis, V. I., J. L. Breslow, G. Utermann, R. W. Mahley, K. H. Weisgraber, R. J. Havel, J. L. Goldstein, M. S. Brown, G. Schonfeld, W. R. Hazzard, and C. Blum. 1982. Proposed nomenclature of apoE isoproteins, apo E genotypes and phenotypes. *J. Lipid Res.* **23:** 911-914.
19. Utermann, G., A. Steinmetz, and W. Weber. 1982. Genetic control of human apolipoprotein E polymorphism: comparison of one- and two-dimensional techniques of isoprotein analysis. *Hum. Genet.* **60:** 344-351.
20. Basu, S. K., Y. K. Ho, M. S. Brown, D. W. Bilheimer, R. G. W. Anderson, and J. L. Goldstein. 1982. Biochemical and genetic studies of the apoprotein E secreted by mouse macrophages and human monocytes. *J. Biol. Chem.* **257:** 9788-9795.
21. Mahley, R. W. 1982. Atherogenic hyperlipoproteinemia – the cellular and molecular biology of plasma lipoproteins altered by dietary fat and cholesterol. *Med. Clin. N. Am.* **66:** 375-402.
22. Basu, S. K., J. L. Goldstein, and M. S. Brown. 1983. Independent pathways for secretion of cholesterol and apolipoprotein E by macrophages. *Science.* **219:** 871-873.
23. Schaefer, E. J., and R. I. Levy. 1985. Pathogenesis and management of lipoprotein disorders. *N. Eng. J. Med.* **312:** 1300-1310.
24. Bouthillier, D., C. F. Sing, and J. Davignon. 1983. Apolipoprotein E phenotyping with a single gel method: application to the study of informative matings. *J. Lipid Res.* **24:** 1060-1069.
25. Gregg, M. E., L. A. Zech, E. J. Schaefer, and H. B. Brewer, Jr. 1981. Type III Hyperlipoproteinemia: defective metabolism of an abnormal apolipoprotein E. *Science.* **211:** 584-586.
26. Ghiselli, G., E. J. Schaefer, P. Gascon, and H. B. Brewer, Jr. 1981. Type III hyperlipoproteinemia associated with apolipoprotein E deficiency. *Science.* **214:** 1239-1241.
27. Schaefer, E. J., R. E. Gregg, G. Ghiselli, T. M. Forte, J. M. Ordovas, L. A. Zech, and H. B. Brewer, Jr. 1986. Familial apolipoprotein E deficiency. *J. Clin. Invest.* In press.
28. Breslow, J. L., V. I. Zannis, T. R. SanGiacomo, J. L. H. C. Third, T. Tracy, and C. J. Glueck. 1982. Studies of familial type III hyperlipoproteinemia using as a genetic marker the apoE phenotype E2/2. *J. Lipid Res.* **23:** 1224-1235.
29. Wardell, M. R., P. A. Suckling, and E. D. Janus. 1982. Genetic variation in human apolipoprotein E. *J. Lipid Res.* **23:** 1174-1182.
30. Menzel, H. J., R. G. Kladetsky, and G. Assmann. 1983. Apolipoprotein E polymorphism and coronary artery disease. *Arteriosclerosis.* **3:** 310-322.
31. Ghiselli, G., R. E. Gregg, L. A. Zech, E. J. Schaefer, and H. B. Brewer, Jr. 1982. Phenotype study of apolipoprotein E isoforms in hyperlipoproteinemic patients. *Lancet.* **2:** 405-407.
32. Cummings, A. M., and F. W. Robertson. 1984. Polymorphism of the apoprotein E locus in relation to risk of coronary disease. *Clin. Genet.* **25:** 310-313.
33. Weisgraber, K. H., S. C. Rall, and R. W. Mahley. 1981. Human E apoprotein heterogeneity. Cysteine-arginine interchanges in the amino acid sequence of the apoE isoforms. *J. Biol. Chem.* **256:** 9077-9083.
34. Weisgraber, K. H., T. L. Innerarity, and R. W. Mahley. 1982. Abnormal lipoprotein receptor-binding activity of the human apoprotein due to cysteine-arginine interchange at a single site. *J. Biol. Chem.* **257:** 2518-2521.
35. Rall, S. C., Jr., K. H. Weisgraber, T. L. Innerarity, and R. W. Mahley. 1982. Structural basis for receptor binding heterogeneity of apolipoprotein E from type III hyperlipoproteinemic subjects. *Proc. Natl. Acad. Sci. USA.* **79:** 4696-4700.
