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Review

Clinical chemistry of common apolipoprotein E isoforms

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Abstract

Apolipoprotein E plays a central role in clearance of lipoprotein remnants by serving as a ligand for low-density lipoprotein and apolipoprotein E receptors. Three common alleles (apolipoprotein E_2, E_3 and E_4) give rise to six phenotypes. Apolipoprotein E_3 is the ancestral form. Common apolipoprotein E isoforms derive from nucleotide substitutions in codons 112 and 158. Resulting cysteine–arginine substitutions cause differences in: affinities for low-density lipoprotein and apolipoprotein E receptors, low-density lipoprotein receptor activities, distribution of apolipoprotein E among lipoproteins, low-density lipoprotein formation rate, and cholesterol absorption. Accompanying changes in triglycerides, cholesterol and low-density lipoprotein may promote atherosclerosis development. Over 90% of patients with familial dysbetalipoproteinaemia have apolipoprotein E_2/E_2. Apolipoprotein E_4 may promote atherosclerosis by its low-density lipoprotein raising effect. Establishment of apolipoprotein E isoforms may be important for patients with diabetes mellitus and several non-atherosclerotic diseases. Apolipoprotein E phenotyping exploits differences in isoelectric points. Isoelectric focusing uses gels that contain pH 4–7 ampholytes and urea. Serum is directly applied, or prepurified by delipidation, lipoprotein precipitation or dialysation. Isoelectric focusing is followed by immunofixation/protein staining. Another approach is electro- or diffusion blotting, followed by protein staining or immunological detection with anti-apolipoprotein E antibodies and an enzyme-conjugated second antibody. Apolipoprotein E genotyping demonstrates underlying point mutations. Analyses of polymerase chain reaction products are done by allele-specific oligonucleotide probes, restriction fragment length polymorphism, single-stranded conformational polymorphism, the primer-guided nucleotide incorporation assay, or denaturating gradient gel electrophoresis. Detection with primers that either or not initiate amplification is performed with the amplification refractory mutation system. Disparities between phenotyping and genotyping may derive from isoelectric focusing methods that do not adequately separate apolipoprotein E posttranslational variants, storage artifacts or faint isoelectric focusing bands.

Keywords: Reviews; Apolipoprotein E; Lipoprotein

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List of abbreviations

apo	Apolipoprotein
apo-ϵ	Apolipoprotein E allele
apo-E	Apolipoprotein E protein
ARMS	Amplification refractory mutation system
ASO-probes	Allele-specific oligonucleotide probes
bp	Base pair
CAD	Coronary artery disease
DGGE	Denaturating gradient gel electrophoresis
DTT	Dithiothreitol
FD	Familial dysbetalipoproteinaemia
gly-Hb	Glycated Hb
Hb	Haemoglobin
HbA_{1c}	β-N-Terminal glycated HbA
HDL	High-density lipoproteins
IDDM	Insulin-dependent diabetes mellitus
IEF	Isoelectric focusing
LDL	Low-density lipoproteins
LRP	LDL-receptor-related protein
NIDDM	Non-insulin-dependent diabetes mellitus
PAA	Polyacrylamide
PCR	Polymerase chain reaction
pI	Isoelectric point
RFLP	Restriction fragment length polymorphism
SSCP	Single-stranded conformational polymorphism
VLDL	Very low-density lipoproteins

1. Introduction

Coronary artery disease (CAD) is the leading cause of death in modern Western societies. CAD is mainly on account of coronary atherosclerosis. Atherosclerosis results from synergistic action of a number of risk factors [1,2], including genetic predisposition, age, gender, blood pressure, smoking, dietary habits, stress and physical activity. Enhanced influx and subsequent accumulation of lipoproteins in the intima of arterial walls is considered to be a prerequisite for atherogenesis. It may partially be explained by lipoprotein accumulation in the circulation. Lipid risk factors include increased total cholesterol, low-density lipoprotein (LDL)-cholesterol, apoli-

poprotein-B (apo-B), triglycerides and lipoprotein (a), and decreased lipid soluble antioxidants, high-density lipoprotein (HDL)-cholesterol, apolipoprotein-AI (apo-AI) and LDL size. Obesity, diabetes mellitus, hypothyroidism, renal disease, liver disease, alcohol abuse and certain medications (e.g. oestrogens, corticosteroids) are among causes of secondary hyperlipidaemia. Since the fate of circulating lipoproteins is to a large extent determined by apolipoproteins, many investigations concentrate on their roles in lipoprotein clearance and catabolism.

Polymorphism of the apolipoprotein E (apo-E) gene is one of the many causes of deviant lipoprotein metabolism. We review clinical chemical aspects of common apo-E polymorphism, including the role of apo-E in lipoprotein metabolism, apo-E biochemistry, apo-E pathophysiology, and current methods for the determination of apo-E phenotypes and genotypes.

2. Apolipoprotein E physiology and biochemistry

Apo-E has various functions in lipoprotein metabolism. Knowledge of apo-E protein and apo-E gene structures is essential to the understanding of apo-E function.

2.1. Role of apolipoprotein E in lipoprotein metabolism

Cholesterol is a structural component of membranes and the precursor of bile acids and steroid hormones. Fatty acids serve as a major source of energy, as building blocks of triglycerides, phospholipids and cholesterol esters, and as precursors of eicosanoids. Because of their hydrophobic properties, lipids are transported as lipid–protein complexes, named lipoproteins. Lipoprotein particles consist of a cholesterol ester and triglyceride-rich core and a cholesterol- and phospholipid-rich coat, in which apolipoproteins are embedded. Serum lipoproteins can be subdivided into four major classes, that differ in function, density, size and compositions of cholesterol esters, triglycerides, phospholipids, cholesterol and apolipoproteins. On the basis of density they are named chylomicrons, very low-density lipoproteins (VLDL), LDL and HDL. Chylomicrons derive from enterocytes, VLDL predominantly from liver, and HDL from liver and enterocytes. Partially digested chylomicrons and VLDL are referred to as remnants. LDL is the final product of VLDL catabolism. Apolipoproteins serve not only as structural components of lipoproteins, but also as cofactors for enzymatic reactions and lipid transfer proteins, and as ligands for selective uptake of lipoproteins by tissues. Examples are apo-A I, II and IV (located in chylomicrons and HDL), apo-B_{100} (VLDL, LDL), apo-B_{48} (chylomicrons, chylomicron remnants) apo-C I, II, and III (chylomicrons, VLDL, some HDL subclasses) and apo-E (chylomicrons, chylomicron remnants, VLDL, VLDL remnants, some HDL subclasses).

Dietary cholesterol and fatty acids are taken up by enterocytes. Fatty acids and cholesterol are esterified to triglycerides and cholesterol esters, respectively. Together with phospholipids, cholesterol and apolipoproteins they are assembled to chylomicrons and secreted into the lymph. Once chylomicrons enter the bloodstream, their triglycerides are hydrolysed by lipoprotein lipase, that resides on the surface of capillary endothelial cells. The process allows fatty acid and glycerol uptake by tissues and gives rise to formation of a chylomicron remnant. Excess chylomicron surface material is transferred to HDL, while apo-E is transferred from HDL to the chylomicron remnant. Apo-E-enriched chylomicron remnants are subsequently taken up in the liver by endocytosis. Recognition takes place by the apo-E receptor [3–6], also referred to as the remnant receptor or the LDL-related protein (LRP).

