

## notes on methodology

### Phenotyping of human apolipoprotein E from whole blood plasma by immunoblotting

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**Summary** Human apolipoprotein E (apoE) exists in the population in three common genetically determined isoforms, apoE-2, E-3, and E-4, that are coded for by three alleles  $\epsilon$ -2,  $\epsilon$ -3 and  $\epsilon$ -4 at the apoE structural gene locus resulting in six phenotypes, three homozygotes (E 2/2, E 3/3, and E 4/4) and three heterozygotes (E 2/3, E 2/4, and E 3/4). A new procedure is described that allows identification of apoE isoforms and phenotypes from whole plasma or serum without the need for isolating apoE-containing lipoproteins or two-dimensional gel electrophoresis of serum. This rapid method combines cysteamine treatment of apoE in plasma, separation in parallel of cysteamine-treated and untreated hydrophobic serum proteins by charge-shift electrophoresis, and isoelectric focusing of apolipoproteins with immunoblotting. Compared to phenotyping of apoE after isolation of VLDL, the new procedure agreed in most cases and may be of special value in detecting apoE mutants that differ in their cysteine residues or either are spun off during isolation of lipoproteins or cofocus with other apoproteins and thus escape detection by conventional one-dimensional techniques. The method provides a simple tool to screen apoE isoforms that are known to have a major impact on individual plasma cholesterol levels. — Steinmetz, A. Phenotyping of human apolipoprotein E from whole blood plasma by immunoblotting. *J. Lipid Res.* 1987. 28: 1364–1370.

**Supplementary key words** genetic polymorphism • cholesterol metabolism

Utermann and coworkers (1–3) first described the genetically determined polymorphism of human apolipoprotein E and the connection of specific apoE phenotype with dysbetalipoproteinemia and hyperlipoproteinemia type III. Zannis and colleagues (4, 5) showed that different apoE isoforms were the result mainly of three alleles occurring at a single gene locus. Structural work on apoE isoforms revealed that apoE-2, E-3, and E-4 isoforms differ in their cysteine-arginine content and apoE-2 and E-4 are thought to be derived from the “wild type” E-3 (112 Cys, 158 Arg) by interchanges at different positions: E-2 (Arg<sub>158</sub>→Cys) and E-4 (Cys<sub>112</sub>→Arg) (6).

According to a proposed nomenclature, the model of apoE inheritance provides three alleles  $\epsilon$ -2,  $\epsilon$ -3,  $\epsilon$ -4 coding for three apoE peptides E-2, E-3, and E-4, and six apoE phenotypes (7). Each apoE isoform additionally occurs in plasma with different degrees of sialylation resulting in more acidic higher molecular weight proteins (4).

Recently, additional rare mutants were described that focus in the E-2 position: E-2 (Arg<sub>145</sub>→Cys) and E-2 (Lys<sub>146</sub>→Gln) or in the E-1 position that is usually only occupied by sialylated derivatives of apoE-2, E-3, and E-4: E-1 (Gly<sub>127</sub>→Asp, Arg<sub>158</sub>→Cys) and apolipoprotein E-Bethesda (8–11). Additionally, a functionally inactive variant focusing in the E-3-position was described by Havekes and coworkers (12) who showed the association of this variant with type III hyperlipoproteinemia. Further mutants of apoE focusing in the E-5-position (13) or E-7-position (14) were detected in Japan. Accordingly, the number of known alleles at the structural apoE gene locus increases with every new mutant characterized.

Several lines of evidence suggest a major role for apolipoprotein E in cholesterol metabolism (for review, see 15). The  $\epsilon$ -2 allele is associated with low plasma- and LDL-cholesterol levels, dysbetalipoproteinemia and hyperlipoproteinemia type III (1, 2, 16, 17; for review, see 18–20). Also, the common apoE-2 isoform and most of the additionally described rare mutants of apoE exhibit defective binding to the LDL-(apoB, E)-receptor (8–10, 21, 22). A high frequency of the E 4/4 phenotype was reported among patients with type V-hyperlipoproteinemia (23) but these reports were not confirmed by others (24). There are, however, quite a few studies suggesting the association of the  $\epsilon$ -4 allele with high plasma- and LDL-cholesterol levels (19, 20, 25, 26). Thus knowledge of individuals' apoE pheno-(geno-)types may be important to judge the risk of developing premature arteriosclerosis.

Determination of apolipoprotein E phenotypes has required the isolation of apoE-containing lipoproteins usually by ultracentrifugation (for review, see 27) or by precipitation (2). Later, two-dimensional gel electrophoresis was introduced to identify apoE patterns from serum (28).