36. Gregg, R. E., G. Ghiselli, and H. B. Brewer, Jr. 1983. Apolipoprotein E Bethesda: a new variant of apolipoprotein E associated with type III hyperlipoproteinemia. *J. Clin. Endocrinol. Metab.* **57:** 969-974.
37. Weisgraber, K. H., S. C. Rall, Jr., T. L. Innerarity, R. W. Mahley, T. Kuusi, and C. Ehnholm. 1984. A novel electrophoretic variant of human apolipoprotein identification and characterization of apolipoprotein E1. *J. Clin. Invest.* **73:** 1024-1033.

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38. Yamamura, T., A. Yamamoto, K. Hiramori, and S. Nambu. 1984. A new isoform of apolipoprotein E—apoE-5—associated with hyperlipidemia and atherosclerosis. *Atherosclerosis.* **50:** 159-172.
39. Yamamura, T., A. Yamamoto, T. Sumiyoshi, K. Hiramori, Y. Nishioeda, and S. Nambu. 1984. New mutants of apolipoprotein E associated with atherosclerotic diseases but not to type III hyperlipoproteinemia. *J. Clin. Invest.* **74:** 1229-1237.
40. Ghiselli, G., E. J. Schaefer, L. A. Zech, L. E. Gregg, and H. B. Brewer, Jr. 1982. Increased prevalence of apolipoprotein E4 in type V hyperlipoproteinemia. *J. Clin. Invest.* **70:** 474-477.
41. Utermann, G., I. Kindermann, H. Kaffarnik, and A. Steinmetz. 1984. Apolipoprotein E phenotypes and hyperlipidemia. *Hum. Genet.* **65:** 232-236.
42. Assmann, G., G. Schmitz, H. J. Menzel, and H. Schulte. 1984. Apolipoprotein E polymorphism and hyperlipidemia. *Clin. Chem.* **30:** 641-643.
43. Sing, C. F., and Davignon. 1985. Role of apolipoprotein E polymorphism in determining normal plasma lipid and lipoprotein variation. *Am. J. Hum. Genet.* **37:** 268-285.
44. Gregg, R. E., L. A. Zech, E. J. Schaefer, D. Stark, D. Wilson, and H. B. Brewer, Jr. 1986. Abnormal in vivo metabolism of apolipoprotein E4 in humans. *J. Clin. Invest.* **78:** 815-821.
45. Warnick, G. R., J. Benderson, and J. J. Albers. 1982. Dextran sulfate-Mg^{2+} precipitation procedure for quantitation of high density lipoprotein cholesterol. *Clin. Chem.* **28:** 1379-1388.
46. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.
47. Marcel, Y. L., M. Bergseth, and A. C. Nestruck. 1979. Preparative isoelectric focusing of apolipoproteins C and E from human very low density lipoproteins. *Biochim. Biophys. Acta.* **573:** 175-183.
48. O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell.* **12:** 1133-1142.
49. Sano, R., Ryuzo, O. Shin-Ichi, F. Yutaka, and T. Takayoshi. 1985. Apolipoprotein E-2/E-3 ratio of very low density lipoprotein in diabetes mellitus. *Tohoku J. Exp. Med.* **146:** 131-136.
50. Holdsworth, G., J. Stocks, P. Dodson, and D. J. Galton. 1982. An abnormal triglyceride-rich lipoprotein containing excess sialylated apolipoprotein C-III. *J. Clin. Invest.* **69:** 932-939.
51. Kashyap, M. L., L. S. Srivastava, B. A. Hynd, P. S. Gartside, and G. Perisutti. 1981. Quantitation of human apolipoprotein C-III and its subspecies by radioimmunoassay and analytical isoelectric focusing: abnormal plasma triglyceride-rich lipoprotein apolipoprotein C-III subspecies concentrations in hypertriglyceridemia. *J. Lipid Res.* **22:** 800-810.
52. Erkelens, D. W., and J. A. J. Mocking. 1982. The CII/CIII ratio of transferable apolipoprotein in primary and secondary hypertriglyceridemia. *Clin. Chim. Acta.* **121:** 59-65.
53. Zannis, V. I., J. M. Ordovas, C. Cladaras, F. S. Cole, G. Forbes, and E. J. Schaefer. 1985. mRNA and apolipoprotein E synthesis abnormalities in peripheral blood monocyte macrophages in familial apolipoprotein E deficiency. *J. Biol. Chem.* **260:** 12891-12894.

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