The liver converts free fatty acids (from adipose tissue), carbohydrates and amino acids to triglycerides. Triglycerides, cholesterol, phospholipids, apo-E and other apolipoproteins are assembled to VLDL and secreted. Like chylomicrons, VLDL particles interact with lipoprotein lipase, causing loss of triglycerides, transfer of excess surface to HDL and formation of a VLDL remnant (also named intermediate-density

lipoprotein). HDL has an important role in the transport of cholesterol from the periphery to the liver (also referred to as reverse cholesterol transport). For this, HDL not only receives cholesterol from excess VLDL and chylomicron surface, but also by exchange of cholesterol in lipoproteins and the plasma membranes of cells. Apo-E is assumed to facilitate uptake of cholesterol by HDL and to act as a ligand for direct uptake of apo-E-enriched HDL by the LDL-receptor. Following local esterification of cholesterol in HDL by lecithin–cholesterol acyltransferase, cholesterol esters are returned to VLDL remnants by the cholesterol ester transfer protein. As a consequence, VLDL remnants become cholesterol ester enriched. VLDL remnants can be processed via three pathways: uptake via the LDL (or apo-B/E) receptor, uptake in the liver via the apo-E receptor, or by further metabolism to LDL. The LDL receptor is located on most mammalian cell types and binds both apo-E and apo-B_{100} containing lipoproteins. In contrast, the apo-E receptor is merely located on hepatocytes and binds only apo-E containing lipoproteins. Conversion of VLDL remnants to LDL comprises hydrolysis of remaining triglycerides by hepatic lipase and the release of apo-E [7]. The resulting LDL particle contains cholesterol esters as the major lipid, with apo-B_{100} as the only apolipoprotein on its surface. LDL particles will be taken up via the LDL receptor pathway, which uses apo-B_{100} as ligand. Modification of LDL by oxidation or glycation prevents recognition by the LDL receptor. Influx of modified LDL into arterial wall intima or local LDL oxidation cause recruitment of macrophages. Uptake of modified LDL by macrophages via the scavenger receptor causes their transformation to cholesterol ester rich foam-cells, which is an early event in atherogenesis.

It is clear that apo-E plays a central role in chylomicron and VLDL remnant clearance by serving as a ligand for both the LDL receptor and the apo-E receptor. In addition, multiple interactions of lipoprotein lipase and hepatic lipase with apo-E have been noted. Digestion of triglycerides by lipoprotein lipase enhances apo-E binding to the apo-E receptor [7], probably by increasing apo-E exposure [8–10], whereas apo-E activates hepatic lipase in vitro [11]. Apo-E has also been shown to play a role in nerve regeneration [12–14], immunoregulation [15–17], modulation of intracellular cholesterol utilization and steroidogenesis in adrenal cells [18].

2.2. Apolipoprotein E protein and gene

In 1973 Shore and Shore [19] described an arginine-rich protein as a major component of VLDL. This protein was designated apo-E by Utermann in 1975 [20]. Apo-E is a 34.2 kDa protein, that is mainly synthesized in the liver, but also in spleen, kidneys, adrenals, gonads, macrophages and brain astrocytes. The protein is encoded by a 3.6 kb gene that contains four exons. It is located on the long arm of chromosome 19, closely linked to the apo-CI and apo-CII genes and more distant from the LDL receptor gene [21–23]. Translation of the 1.2 kb apo-E mRNA results in synthesis of a 317 amino acid precursor polypeptide (pre-apo-E). Post-translational modification comprises glycosylation (including sialic acid attachment) and removal of an 18 amino acid signal peptide prior to secretion [24,25]. The receptor binding domain of the mature 299 amino acid apo-E protein lies in the lysyl- and arginyl-rich region between amino acids 130 and 150. Residues 1–183 are necessary for maximal receptor-binding activity [26–29]. Amino acids 216–299 at the carboxy terminus constitute the lipid-binding domain [30,31]. The structure–function relationships of apo-E have recently been reviewed by Weisgraber [32].

The apo-E gene is polymorphic. Three common alleles, denoted apo-ϵ_2, ϵ_3 and ϵ_4, code for 3 major apo-E isoforms, named apo-E_2, E_3 and E_4, respectively [33,34]. Apo-E_3 is considered as the ancestral isoform. The alleles are codominantly inherited, which implies that the six possible genotypes correspond with six phenotypes, denoted apo-E_2/E_2, E_3/E_3, E_4/E_4 (homozygotes) and E_2/E_3, E_2/E_4, E_3/E_4 (heterozygotes). Frequency distributions of apo-E isoforms differ among populations. For instance, apo-ϵ allele frequency (in %) in the Dutch population is: apo-ϵ_2 8.2, apo-ϵ_3 75.1, and apo-ϵ_4 16.8 [35]. The Nigerian [36] and Finnish [37] populations have higher apo-ϵ_4 and lower apo-ϵ_2 frequencies,

Table 1
Genotypic and phenotypic differences between common isoforms of apolipoprotein E

Protein	Allele	Position 112 codon/amino acid	Position 158 codon/amino acid	Apparent p*I*	
apo-E_2	apo-ϵ2	5′-TGC-3′/Cysteine	5′-TGC-3′/Cysteine	5.9[a]	5.68[b]
apo-E_3	apo-ϵ3	5′-TGC-3′/Cysteine	5′-CGC-3′/Arginine	6.0	5.81
apo-E_4	apo-ϵ4	5′-CGC-3′/Arginine	5′-CGC-3′/Arginine	6.1	5.95

[a] Warnick et al. [80].
[b] Eto et al. [85].

whereas the Chinese population has lowest apo-ϵ_4 frequency. In contrast, the Japanese population has low frequencies of both apo-ϵ_2 and apo-ϵ_4, and consequently higher apo-ϵ_3 [37].

The three major apo-E isoforms differ in amino acid substitutions at sites 112 and/or 158 of the polypeptide chain. The substitutions are located in the domain that determines maximal receptor binding activity. Apo-E_3 has cysteine at 112 and arginine at 158; apo-E_2 contains cysteine at both sides, whereas apo-E_4 contains arginine at both sides. Consequently, they differ in isoelectric point (pI). Sialylated apo-E forms bear additional negative charges and therefore have lower pI than non-sialylated counterparts. The amino acid substitutions derive from single nucleotide substitutions. At positions 112 and 158 the codon for cysteine is TGC and that of arginine CGC. Table 1 summarizes phenotypic and genotypic differences between the common apo-E isoforms.

In addition to the common apo-E isoforms, other (rare) mutations have been described and recently reviewed [38,39]. These mutations are usually detected by charge differences of the resulting apo-E proteins, compared with those of the common isoforms. Most of them are associated with familial dysbetalipoproteinaemia (see below). Mutations that affect the receptor binding domain are likely to be associated with this disease in a dominant fashion.

3. Apolipoprotein E pathophysiology

Differences in amino acid sequences of common apo-E isoforms alter lipoprotein metabolism. These alterations are associated with development of both atherosclerotic and non-atherosclerotic diseases. Reviews can be found in Refs. [32,37,39–43].

3.1. Apolipoprotein E polymorphism alters lipoprotein metabolism

The common apo-E isoforms have different affinities for the LDL (apo-B/E) and remnant (apo-E) receptors, are differently distributed among circulating lipoproteins, cause differences in VLDL to LDL conversion rate, and cause differences in dietary cholesterol absorption. These properties generate secondary differences in LDL receptor activities.