The present report describes a rapid procedure for determination of apoE phenotypes from whole serum or plasma and detection of isoelectric apoE-peptide variants.

### MATERIAL AND METHODS

Plasma or serum samples were drawn from an antecubital vein from normolipemic subjects or from patients with various forms of hyperlipoproteinemia seen at the lipid clinic of the University of Marburg.

Throughout the report, serum or plasma is used synonymously as both could be used in the experiments.

CTAB, Triton X-100, SDS, N, N, N', N'-tetramethylethylenediamine, N, N'-methylenebisacrylamide, and urea were obtained from Serva, Heidelberg, F. R. G., carrier ampholytes were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Acrylamide, four times recrystallized, was obtained from Roth KG, Karlsruhe, F. R. G., Nitrocellulose sheets (0.1  $\mu\text{m}$ ) were purchased from Schleicher and Schuell, Dassel, F. R. G. Goat anti-rabbit IgG (peroxidase-conjugated) antibody, 4-chloro-1-naphthol, and  $\beta$ -mercaptoethylamine (cysteamine) were obtained from Sigma Chemie, Taufkirchen, F. R. G.

Very low density lipoproteins were isolated by ultracentrifugation according to standard techniques (29), delipidated in ethanol-ether 3:1(v/v) at  $-20^{\circ}\text{C}$ , finally extracted with cold ether, and dissolved in 10 mM Tris, pH 8, 8M urea for further analysis.

### Production of anti-human apolipoprotein E antiserum in rabbits

Apolipoprotein E was isolated from very low density lipoproteins of subjects homozygous for E-2, E-3, or E-4 by preparative SDS polyacrylamide gel electrophoresis as described before (30). ApoE-containing fractions were extensively dialyzed against 0.02 M ethylmorpholine, pH 8.6, and lyophilized. Aliquots of apoE of each of the isoforms were subjected to isoelectric focusing in rod gels (31). After electrophoresis, one gel was stained to visualize the apoE positions and apoE isoforms were cut from the additional gels. The gel slices containing different apoE isoforms were ground into fine pieces with a mortar and pestle, mixed with equal volumes of distilled water and complete Freund's adjuvant, and emulsified by forcing the mixture several times through a 25-gauge needle. The emulsified gels (containing all three isoforms) were injected subcutaneously in rabbits in the upper back and neck and intramuscularly into the thigh. The rabbits were given booster injections 7, 14, and 30 days after the initial immunization with apoE isoforms emulsified with complete Freund's adjuvant. Rabbits were bled from the ear, blood was allowed to clot at  $4^{\circ}\text{C}$  overnight, and antiserum was obtained by low speed centrifugation.

### Cysteamine treatment

Cysteamine treatment of plasma or serum samples was carried out modifying the procedure of Weisgraber, Rall, and Mahley (6). Specifically, 20  $\mu\text{l}$  of plasma or serum was mixed with an equal volume of a 0.2 M Tris buffer, pH 8.0, containing 25 mg/ml of cysteamine and incubated overnight at room temperature. Thereafter, the entire mixture was used to perform charge-shift electrophoresis as described below for untreated plasma, with the exception that DTT was omitted in the charge-shift incubation buffer.

### Electrophoretic procedure

Twenty-microliter aliquots of plasma or serum or 40  $\mu\text{l}$  of the cysteamine-treated mixture (see above) were incubated for 1 hr at  $4^{\circ}\text{C}$  in the presence of excess CTAB, Triton X-100, and 25 mM DTT for the plasma or serum samples, and with CTAB and Triton X-100 but without DTT in the case of the cysteamine-treated mixture. The samples were then electrophoresed into detergent-containing agarose gels, by a modification of the procedure described for membrane proteins (32, 33) as outlined in detail for group A apolipoproteins (34). After agarose gel electrophoresis, isoelectric focusing was performed essentially as outlined before (34), modifying the pH gradient by using an ampholyte mixture (1/3 pH 5-7, 2/3 pH 4-6.5).