Compared with ancestral apo-E_3, apo-E_2 has 100 times lower affinity for the LDL receptor [44]. Low affinity causes delayed clearance of chylomicron and VLDL remnants that carry apo-E_2. Conversion of VLDL remnants to LDL is dependent on apo-E and occurs at a lower rate in subjects with apo-E_2/E_2 than in counterparts with apo-E_3/E_3 [45]. In addition, subjects with apo-E_2/E_2 have lower cholesterol absorption from the diet, compared with those with the apo-E_3/E_3 genotype [46]. LDL receptor expression is regulated by the intracellular cholesterol content. Delayed hepatic influx of (cholesterol ester rich) remnants and LDL in subjects with apo-E_2/E_2 generates compensatory upregulation of the LDL receptor. Subjects with apo-E_2/E_2 are therefore characterized by higher circulating levels of apo-E and remnants, and lower LDL levels.

In contrast to remnants that carry apo-E_2, those that carry apo-E_4 are more rapidly cleared

from the circulation, compared with those that contain apo-E_3. This may be due to preferential association of apo-E_4 with VLDL and chylomicrons [47,48]. Enhanced remnant clearance, rapid conversion of VLDL to LDL, and higher dietary cholesterol absorption [46] in subjects with apo-E_4/E_4 downregulates the LDL receptor. Consequently, subjects with apo-E_4/E_4 have lower levels of circulating apo-E and remnants, and higher LDL levels.

3.2. Apolipoprotein E polymorphism and atherosclerosis

Apo-E polymorphism influences plasma triglycerides and may explain 20, 12 and 4% of interindividual variation of plasma apo-E, apo-B and total cholesterol, respectively [49]. Since abnormal serum lipid profiles may predispose to atherosclerosis, it is reasonable to assume that apo-E polymorphism-induced changes in circulating lipoprotein levels are involved in CAD development. Pathogenesis by deposition of cholesterol-rich remnants (apo-E_2/E_2) and LDL (apo-E_4/E_4) into vascular walls is conceivable. Studies in patients with angiographically documented CAD have, however, been conflicting [50–57]. For instance, Utermann et al. [50] found no increase of the apo-ϵ_2 allele in CAD patients, as compared with controls. On the other hand, all CAD patients with the apo-E_2/E_2 genotype had features of familial dysbetalipoproteinaemia (FD; Fredrickson type III hyperlipoproteinaemia). FD is characterized by accumulation of cholesterol-enriched chylomicron and VLDL remnants, and development of peripheral, cerebral and coronary atherosclerosis [37,40,41,58,59]. Palmar striae are pathognomonical. Impaired removal of remnants, reduced conversion of VLDL remnants to LDL, and VLDL overproduction account for elevated levels of both cholesterol and triglycerides and lower LDL and HDL. Over 90% of FD patients are homozygous for the apo-ϵ_2 gene. However, only 1–10% of subjects with apo-E_2/E_2, develop FD [39]. It has been suggested that the apo-E_2/E_2 genotype is a necessary but insufficient requirement of FD development. Impaired remnant removal (like in hypothyroidism; Ref. [60]), low LDL receptor expression (hypothyroidism, familial hypercholesterolaemia, oestrogen withdrawal), increased VLDL synthesis (obesity, diabetes mellitus, familial combined hyperlipoproteinaemia, alcohol consumption) and age are among additional requirements [37,40,41,58,59].

Several investigators reported association of the apo-ϵ_4 allele with CAD [51–57], which may be due to its LDL-cholesterol raising effect. Recent data indicate that the apo-ϵ_4 allele is associated with CAD development, even after adjusting for traditional coronary risk factors and lipids [61]. The frequency of the apo-ϵ_4 allele tends to be higher in populations with increased CAD mortality rates (e.g. Finnish population), and lower in those with reduced rates (e.g. Japanese population). The apo-ϵ_4 allele is assumed to shorten life expectancy, presumably by its influence on lipoprotein levels [62–64]. Apo-ϵ_4 frequencies are high in many populations. The impact of the apo-ϵ_4 allele in CAD epidemiology is, therefore, likely to exceed that of the relatively rare FD in subjects with apo-ϵ_2/ϵ_2.

Apo-E_4 is associated with development of sporadic and late-onset Alzheimer's disease [65–67]. This disease is characterized by deposition of amyloid β-peptide in senile plaques and cerebral vessels. Apo-E_4 has high affinity for the amyloid β-peptide both in vitro [68,69] and in vivo [70]. Because of its demonstration in amyloid plaques apo-E_4 is believed to take part in the pathogenetic cascade. Apo-E_4 is also associated with Creutzfeldt–Jakob disease [71].

It is clear that determination of the apo-E isoform is necessary for FD diagnosis. FD diagnosis is important, since the condition strongly predisposes to atherosclerosis and responds excellently to treatment with diet and fibrates [37]. Establishment of apo-E genotypes of patients with diabetes mellitus might be useful, because of its high prevalence and the associated risk of atherosclerosis. Determination of apo-E isotypes may become useful in the choice of lipid lowering treatment strategies, since several studies suggest that apo-E polymorphism influences the magnitude of lipid changes following treatment

with lipid-lowering drugs [72–74]. Subjects with apo-E_4/E_4 are more susceptible to cholesterol lowering dietary intervention [75,76].

4. Apolipoprotein E phenotyping

Since common apo-E isoforms differ in pI (Table 1), they can be separated by methods that take advantage of charge differences.

4.1. Early methods

Apo-E, isolated from VLDL, was investigated by mono- and two-dimensional sodium dodecylsulfate–polyacrylamide gel electrophoresis [77,78] and isoelectric focusing (IEF) [79]. One isoform was found to exhibit defective binding to lipoprotein receptors. On basis of their IEF characteristics the three major isoforms were denoted apo-E_2, apo-E_3 and apo-E_4. IEF methods were used for FD diagnosis by demonstration of 'VLDL apo-E_3 deficiency' [79,80]. An example is the method of Warnick et al. [80]. They isolated VLDL from 5 ml EDTA-plasma by two ultracentrifugation steps. Following delipidation by extraction with acetone–methanol and diethyl ether, the protein was redissolved in a Tris-HCl buffer, pH 8.6, that contained urea for denaturation and dithiothreitol (DTT) for reduction of cystine disulfides. IEF was performed in cylindrical polyacrylamide (PAA) gels that contained pH 4–6 ampholytes and urea. Proteins were stained with Coomassie Brilliant Blue and quantified by densitometric scanning.

The gels showed multiple bands that besides apo-E isoforms and their sialylated forms [80,81] were assigned to albumin [80], apo-C [80,81] and apo-AI [80]. Diagnosis was made by comparison of apo-E_3 intensity relative to that of the other apo-E isoforms, notably apo-E_2. A drawback of aspecific protein staining was the presence of contaminating proteins in VLDL [80], such as pro-apo-AI [82] and serum amyloid A [83], which produce bands in the apo-E IEF region. Together with the presence of the more acidic sialylated apo-E forms [81,84] they constituted a source of possible misinterpretation.

4.2. Improvements

Various improvements have subsequently been made. Introduction of flat gels in combination with VLDL isolation [85] augmented the number of samples that could simultaneously be analyzed by IEF. Further improvements were the use of: prepurification methods that do not need VLDL isolation by ultracentrifugation, direct sample application, use of small samples sizes (10–50 μl range; except for Ref. [87]: 1 ml), and detection following immunofixation or (immuno)blotting. Some of these will be described in more detail [84,86–92]

Serum [84,89] and plasma [92] were directly applied after addition of DTT [84,89,92], Tween-20 [89,92], urea [84,89], ampholytes pH 5–7 [84] and guanidine/HCl [84]. Prepurification methods are delipidation by organic solvent extraction [88,89,91], lipoprotein precipitation with polyethylene glycol [90], $MgCl_2$/phosphotungstate lipoprotein precipitation/organic solvent extraction [87], or dialysation [86]. After prepurification, most investigators add DTT [81,87–90,92], detergents like Tween-20 [89,90,92] or decylsulfate [81,88], and urea [81,84,87–90]. IEF is performed in 5% PAA [81,84,86,91,92] or about 2% agarose [87–90] with usually 2–2.8% ampholytes, under urea denaturing conditions [81,84,86–92]. Ampholyte pH range is usually 4.5–6.5 [87,88] or 5–8 [84,86,91,92], and occasionally 3–10 [89,90]. Focusing time is mostly about 3 h [86–90,92] and occasionally 45 min [91] or 17.5 h [84].

Apo-E is detected by immunofixation or (immuno)blotting. Following immunofixation with polyclonal anti-apo-E antibodies [90,91] the gels are stained with Serva Blue [90] or silver [91]. After electroblotting [84] or diffusion blotting facilitated by pressure [84,86–88,92] the proteins are transferred to nitrocellulose [84,86,87,92] or polyvinylidenedifluoride [88] membranes. Immediate staining was done with amido black [87]. For immunological detection blots are initially saturated with low-fat milk proteins [86,88,92] or bovine serum albumin [84]. The first (anti-apo-E)

antibody may be monoclonal [84,88] or polyclonal [84,86,91], whereas the second (directed against first) is conjugated with alkaline phosphatase [86,92] or horseradish peroxidase [84,88]. Subsequent staining is done by incubation with 4-chloro-1-naphthol/H_2O_2 [84] or diaminobenzidine/$NiCl_2$ [88] for visualization of peroxidase activity and β-naphthyl phosphate/Fast Blue BB [86,92] for alkaline phosphatase activity. Application of immobilized pH gradients improves separation between parent apo-E isoforms and their posttranslational variants [93–95].

4.3. Interpretation

Interpretation difficulties that may arise from the presence of sialylated apo-E forms and the need to confirm the amino acid substitutions in the observed apo-E isoforms led to the use of neuraminidase (sialidase) and cysteamine pretreatments. Neuraminidase removes sialic acid residues, causing simplified patterns. The cysteamine sulfhydryl function reacts specifically with that of cysteines, giving rise to attachment of a disulfide-bridged ethylamino group. The net result is that apo-E_3 (^{112}Cys/^{158}Arg) and apo-E_2 (^{112}Cys/^{158}Cys), will carry the same charge as apo-E_4 (^{112}Arg/^{158}Arg).

Illustrative examples of neuraminidase and cysteamine pretreatments were presented by McDowell et al. [88]. They used untreated, neuraminidase-pretreated, cysteamine-pretreated and neuraminidase/cysteamine-pretreated serum. The samples were delipidized by organic solvent extraction and redissolved in Tris–decylsulfate–urea, that was DTT-supplemented (untreated and neuraminidase-pretreated samples) or DTT-unsupplemented to avoid reduction of cysteine-attached cysteamine (cysteamine-pretreated and neuraminidase/cysteamine-pretreated samples). IEF was done in agarose with pH 4.0–6.5 ampholytes, followed by immunoblotting on polyvinylidenedifluoride membranes. They tested a monoclonal antibody and two polyclonal anti-apo-E antibodies for the reaction with apo-E and various peroxidase-conjugated polyclonal counterparts as the second detecting antibody. Peroxidase was visualized with diaminobenzidine/$NiCl_2$.

Fig. 1 shows their results of untreated (A) and neuraminidase-pretreated (B) serum for the six common apo-E phenotypes. Sialylated apo-E forms (panel A) may complicate interpretation, since they focus close to parent apo-E bands. For instance, distinction between apo-E_3/E_3 and apo-E_2/E_3 phenotypes is usually based on visual or densitometric judgement of relative band intensities of apo-E_2 and apo-E_3. Apo-E_3/E_3 is usually assigned when apo-E_3 band intensity exceeds that of apo-E_2 (i.e. sialylated apo-E_3), whereas band intensity of apo-E_2 exceeds that of apo-E_3 in apo-E_2/E_3. Similar difficulties may arise in distinction between E_3/E_4 and E_4/E_4. Neuraminidase may effectively remove sialic acid residues (panel B). The resulting patterns greatly diminish the risk of misinterpretation. Treatment with cysteamine (Fig. 2B) shifts unsialylated apo-E_2 and apo-E_3 to the position of unsialylated apo-E_4 and, analogously, aligns their sialylated forms according to the number of sialic acid residues. Combined pretreatment with neuraminidase/cysteamine (Fig. 2C) confirms that additional bands in panels A and B are caused by sialylated apo-E and that the identified apo-E isoforms carry Arg/Cys substitutions characteristic for the common apo-E phenotypes.

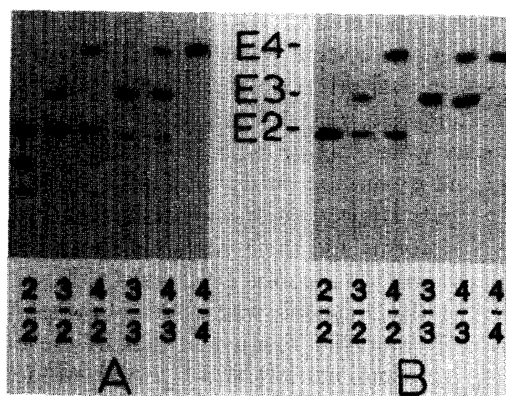


Fig. 1. Influence of neuraminidase pretreatment on phenotyping of common apolipoprotein E isoforms by isoelectric focusing and immunoblotting. (A) Untreated serum; (B) pretreated with neuraminidase. Cathode, top; anode, bottom. Reproduced from McDowell et al. [88], with permission.

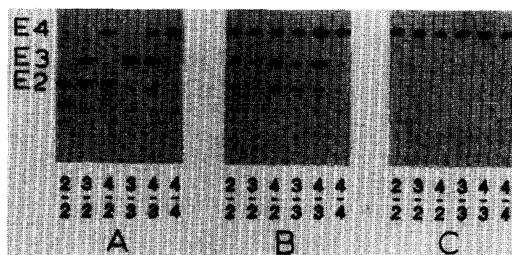


Fig. 2. Influence of neuraminidase and cysteamine pretreatments on phenotyping of common apolipoprotein E isoforms by isoelectric focusing and immunoblotting. (A) Untreated serum; (B) pretreatment with cysteamine; (C) pretreatment with neuraminidase and cysteamine. Reproduced from McDowell et al. [88], with permission.

5. Apolipoprotein E genotyping

Establishment of mutations on the DNA level require molecular biological techniques that, in case of the common apo-E isoforms, allow detection of underlying point mutations (Table 1).

5.1. Early methods

Apo-E genotypes were initially determined by incubation of genomic DNA with restriction endonucleases, fragment separation by electrophoresis, transfer to a membrane, hybridization with radiolabelled allele-specific oligonucleotides (probes) and autoradiographical detection [96]. Development of the polymerase chain reaction (PCR) facilitated detection of mutations by amplification of the fragment of interest. At first, nested PCR reactions [97,98] were applied to increase PCR fidelity. However, this proved unnecessary and was subsequently replaced by one stage PCR [99–110].