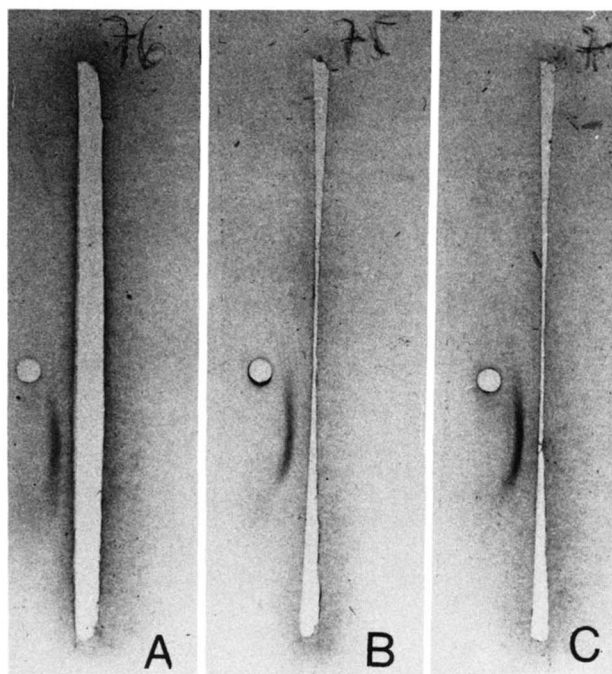
After isofocusing, proteins were electrophoretically blotted onto nitrocellulose sheets in a Transblott apparatus (LKB, Bromma, Sweden) essentially as previously outlined (35) with some modification. Blotting was carried out for a total of 2 hr, 0.5 hr at 250 mA and then 1.5 hr at 400 mA at  $+4^{\circ}\text{C}$ . Blotting buffer consisted of 25 mM Tris, 192 mM glycine containing 20% (v/v) methanol. A reference strip of the nitrocellulose sheet was stained with Amido Black (0.1% Amido Black in 45 parts of methanol, 10 parts of acetic acid, and 45 parts of water) and destained in 10% acetic acid.

The sample strips (10 cm  $\times$  0.9 cm) were first soaked for 30 min at room temperature in 50 mM Tris buffer, pH 8.0, containing 90 mM NaCl and 50 mg/ml BSA (buffer A) to saturate nonspecific binding sites. Thereafter sheets were incubated and gently agitated for 1 hr at room temperature in buffer A containing 1  $\mu\text{l}/\text{ml}$  of rabbit anti-human apoE antibody. The sheets were then washed (with three changes) in 50 mM Tris, pH 8, containing 90 mM NaCl (buffer B). Incubation was then continued in buffer A containing 10  $\mu\text{l}/\text{ml}$  of goat anti-rabbit IgG (peroxidase-conjugated) for 90 min at room temperature under gentle agitation and sheets were again washed with three changes of buffer B. ApoE bands were then visualized at  $37^{\circ}\text{C}$  in 50 mM Tris buffer, pH 6.4, containing 90 mM NaCl, 0.5  $\mu\text{l}/\text{ml}$  of  $\text{H}_2\text{O}_2$ , and 10  $\mu\text{l}/\text{ml}$  of 1.8% 4-chloro-1-naphthol dissolved in ethanol. Strips were dried and kept dark for proper documentation. Two-dimensional SDS-electrophoresis was carried out in 10%-22.5% polyacrylamide linear gradient slab gels as outlined earlier (34). After the run, apoproteins were blotted onto nitrocellulose sheets and apoE was visualized as described above for IEF gels.

Scanning densitometry was carried out in an Elscript 400 scanning densitometer (Fa. Hirschmann, München, West Germany). Coomassie blue-stained IEF gels were scanned at 546 nm, transmitted light; nitrocellulose sheets were read at 366 nm, reflected light.

## RESULTS

All major apoE isoforms undergo a charge shift in a very similar way, moving towards the cathode in the presence of the nonionic detergent Triton X-100 and the cationic detergent CTAB. All detectable apoE from whole serum underwent the cathodic shift (**Fig. 1**). Complete shift of the apoE peptides could be demonstrated also in grossly lipemic samples where triglycerides exceeded 1000 mg/dl and cholesterol exceeded 500 mg/dl (data not shown). By transferring serum apoprotein-containing agarose gel pieces after charge-shift electrophoresis onto isofocusing gels, apoproteins are separated according to their isoelectric point as shown earlier for group A apoproteins (34). Group A apoproteins are in relatively high concentration in plasma (36, 37) and can therefore be visualized by Coomassie staining (34). ApoE isoforms were not detectable by conventional staining techniques (34) probably because of low plasma levels (38, 39). Furthermore, isofocusing positions of apoE-peptides interfere with pro-apoA-I (data not shown). After transfer of isofocused apoproteins to nitrocellulose paper, apoE could be specifically recognized by apoE antibodies and visualized by peroxidase-conjugated second antibodies in the pres-



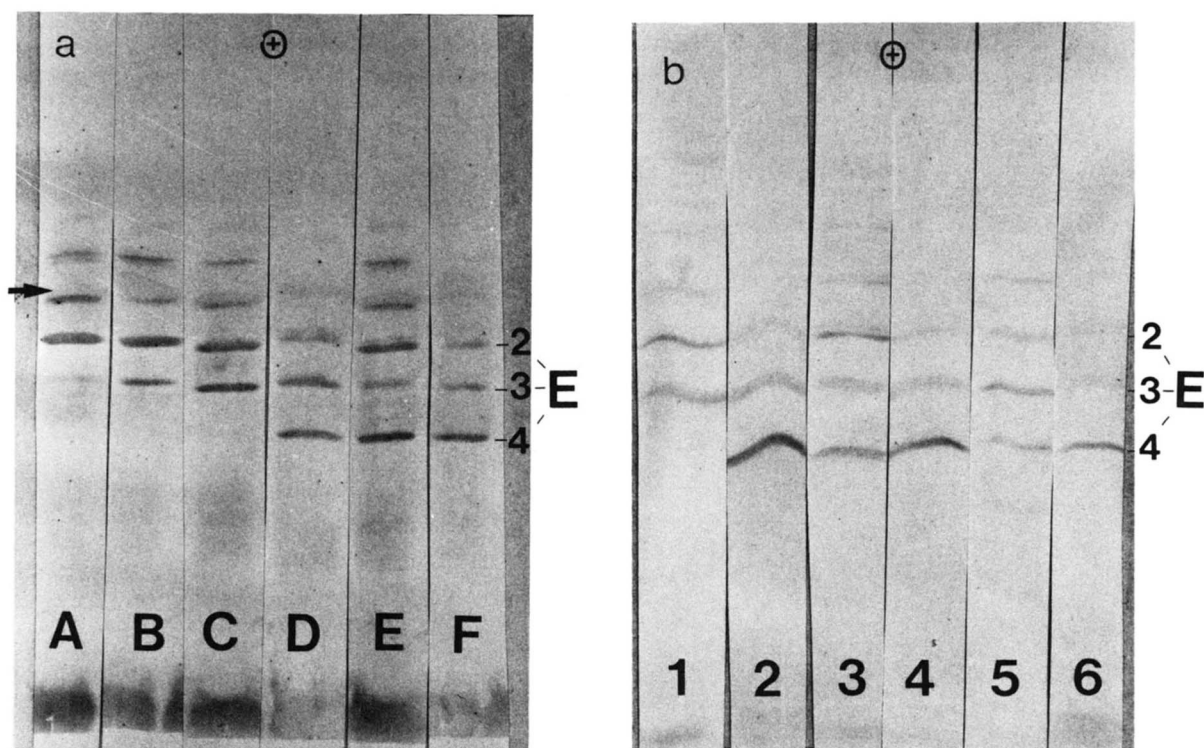
**Fig. 1.** Agarose gel immunoelectrophoresis in Triton X-100 and CTAB of whole serum preincubated with Triton X-100 and CTAB. Troughs contain monospecific antiserum against apoE. Sera from subjects exhibiting apoE-2 (lane A), apoE-4 (lane B), and apoE-3 (lane C) were used. Note that all three isoforms undergo similar charge shift in the detergent mixture. Anode is at the top.

ence of 4-chloro-1-naphthol. **Fig. 2a** shows the results of apoE phenotyping obtained by this procedure. Separation of apoproteins from the bulk of hydrophilic serum proteins by charge-shift electrophoresis did not alter the behavior of apoE isopeptides upon isofocusing as compared to delipidated apoE from VLDL. Isopeptides isolated by ultracentrifugation and by charge-shift electrophoresis exhibited the same isoelectric points.

Phenotyping of apoE from plasma by immunoblotting was compared to a conventional technique via isolation of VLDL by ultracentrifugation. Eighty-three samples (7 E 2/2, 19 E 2/3, 34 E 3/3, 13 E 3/4, 5 E 2/4, and 5 E 4/4), some of them with previously identified patterns from family studies, were analyzed in parallel. In general, the apoE pattern from whole plasma showed a relative enrichment of sialylated apoE peptides as compared with IEF of isolated VLDL. The reasons for the relative enrichment of the plasma or depletion of the VLDL pattern of sialylated apoE peptides is not obvious. It could be due to a preferential loss of the sialylated peptides by ultracentrifugation or an accumulation of hinger sialylated apoE peptides in other than VLDL lipoprotein fractions. To clearly discriminate between homozygous and heterozygous patterns, each plasma sample was run in duplicate with and without cysteamine treatment. As cysteamine treatment affects the mobility of apoE according to the number of cysteine residues, all unsialylated apoE isoforms move to the E-4 position. Thus the ratio of successive bands to one another would not change for homozygous but would for heterozygous patterns. **Fig. 2b** shows the changes in mobility and ratio of three heterozygous patterns. These changes could be used to discriminate between homozygous and heterozygous samples.