5.2. DNA isolation and polymerase chain reaction

First step in apo-E genotyping is DNA isolation. Various methods have been used. They vary in sample source (e.g. mouth wash, whole blood, leucocytes) and DNA isolation/purification technique (e.g. boiling, salting out procedures, lysis followed by extraction with organic solvents, spin columns, proteinase K digestion; or certain combinations). Current methods for apo-E genotyping are either based on analysis of a PCR product, or the use of specific primers that either or not initiate amplification.

PCR of common apo-E isoforms [97–103,106–110] usually employs amplification of a 200–300 bp fragment of exon 4, that contains codons 112 and 158. For instance, Hixson et al. [101] used two primers named F4 and F6 (Fig. 3). Primer F6 is a 25-base oligonucleotide that anneals downstream to anti-codon 112, whereas F4 measures 26 bases that anneal upstream to codon 158. PCR with the primer set results in a 244 bp PCR product. After PCR, the nucleotide substitutions of the common apo-E alleles at positions 112 and 158 can be detected with: (1) labelled allele-specific oligonucleotide probes (ASO-probes; Refs. [97,99,100,102,110]); (2) restriction fragment length polymorphism (RFLP; Refs. [97,101,103,107,108]); (3) single-stranded conformational polymorphism (SSCP; Ref. [106]); (4) the primer-guided nucleotide incorporation assay [98]; or (5) denaturating gradient gel electrophoresis (DGGE; Ref. [109])

5.3. Allele-specific oligonucleotide probes

Detection with ASO-probes [97,99,100,102,110] makes use of radiolabelled oligonucleotides that either or not hybridize with complementary sequences in the single-stranded PCR product. Two probes are used to detect a single mutation: one hybridizes with the mutant, the other with the non-mutant. After PCR, the product is rendered single stranded and transferred to blotting membranes in duplicate. One of the membranes is incubated with the probe that hybridizes with the mutant, the other is incubated with the probe that recognizes the non-mutant. Incubation and subsequent washing is done under strict conditions to allow specific hybridization. The probe that causes hybridization can be detected by autoradiography and thereby detects the nucleotide sequence. In case of the common apo-E alleles, four probes are necessary to detect the mutations in codons 112 and 158. Each of the six genotypes is characterized by a unique combination of positively reacting probes. The major

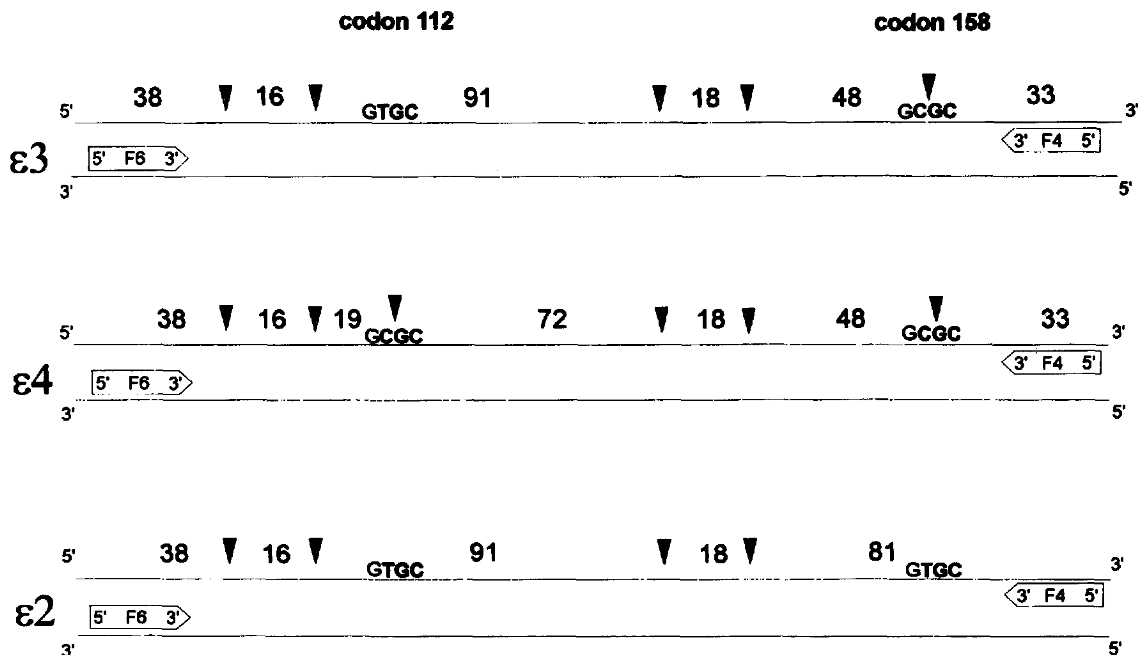


Fig. 3. Polymerase chain reaction and Hha I restriction sites for genotyping of the six common apolipoprotein E genotypes by restriction fragment length polymorphism. PCR with primers F6 and F4 aims at amplification of part of exon 4, that includes codons 112 and 158. Grey arrows, common Hha I restriction sites; black arrows, Hha I restriction sites in codons 112 and 158; arrows in primers denote directions of amplification. Numbers of base pairs of Hha I fragments are indicated between restriction sites. Method is according to Hixson and Vernier [101].

drawback of ASO-probes is aspecific hybridization that is caused by the high GC content of the apo-E gene.

Nowadays, enzyme-conjugated probes or other probes are used. Hybridized probes can be detected by colour development, following incubation with an appropriate substrate. For instance, the commercially available kit of Innogenetics (INNOLiPA ApoE test; Nijmegen, Netherlands) uses a biotin-conjugated primer, an unlabelled primer, four membrane-bound allele-specific probes and an alkaline phosphatase-avidin conjugate. The four probes have been applied on membrane strips in lines ('line probe assay'). PCR results in a biotin-conjugated PCR product that is subsequently rendered single stranded and incubated with the membrane. Dependent on the present apo-ϵ alleles, the biotin-labelled PCR product hybridizes with a unique combination of two, three or four probes. Hybridization is visualized by subsequent incubation with the alkaline phosphatase–avidin conjugate, during which the avidin moiety binds to the biotin moiety of the primer. Incubation of the membrane with alkaline phosphatase substrate leads to formation of a coloured precipitate.

5.4. Restriction fragment length polymorphism

Determination of common apo-E genotypes with RFLP [97,101,103,107,108] makes use of the specificity of the restriction endonuclease Hha I (Cfo I), which recognizes the sequence 5′-**GCGC**-3′ (bold: codon 112 in ϵ_4, and codon 158 in ϵ_3 and ϵ_4), but not 5′-**GTGC**-3′ (bold: codon 112 in ϵ_3 and ϵ_2, and codon 158 in ϵ_2). Cleavage of the PCR product with Hha I leads to a combination of restriction fragments that is unique for each of the 6 common apo-ϵ genotypes. Fig. 3 shows common and altered restric-

tion sites together with the resulting fragment lengths. The PCR product derived from the use of primers F4 and F6, as described by Hixson and Vernier [101]. Fig. 4A shows expected detectable restriction fragments for each apo-ϵ genotype, following separation of the fragments by electrophoresis. Briefly, the procedure is as follows. After PCR, Hha I is added to the reaction mixture. Restriction fragments are separated with electrophoresis under non-denaturating conditions. PAA gels [97,101,103,107] or agarose gels suitable for separation and detection of small DNA fragments [108] can be used. Restriction patterns become visible following staining with ethidium bromide and UV illumination. Fragment lengths are estimated by additional application of DNA size markers. Fig. 4B shows the result for each of the six common apo-E genotypes, as obtained in our laboratory. Since small fragments are relatively difficult to detect, those that contain 48, 38 and 33 bp may give rise to faint bands. Intensity of the common 38 bp fragment can be used to avoid misinterpretation due to faint bands in the low fragment size region.