When the phenotypes thus obtained were compared to IEF of isolated apoVLDL of the 83 samples, only 3 phenotypes disagreed. One case turned out to be a false E 3/4 in VLDL caused by apoSAA (serum amyloid A); the real pattern was E 3/3 as found by immunoblotting. The other 2 discrepant samples were subjected to two-dimensional electrophoresis. In both cases the patterns suggested by blotting could be confirmed, apoE 3/3 and E 3/4 rather than E 2/3 and E 4/4 as found by apoVLDL focusing. Thus analysis of apoE phenotypes by immunoblotting in parallel samples with and without cysteamine treatment is a reliable technique to phenotype apoE patterns.

The new procedure of focusing apoE from plasma also allows two-dimensional analysis of the apoE phenotypes. Therefore a strip of the IEF gel was subjected to SDS gel electrophoresis and immunoblotting of the second gel was carried out. Examples of this technique are shown in **Fig. 3**.



**Fig. 2.** Phenotyping of apoE from whole serum by immunoblotting. Apoproteins were separated from the bulk of serum-proteins by charge-shift electrophoresis and blotted onto nitrocellulose sheets after isofocusing. ApoE bands were visualized with peroxidase-conjugated antibodies to rabbit (anti-human apoE) immunoglobulins. a: ApoE phenotypes E-2/2 (lane A), E-2/3 (lane B), E-3/3 (lane C), E-3/4 (lane D), E-2/4 (lane E), and E-4/4 (lane F). Arrow indicates focusing position of the major apoA-I plasma isoform. b: Cysteamine treatment of apoE to identify apoE phenotypes. Sera from apoE-2/3, E-2/4, and E-3/4 phenotypes in lanes 1, 3, and 5, respectively, were processed as outlined in Fig. 2a. Lanes 2, 4, and 6 show the corresponding samples immunoblotted after cysteamine treatment. Note that almost all apoE peptides move to the E-4 position changing the ratio of neighboring hands in the heterozygous samples that are shown.

## DISCUSSION

The new procedure outlined in this report describes the determination of apoE phenotypes from whole serum or plasma without the need to first isolate apoE-containing lipoproteins or run two-dimensional gels in most cases. The procedure is based on the ability of hydrophobic apolipoproteins to bind large amounts of nonionic detergent and undergo a "charge-shift" following the addition of ionic detergent and electrophoresis in the presence of the detergent mixture (40). The first two steps of the procedure are fundamentally similar to the method described earlier in our laboratory to screen for genetic variants of group A apolipoproteins (34). In addition, the cysteamine modification was introduced and samples were run in parallel to clearly discriminate between homozygous and heterozygous patterns. The additional step, to transfer apoproteins to nitrocellulose paper after isoelectric focusing and identify by specific antibodies, became necessary in the case of apoE for several reasons. ApoE occurs in plasma in much lower concentrations than all A-

apolipoproteins and exhibits, in addition, several isoforms, and thus is poorly stained with Coomassie blue. Application of a more sensitive protein staining procedure did not seem feasible as pro-apoA-I, which cofocuses in the apoE region, is picked up by our procedure (data not shown) and would interfere with apoE phenotype determination. Furthermore, during defined disease states, Stuyt et al. (41) demonstrated the appearance of additional proteins in very low density lipoproteins cofocusing in the apoE-4 position. Also, in patients exhibiting serum amyloid A(SAA), one of the serum forms of this acute phase reactant can confuse apoE phenotype determination (Steinmetz, A., unpublished observation). In addition, apoE charge variants cofocusing with other apoproteins would escape detection in the first dimension if not immunolocalized.