Various primer sets have been used to amplify the fragment of interest. Differences in primer annealing sites alter the sizes of terminal restriction fragments. Rare mutations may alter restriction fragment lengths by introduction/removal of restriction sites, or by insertion/deletion. For instance, Richard et al. [107] demonstrated that RFLP additionally allows detection of some of them. Digestion with Hha I also detects the Christchurch allele and the E_4 Cys 142-allele (both removal of common restriction site), and apo-E_3 Leiden (insertion). Digestion of the same PCR product with Taq I detects the Weisgraber allele (introduction of Taq I restriction site).

5.5. Single-stranded conformational polymorphism

SSCP [106] determines not only common apo-E genotypes, but may also detect rare mutations. After dissociation of the double-stranded PCR product, each single strand assumes a folded conformation. Conformational changes may occur in one or both strands when DNA sequence is altered by mutation. They can be detected as changes in PAA electrophoretic mobility. In case of apo-E, the slowly moving single strands that derive from the apo-E PCR product do not show differences in mobility. However, among the faster moving strands, the apo-ϵ_3 allele moves fastest, followed by the ϵ_2

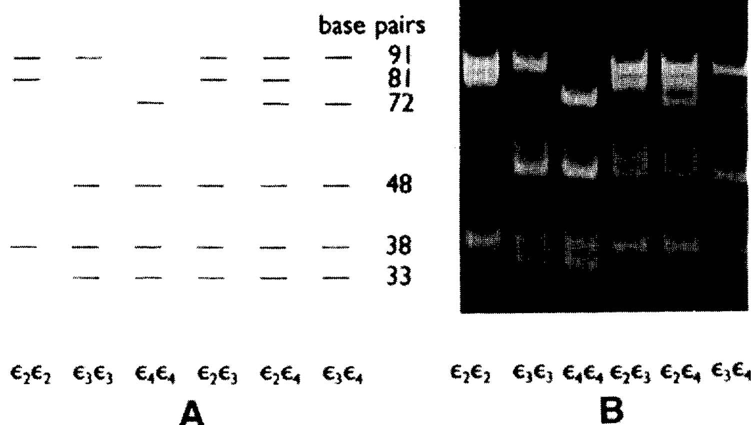


Fig. 4. Hha I fragment patterns for the six common apolipoprotein E genotypes, as obtained by electrophoresis. The PCR products were obtained with primers F4 and F6, and digested with Hha I (see Fig. 3). Fragments were separated by electrophoresis and stained with ethidium bromide. (A) Expected pattern; (B) example from own laboratory. Method is according to Hixson and Vernier [101].

and ϵ_4 alleles. Differences in mobility of fast-moving strands allow identification of the six common apo-E genotypes. To our knowledge, the method has not been applied for detection of rare apo-E mutants as yet. SSCP is very sensitive to environmental conditions, like salt concentration and temperature.

5.6. Primer-guided nucleotide incorporation assay

Another option is detection of common apo-E genotypes with the primer-guided nucleotide incorporation assay [98]. PCR is performed with two primers. The one that anneals with the coding strand is conjugated with biotin at its 5′-end. The biotin moiety of the PCR product is bound to avidin, that in its turn is bound to a matrix. The PCR product is subsequently denatured to a single-stranded product. Following washing, only the non-coding strand of the PCR product is retained on the matrix. Two detecting primers are subsequently used to determine the six common apo-E genotype. The first (P1) anneals downstream of anti-codon 112. The position is such (see Table 1) that the first nucleotide that is subsequently incorporated is dTTP (in case of apo-ϵ_2 and apo-ϵ_3) or dCTP (in case of apo-ϵ_4). The second detecting primer (P2) aims at codon 158. Its position is such that the first nucleotide to be incorporated is dTTP (in case of apo-ϵ_2) or dCTP (in case of apo-ϵ_3 or apo-ϵ_4). PCR with either P1 or P2 is performed with either radiolabelled dTTP or dCTP as the only nucleotides (i.e. four PCR reactions). Combinations of dTTP or dCTP incorporation with P1 and P2 are unique for the six apo-E genotypes.

In its non-radioactive form, the technique is applied in the commercially available kit of Sangtec Medical (AffiGene; Bromma, Sweden). A biotinylated PCR product is bound to streptavidine-coated microplate wells. Following its denaturation, detecting primers anneal to the single-stranded PCR product. Dinitrophenyl (DNP)-modified nucleotides are either or not incorporated in a single polymerase extension step. Detection takes place with an enzyme-conjugated anti-DNP antibody, followed by colour development by the addition of substrate.

5.7. Denaturing gradient gel electrophoresis

Dependent on the base pair sequence, double-stranded DNA partially dissociates (melts) under denaturing conditions. Dissociation occurs stepwise in domains, according to the strength of the base pair bonds in each of those domains. Electrophoresis of a DNA fragment in a PAA gel that contains a linear gradient of denaturant causes the fragment to arrest in a position at which the concentration of the denaturant dissociates the domain with the lowest melting point. Single base pair substitutions in DNA fragments may alter melting points of domains. These fragments can therefore potentially be separated with DGGE.