Avoiding ultracentrifugation and two-dimensional electrophoresis to determine apoE phenotypes seems to be a big advantage of this new rapid procedure and may help to avoid artifacts. It is known from several studies that apoE dissociates from lipoprotein particles upon stress

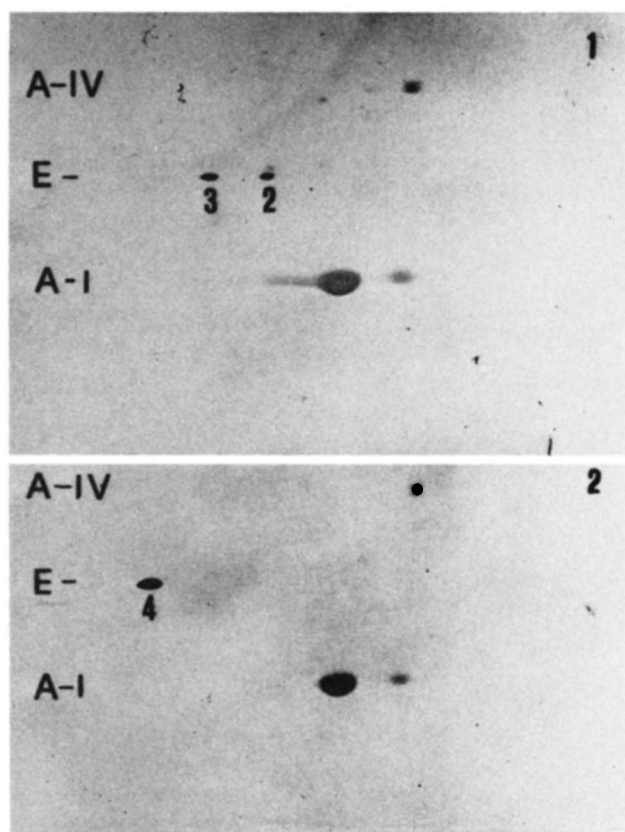


Fig. 3. Phenotyping of apoE from whole serum by immunoblotting after two-dimensional SDS-gel electrophoresis. Following charge-shift electrophoresis and isofocusing, apoproteins were subjected to two-dimensional SDS-gel electrophoresis in 10–22.5% acrylamide gels and then blotted onto nitrocellulose sheets. ApoE spots were visualized as outlined in the Methods section. For reference, trace amounts of anti-A-I and anti-A-IV antibody were added to localize the apoE isopeptide positions. Only the area of the gel used to identify apoE is shown. Upper gel shows an apoE-2/3-, lower gel and apoE-4/4-phenotype.

forces induced by ultracentrifugation and hence only apoE tightly bound to lipoprotein is characterized after isolation (38, 39, 42). Furthermore, mainly very low and intermediate density lipoproteins have been isolated and used to characterize apoE phenotypes, whereas in normal individuals the bulk of apoE is located within the high density lipoprotein region (38). Theoretically one could think of mutations of apoE affecting lipid binding of the apoprotein in a way that all of the mutant or the normal apoprotein could come off during centrifugation, or that one form would displace the other from the particle. These mutants would be likely to escape detection after ultracentrifugation.

The method outlined in this report presents a feasible tool for large scale determination of apoE phenotypes in serum or plasma and provides good access for questions associated with apoE phenotypes with respect to cholesterol metabolism. It is known, for example, that apoE-2 has a profound hypocholesterolemic effect (16, 19, 20) and causes dysbetalipoproteinemia in the homozy-

gous state (2). The lower incidence of the  $\epsilon$ -2 allele in a random sample of survivors of myocardial infarction (43) and in a population with angiographically proven absence of coronary artery disease (44) suggests a protective influence of the E-2 peptide.

Furthermore, apoE-4 was found more frequently among patients with hypercholesterolemia (25, 26) and, in addition, E-4 led to a statistically significant increase of LDL-cholesterol in women (19). Sing and Davignon (20) concluded from their studies and from analysis of data from the literature that the  $\epsilon$ -4 allele raised total cholesterol, LDL-cholesterol, and LDL-apoB levels.

With respect to coronary artery disease, there are still conflicting data in terms of apoE-4. It was found more frequently than expected among survivors of a myocardial infarction (43) but also a lower incidence of E-3/4 in a similar population was reported (45). Thus larger population studies are needed to establish the apoE phenotype as predictor of individuals' risk for the development of coronary artery disease. The method described here could be helpful in determining large scale apoE phenotypes. ■