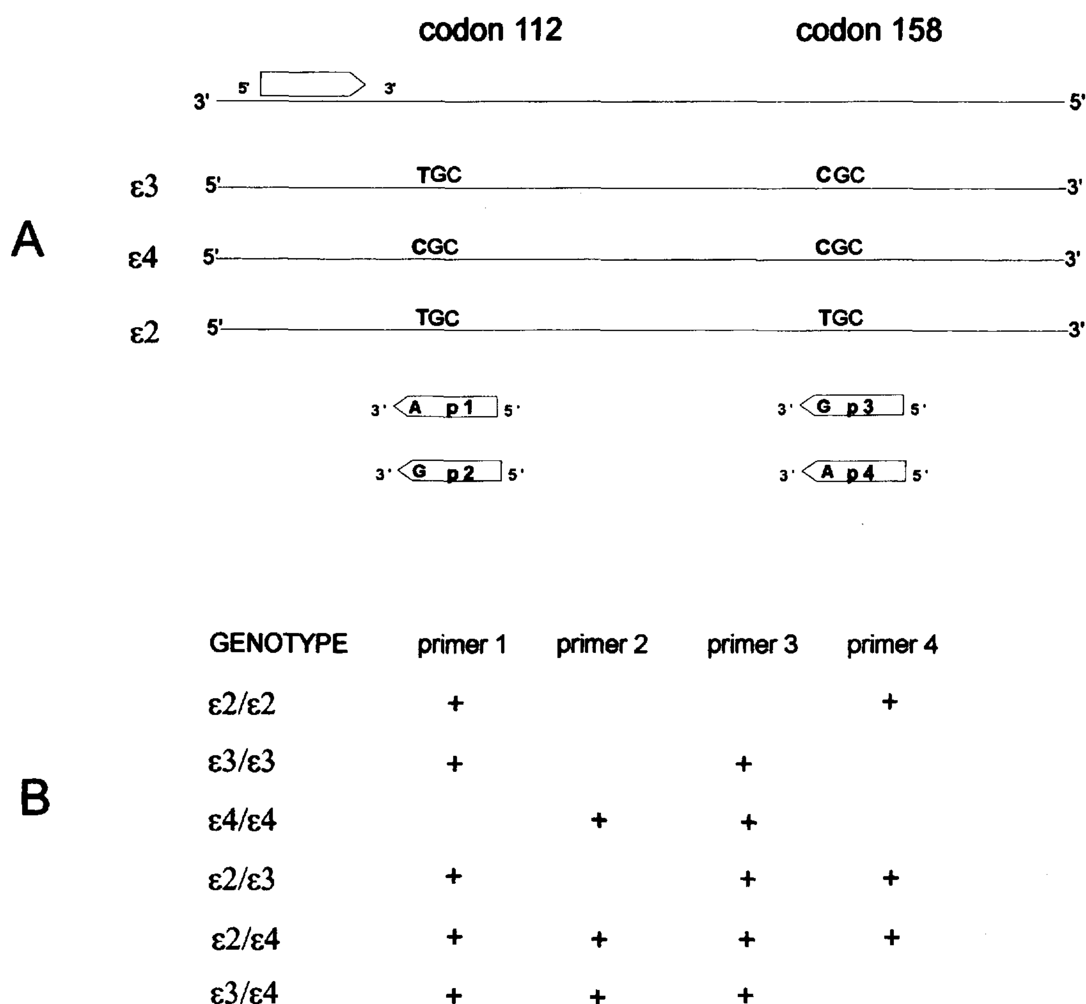
Parker et al. [109] demonstrated that DGGE enables separation of double-stranded DNA fragments that derive from the three common apo-ϵ isoforms. A 641 bp PCR product was initially incubated with a restriction enzyme. One of the resulting fragments measured 467 bp and contained codons 112 and 158. Consistent with lower melting point of T=A, compared with C≡G, the ϵ_2 fragment (^{112}T=A/^{158}T=A) arrested at a denaturant concentration of 71%; ϵ_3 (^{112}T=A/^{158}C≡G) at 72%; and ϵ_4 (^{112}C≡G/^{158}C≡G) at 73% (see Table 1). These properties enabled identification of the six common apo-E genotypes. Improvement by direct amplification of the low-melting domain circumvents the necessity to isolate the fragment of interest by restriction enzyme digestion. DGGE is technically demanding, but enables detection of rare mutants.

5.8. Amplification refractory mutation system

Detection of common apo-E genotypes with primers that either or not initiate amplification is performed with ARMS [104,105]. The principle is based on amplification with allele-specific primers. Use of a primer that is mismatched at the 3′-nucleotide prevents amplification. The

allele-specific primer is synthesized in two forms. One anneals with the mutant sequence, the other with the non-mutant sequence. Amplification will only occur when the primer matches with the template DNA. In practice, an additional base mismatch is introduced near the primer 3′-end to enhance specificity.

Fig. 5 illustrates the principle, based on the method of Main et al. [105]. A common primer is located downstream of the mutation. Four specific primers are used in four PCR reactions. Primer 1 is directed at codon 112. Since its 3′-end is adenosine, primer 1 anneals with both apo-ϵ_2 and apo-ϵ_3. The 3′-end of primer 2 is guanosine.



GENOTYPE	primer 1	primer 2	primer 3	primer 4
ε2/ε2	+			+
ε3/ε3	+		+	
ε4/ε4		+	+	
ε2/ε3	+		+	+
ε2/ε4	+	+	+	+
ε3/ε4	+	+	+	

Fig. 5. Principle of the amplification refractory mutation system as applied for detection of the six common apolipoprotein E genotypes. The common primer is located on the non-coding strand (A). Primers 1 and 2 aim at codon 112; primers 3 and 4 at codon 158. Primer 1 anneals with apo-ϵ_2 and apo-ϵ_3; primer 2 with apo-ϵ_4; primer 3 with apo-ϵ_3 and apo-ϵ_4; and primer 4 with apo-ϵ_2. Arrows in primers denote directions of amplification. Combinations of positive PCR reactions, demonstrable by different fragment sizes upon electrophoresis, are unique for the six apo-E genotypes (B). Method is according to Main et al. [105].

It merely anneals with apo-ϵ_4. Analogously, primers 3 and 4 are directed at codon 158 and anneal with apo-ϵ_3 and apo-ϵ_4 (primer 3), and apo-ϵ_2 (primer 4). Combinations of positive PCR reactions, demonstrable by different fragment sizes upon electrophoresis, are unique for the six apo-E genotypes. The advantage of this method is that it merely necessitates detection of a PCR product. However, both annealing temperature and composition of the PCR mixture are critical in specific amplification.

6. Phenotyping or genotyping?

Presence of sialylated apo-E, but also glycated apo-E may confound phenotype interpretation. Knock-out of a primary nitrogen charge causes decreased pI and, like sialylated forms, focusing at lower pH. Assignment of apo-E posttranslational variants to parent apo-E isoforms may, therefore, be expected to overestimate apo-E isoforms at more anodal IEF positions. More specifically, apo-E_4 posttranslational variants may erroneously be classified as apo-E_3 or apo-E_2, whereas those of apo-E_3 may be classified as apo-E_2 (see Fig. 1). Another source of posttranslational modification is by carbamylation, either by high circulating urea (e.g. diabetic nephropathy) or by addition of urea in IEF methods. McDowell et al. [88] and Luley et al. [90] reported that the latter is no source of misclassification. To our knowledge there are no reports on in vivo apo-E carbamylation and acetylation. Like HbA, apo-E may acetylate by high intracellular acetyl–CoA contents (e.g. poorly controlled patients with diabetes mellitus) or extracellularly by aspirin treatment. Acetylated apo-E may be expected to behave similar to sialylated and carbamylated apo-E upon IEF.

Several authors [92,111–116] compared results of phenotypically and genotypically determined common apo-E isoforms. Comparing those of non-insulin-dependent patients with diabetes mellitus (NIDDM), Snowden et al. [111] observed 16% discrepancies (Table 2). Consistent with misclassification due to posttranslational variants, discrepancies were on account of the apo-ϵ_4 allele being observed as the apo-E_3 protein and the apo-ϵ_3 allele being held for apo-E_2. In their hands, neuraminidase-pretreatment had little effect on simplification of phenotype interpretation and was therefore not employed. The authors suggested that the discrepancy might result from resistance of diabetic apo-E to neur-

Table 2
Comparison of phenotypically and genotypically established results for common apolipoprotein E isoforms

Reference	*n*	Study group	Phenotyping	Genotyping	Discrepancy (%)
Snowden et al. [111]	95	NIDDM[a]	VLDL/IEF/Coom BB	PCR/ASO-probe	16
Wenham et al. [112]	52	IDDM[b]	Neuram/del/IEF/immunoblot	PCR/RFLP	13
	58	control[c]			17
Mailly et al. [113]	195	random	dialysation/IEF/immunoblot	PCR/ASO-probes	4
Stavljenic-Rukavina et al. [114]	50	IDDM	VLDL/Neuram/del/IEF/Coom BB	PCR/ASO-probe	24
				ARMS	16
James et al. [115]	151	NIDDM[d]	IEF/Immunoblot	PCR/RFLP	1
Hansen et al. [116]	460	random	del/IEF/immunoblot	PCR/RFLP	2
Kataoka et al. [92]	431	random	IEF/immunoblot	PCR/RFLP	2

[a] Gly-Hb 8.4 ± 0.2% (mean ± SEM); [b] HbA_1 10.4 ± 0.3% (mean ± SEM); [c] HbA_1 7.1 ± 0.2 (mean ± SEM); [d] HbA_{1c} 8.0 ± 1.8 (mean ± SD).
NIDDM, non-insulin-dependent diabetes mellitus; IDDM, insulin-dependent diabetes mellitus; VLDL, very low-density lipoproteins; IEF, isoelectric focusing; Coom BB, Coomassie Brilliant Blue; Neuram, neuraminidase; del, delipidation; PCR, polymerase chain reaction; ASO-probe, allele-specific oligonucleotide as probe; RFLP, restriction length polymorphism; ARMS, amplification refractory mutation system.

aminidase, or the presence of glycated or peroxidized apo-E. Discrepancies were confirmed and extended by Wenham et al. [112], who successfully employed neuraminidase treatment and used the more specific immunoblotting for detection. Both patients with insulin-dependent diabetes mellitus (IDDM) and controls showed high discrepancy percentages that were, however, mutually not significantly different. Observed disparities were mainly on account of the apo-ϵ_4 allele being observed for the apo-E_3 protein. The data suggest that nor sialylated apo-E nor glycated apo-E explain the observation.

Results of Snowden et al. [111] and Wenham et al. [112] were confirmed by Stavljenic-Rukavina et al. [114], but not in four other investigations [92,113,115,116] that comprised larger study groups (Table 2). Mailly et al. [113] explained the majority of discrepancies by IEF superimposition of glycosylated apo-E isoforms. Other possible reasons were wrong sample identification and the presence of a rare mutant. The low percentage discrepancies in NIDDM patients observed by James et al. [115] does not seem to be associated with better glucose homeostasis, because indices of long-term diabetic control (HbA_{1c}) were similar to those of NIDDM and IDDM patients studied by Snowden et al. (glyc-Hb) and Wenham et al. (HbA_1), respectively. James et al. [115] explained differences in percentage discrepancies by their better separation of sialylated and non-sialylated forms, and the use of serum, instead of isolated VLDL. Most of the discrepancies reported by Hansen et al. [116] were on account of faint IEF bands, the others possibly by errors in sample identification or rare mutations. Faint immunoblots were recommended to be re-run using higher sample volumes [84,116]. Incomplete digestion of the PCR product by the restriction enzyme was considered unlikely [116].

Besides disparities between phenotypically and genotypically determined apo-E isoforms, Stavljenic-Rukavina et al. [114] found 8% discrepancies between genotypes as measured by PCR/ASO-probes and ARMS analyses. Mailly et al. [113] reported 3% discrepancies between analyses by PCR/ASO-probes and PCR/RFLP. Deviant results were attributed to poor amplification, resulting in poor discrimination between ASO-probe signals. Improvement of ASO-probe design resulted in 100% concordance with PCR/RFLP.

Posttranslational variants seem the principal drawback of apo-E phenotyping, although some authors consider sialylated forms useful for the identification of apo-E isoforms [81] and rare mutants [86]. Moreover, additional IEF bands appear during serum storage [81,84,86,88] and freezing–thawing [88], possibly due to proteolytic activity of thrombin [81]. In contrast, genotyping is obviously not affected by posttranslational variants and generally less laborious than phenotyping. It requires only small cell numbers. DNA can safely be stored for prolonged periods and used for other (e.g. retrospective) purposes. This seems especially important for cohort studies and retrospective diagnosis, given the rapidly expanding knowledge on the relation between altered genes and diseases.

Because of the specificity of genotyping with PCR/ASO-probes, PCR/primer-guided nucleotide incorporation assay and ARMS to merely detect common apo-ϵ alleles, IEF profiling of apo-E isoforms seems superior for the detection of rare mutants. This is, however, not necessarily the case with less specific genotyping with PCR/RFLP [107] and possibly PCR/SSCP and PCR/DGGE. Phenotyping may identify rare mutants if the mutation clearly changes pI and the resulting pI is in the employed IEF pH range. For instance, Hackler et al. [91] showed illustrative examples of the IEF positions of apo-E_1 and apo-E_5. On the other hand, Rall et al. [39] reported that a sizeable number of rare mutations that cause FD in a dominant fashion, exhibit pI similar to apo-E_3 and E_4. Moreover, the choice of method for routine application is not based on its ability to identify rare mutants.

7. Conclusions

Common apo-E polymorphism influences serum triglycerides, and may account for as much as 12 and 4% of interindividual variance of

circulating apo-B and total cholesterol, respectively. Establishment of apo-E_2/E_2 confirms FD diagnosis. This is important since FD responds well to established therapy. Apo-E isotyping may be of importance for patients with diabetes mellitus (who are at risk of atherosclerosis), choice of lipid lowering strategies, and several non-atherosclerotic diseases (e.g. Alzheimer's disease). Present analyses of apo-E isoforms are based on phenotyping or genotyping.

Phenotyping is done with IEF in flat PAA or agarose gels that contain pH 4–7 ampholytes and urea. IEF is preceded by direct serum application, or prepurification methods like delipidation by organic solvent extraction, lipoprotein precipitation and dialysation. IEF is followed by immunofixation/protein staining. Another option is electro or diffusion blotting, followed by protein staining or immunological detection with a specific anti apo-E antibody and a second polyclonal antibody that is conjugated with a detection enzyme. Neuraminidase and cysteamine pretreatments may add to identification of parent apo-E isoforms. Alternatively, IEF with immobilized pH gradients may be employed to enhance separation between sialylated and parent apo-E forms. Faint bands can be avoided by application of sample sizes that contain comparable amounts of triglycerides, rather than volumes.

Current methods for apo-E genotyping are either based on analysis of a PCR product, or the use of specific primers that either or not initiate amplification. PCR of common apo-E isoforms amplify a 200–300 bp fragment of exon 4, that contains codons 112 and 158. Analyses of PCR products are done by ASO-probes, RFLP, SSCP, the primer-guided nucleotide incorporation assay, or DGGE. Detection of common apo-E genotypes with primers that either or not initiate amplification is performed with ARMS. The 8% discrepancy between results from PCR/ASO-probe and ARMS, reported in one study, is alarming and underlines the need to implement systems for internal quality control and external quality assessment.

Detection of rare mutants is important, since most of those that are presently known cause FD. Selection of a routine method is not based on analyses of rare mutants and neither phenotyping nor genotyping guarantee their detection. Combination of techniques may be the first choice for further investigation of patients with type III hyperlipoproteinaemia when, on the basis of a single technique, the condition can not be explained by the apo-E_2/E_2 isoform. Sequencing in specialized laboratories provides ultimate proof.

The discussion on discrepancies between the outcomes of phenotyping and genotyping does not seem settled. It appears that the disparity is caused by: IEF methods that do not adequately separate posttranslational apo-E variants, storage artifacts or faint IEF bands. Genotyping is usually less laborious than phenotyping and unaffected by posttranslational variants. DNA can safely be stored and used for retrospective purposes. PCR/RFLP is preferred for routine applications, since it does not necessitate multiple PCR reactions (as with primer-guided nucleotide incorporation assays, ARMS), does not require detection by hybridization (ASO-probes), allows less stringent experimental conditions (SSCP, DGGE, ARMS), and provides an internal control (38 bp fragment) that minimizes the risk of misinterpretation caused by faint bands. If specific antibodies that detect common apo-E isoforms [117] become widely available, both phenotyping by IEF and genotyping will be referred to specialized laboratories for detection of rare mutants.

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