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## REFERENCES

1. Utermann, G., M. Jaeschke, and H. J. Menzel. 1975. Familial hyperlipoproteinemia type III: deficiency of a specific apolipoprotein (apo E-III) in the very low density lipoproteins. *FEBS. Lett.* **56**: 352–355.
2. Utermann, G., M. Hees, and A. Steinmetz. 1977. Polymorphism of apolipoprotein E and occurrence of dysbetalipoproteinemia in man. *Nature (London)*. **269**: 604–607.
3. Utermann, G., U. Langenbeck, U. Beisiegel, and W. Weber. 1980. Genetics of the apolipoprotein E system in man. *Am. J. Hum. Genet.* **32**: 339–347.
4. Zannis, V. I., and J. L. Breslow. 1981. Human VLDL apolipoprotein E isoprotein polymorphism is explained by genetic variation and post-translational modification. *Biochemistry*. **20**: 1033–1041.
5. Zannis, V. I., P. W. Just, and J. L. Breslow. 1981. Human apolipoprotein E isoprotein subclasses are genetically determined. *Am. J. Hum. Genet.* **33**: 11–24.
6. Weisgraber, K. H., S. C. Rall, Jr., and R. W. Mahley. 1981. Human E apoprotein heterogeneity: cysteine-arginine interchanges in the amino acid sequence of apoE isoforms. *J. Biol. Chem.* **256**: 9077–9083.
7. 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. B. Blum.

1982. Proposed nomenclature of apoE isoproteins, apoE genotypes and phenotypes. *J. Lipid Res.* **23**: 911-914.
8. 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.
9. Rall, S. C., Jr., K. H. Weisgraber, T. L. Innerarity, T. P. Bersot, R. W. Mahley, and C. B. Blum. 1983. Identification of a new structural variant of human apolipoprotein E, E2 (Lys<sub>146</sub>-Gln), in a type III hyperlipoproteinemic subject with the E 3/2 phenotype. *J. Clin. Invest.* **72**: 1288-1297.
10. 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 E: identification and characterization of apolipoprotein E-1. *J. Clin. Invest.* **73**: 1024-1033.
11. Gregg, R. W., 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.
12. Havekes, L. M., J. A. Gevers-Leuven, E. van Corven, E. de Wit, and J. J. Emeis. 1984. Functionally inactive apolipoprotein E in a type III hyperlipoproteinaemic patient. *Eur. J. Clin. Invest.* **14**: 7-11.
13. Yamamura, T., A. Yamamoto, K. Hiramori, and S. Nambu. 1984. A new isoform of apolipoprotein E—apo E-5—associated with hyperlipidemia and atherosclerosis. *Atherosclerosis.* **50**: 159-172.
14. 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.
15. Mahley, R. W. 1983. Apolipoprotein E and cholesterol metabolism. *Klin. Wochenschr.* **61**: 225-232.
16. Utermann, G., N. Pruin, and A. Steinmetz. 1979. Polymorphism of apolipoprotein E. III. Effect of a single polymorphic gene locus on plasma lipid levels in man. *Clin. Genet.* **15**: 63-72.
17. Zannis, V. I., and J. L. Breslow. 1980. Characterization of a unique human apolipoprotein E variant associated with type III hyperlipoproteinemia. *J. Biol. Chem.* **255**: 1759-1762.
18. Mahley, R. W., and B. Angelin. 1984. Type III hyperlipoproteinemia: recent insights into the genetic defect of familial dysbetalipoproteinemia. *Adv. Int. Med.* **29**: 385-411.
19. Robertson, F. W., and A. M. Cumming. 1985. Effects of apoprotein E polymorphism on serum lipoprotein concentration. *Arteriosclerosis.* **5**: 283-292.
20. Sing, C. F., and J. Davignon. 1985. Role of the apolipoprotein E polymorphism in determining normal plasma lipid and lipoprotein variation. *Am. J. Hum. Genet.* **37**: 268-285.
21. Schneider, W. J., P. T. Kovanen, M. S. Brown, J. L. Goldstein, G. Utermann, W. Weber, R. J. Havel, L. Kotite, J. P. Kane, T. L. Innerarity, and R. W. Mahley. 1981. Familial dysbetalipoproteinemia: abnormal binding of mutant apoprotein E to low density lipoprotein receptors of human fibroblasts and membranes from liver and adrenal of rats, rabbits and cows. *J. Clin. Invest.* **68**: 1075-1085.
22. Weisgraber, K. H., T. L. Innerarity, and R. W. Mahley. 1982. Abnormal lipoprotein receptor-binding activity of human E apoprotein due to cysteine-arginine interchange at a single site. *J. Biol. Chem.* **257**: 2518-2521.
23. Ghiselli, G., E. J. Schaefer, L. A. Zech, R. E. Gregg, and H. B. Brewer, Jr. 1982. Increased prevalence of apolipoprotein E-4 in type V hyperlipoproteinemia. *J. Clin. Invest.* **70**: 474-477.
24. Stuyt, P. H. J., A. F. H. Stalenhoef, P. N. M. Demacker, and A. Van 't Laar. 1982. Hyperlipoproteinemia Type V and apolipoprotein E-4. *Lancet.* **2**: 934.
25. Utermann, G., I. Kindermann, H. Kaffarnik, and A. Steinmetz. 1984. Apolipoprotein E phenotypes and hyperlipidemia. *Hum. Genet.* **65**: 232-236.
26. Assmann, G., G. Schmitz, H. J. Menzel, and H. Schulte. 1984. Apolipoprotein E polymorphism and hyperlipidemia. *Clin. Chem.* **30**: 641-643.
27. Zannis, V. I., and J. L. Breslow. 1982. Apolipoprotein E. *Mol. Cell. Biochem.* **42**: 3-20.
28. Borresen, A. L., and K. Berg. 1981. The apoE polymorphism studied by two-dimensional, high resolution gel electrophoresis of serum. *Clin. Genet.* **20**: 438-448.
29. Havel, R. J., H. A. Eder, and J. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34**: 1345-1353.
30. Utermann, G. 1975. Isolation and partial characterization of an arginine-rich apolipoprotein from human very-low-density lipoproteins: apolipoprotein E. Hoppe-Seyler's Z. *Physiol. Chem.* **356**: 1113-1121.
31. Warnick, G. R., C. Mayfield, J. J. Albers, and W. R. Hazard. 1979. Gel isoelectric focusing method for specific diagnosis of familial hyperlipoproteinemia type III. *Clin. Chem.* **25**: 279-284.
32. Helenius, A., and K. Simons. 1977. Charge shift electrophoresis: simple method for distinguishing between amphiphilic and hydrophilic proteins in detergent solution. *Proc. Natl. Acad. Sci. USA.* **74**: 529-532.
33. Bhakdi, S., B. Bhakdi-Lehnen, and O. J. Bjerrum. 1977. Detection of amphiphilic proteins and peptides in complex mixtures. Charge-shift crossed immunoelectrophoresis and two-dimensional charge-shift electrophoresis. *Biochim. Biophys. Acta.* **470**: 35-44.
34. Utermann, G., G. Feussner, G. Franceschini, J. Haas, and A. Steinmetz. 1982. Genetic variants of group A apolipoproteins. Rapid methods for screening and characterization without ultracentrifugation. *J. Biol. Chem.* **257**: 501-507.
35. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* **76**: 4350-4354.
36. Cheung, M. C., and J. J. Albers. 1977. The measurement of apolipoprotein A-I and A-II-levels in men and women by immunoassay. *J. Clin. Invest.* **60**: 43-50.
37. Beisiegel, U., and G. Utermann. 1979. An apolipoprotein homolog of rat apolipoprotein A-IV in human plasma. Isolation and partial characterization. *Eur. J. Biochem.* **93**: 601-608.
38. Blum, C. B., L. Aron, and R. Sciocca. 1980. Radioimmunoassay studies of human apolipoprotein E. *J. Clin. Invest.* **66**: 1240-1250.
39. Havel, R. J., L. Kotite, J. L. Vigne, J. P. Kane, P. Tun, N. Philipps, and G. C. Chen. 1980. Radioimmunoassay of human arginine-rich apolipoprotein, apoprotein E. *J. Clin. Invest.* **66**: 1351-1362.
40. Utermann, G., and U. Beisiegel. 1979. Charge shift electrophoresis of apolipoproteins from normal humans and patients with Tangier disease. *FEBS Lett.* **97**: 245-248.
41. Stuyt, P. M. J., P. N. M. Demacker, B. J. Brenninkmeijer, and A. Van 't Laar. 1984. Apoprotein E-4-like isoprotein in

hypertriglyceridemic patients during acute pancreatitis. *Atherosclerosis*. **52**: 353-355.

42. Castro, G. R., and C. J. Fielding. 1984. Evidence for the distribution of apolipoprotein E between lipoprotein classes in human normocholesterolemic plasma and for the origin of unassociated apolipoprotein E (Lp-E). *J. Lipid Res.* **25**: 58-67.
43. Cumming, A. M., and F. W. Robertson. 1984. Polymorphism at the apoprotein E locus in relation to risk of coronary disease. *Clin. Genet.* **25**: 310-313.
44. Menzel, H. J., R. G. Kladetzki, and G. Assmann. 1983. Apolipoprotein E polymorphism and coronary artery disease. *Arteriosclerosis*. **4**: 310-315.
45. Utermann, G., A. Hardewig, and F. Zimmer. 1984. Apolipoprotein E phenotype in patients with myocardial infarction. *Hum. Genet.* **65**: 237-241.