APOE Variants E2, E3, and E4 Can Be Miscalled By Classical PCR-RFLP When The Christchurch Variant Is Also Present

Beatriz Candás-Estébanez,^{1*} Ariadna Padró-Miquel,¹ Cristina Ruiz-Iruela,¹ Emili Corbella-Inglés,² Xavier Pintó-Sala,² and Pedro Alía-Ramos¹

¹Hospital Universitari de Bellvitge, Laboratori Clínic, L'Hospitalet de Llobregat, Catalunya, Spain

²Cardiovascular Risk Unit, Internal Medicine, Hospital Universitari de Bellvitge, L'Hospitalet de Llobregat, Catalunya, Spain

Background: The *APOE* Christchurch (*APOECh*) is a rare variant (c.543C>A) in codon 154. It was first described in an E2 patient with type III dyslipidemia, and thus initially called *E2Ch*. Its prevalence and the lipid profile of carriers remain unclear. **Methods:** E2, E3, and E4 screening for the *APOE* gene was performed by PCR-RFLP. The rare *APOECh* variant was firstly found after detecting an unexpected 109 base-pair band in the high-resolution agarose gel electrophoresis leading to a genotype misinterpretation: the presence of *APOECh* alters the restriction-bands pattern. To confirm the Ch variant, a second PCR-RFLP method was specifically designed to detect this variant and Sanger sequencing was also performed for all positive samples. **Results:** We identified 12 unrelated subjects for the *APOECh*

among a cohort of 2,560 patients: nine E3/E3Ch, two E3Ch/E4, and one E2/E3Ch or E2Ch/E3. The frequency of the variant is 0.4% in our study population, which represents the highest percentage published so far. If there is a 109 bp band, it is easy to recognize the presence of the variant. However, in *APOE* routine genotyping, an E4Ch allele is indistinguishable from a standard E3. Therefore, E4Ch alleles might be under-represented in the results. **Conclusion:** We recommend *APOE* exon 4 sequencing to unequivocally detect the common three variants E2, E3, and E4 and the rare variants as well, to find out the real role they play in atherosclerosis and to estimate its real frequency which is nowadays unclear, in part by the small number of cases identified. *J. Clin. Lab. Anal.* 31:e22040, 2017. © 2016 Wiley Periodicals, Inc.

Key words: *APOE* variants; Christchurch variant

INTRODUCTION

Apolipoprotein E gene presents a great number of sequence variants (1). Among them, the most widely studied ones are c.388T>C and c.526C>T that lead to the *APOE3*, *APOE2*, and *APOE4* isoforms.

These *APOE* variants have been associated with disorders in lipid metabolism such as familial dysbetalipoproteinemia (E2/E2 homozygotes) (3), or other dyslipidemias (4–6), and with the development of Alzheimer's disease as well (7, 8).

On the other hand, the so-called *APOE* Christchurch (*APOECh*) is a rare variant very close to the previously mentioned sequence variants, resulting from a C to A substitution (c.543C>A) in codon 154. This change involves a missense mutation from Arginine to Serine. It was first described in an E2 patient with type III dyslipidemia (9), and thus initially called *APOE2* Christchurch

(*E2Ch*). Since its discovery in 1987, the great majority of *APOECh* cases were associated to E2 allele. The lipid profile of carriers remains unclear as some works support the theory that *APOECh* presence is not enough to develop a lipid disorder (9, 10), whereas others have suggested an association between *APOECh* and the development of type III hyperlipoproteinemia and familial dysbetalipoproteinemia when *APOECh* was present together with E2 (11). Its frequency is nowadays unknown, therefore their involvement in the development of atherosclerosis is even more uncertain.

*Correspondence to: Beatriz Candás-Estébanez, Hospital Universitari de Bellvitge, Laboratori Clínic, L'Hospitalet de Llobregat, Catalunya, Spain. E-mail: beatrizcandas@gmail.com

Received 20 April 2016; Accepted 28 July 2016

DOI 10.1002/jcla.22040

Published online in Wiley Online Library (wileyonlinelibrary.com).

Our laboratory routinely performed *APOE* genotyping (E2, E3, and E4 typing) for certain patients attending either the Cardiovascular Risk Unit or the Neurology Department. E2, E3, and E4 typing for the *APOE* gene was traditionally determined by PCR-RFLP, as described by Hixson and Vernier (2). Although this method is being replaced by real-time PCR, it is still widely used in many small laboratories. Briefly, genomic DNA was extracted from fresh blood using a standard protocol (High Pure PCR Template Preparation Kit, Roche, Mannheim, Germany). After amplification of the region of interest, digestion by *CfoI* enzyme and high-resolution agarose gel electrophoresis were performed. The final fragments sized 91, 83, 72, or 48 bp allowed the classification into the corresponding isoforms. The rare *APOE*Ch variant was firstly found after detecting an abnormal 109 base-pair band in the high-resolution agarose gel electrophoresis (Fig. 1).

PCR-RFLP methods for *APOE* genotyping (similar to that described by Hixson and Vernier) have been widely used for years in many laboratories. However, the expected agarose gel band patterns may be affected by other—unexpected—*APOE* variants altering restriction *CfoI* (or *HhaI*) targets, thus leading to a genotype misinterpretation. That is the case with Christchurch variant (c.543C>A): the detection of *APOE*Ch variant was possible because the C>A substitution abolishes a CGCG restriction site for the *CfoI* enzyme, altering the restriction-bands pattern in a way illustrated in Table 1. If we pay attention to the Table 1, we can observe that it is possible that a misclassification takes place depending on which combination between the *APOE*Ch and *APOE* allele occurs. Professionals could suspect from *APOE*Ch variant if an unexpected 109 band is present. However, there are cases where

TABLE 1. Comparison Between Standard *APOE* Band Patterns And Those Obtained In Presence Of The Christchurch Mutation When Hixson and Vernier Method Is Used

<i>APOE</i> genotype	Standard pattern (bp)	Christchurch	
		Homocygosis	Heterocygosis
E3E3	91, 48 ^b	109, 48	109, 91, 48
E2E2	91, 81	109, 81	109, 91, 81
E4E4	72, 48	91, 90, 48 ^b	91, 90, 72, 48 ^a
E2E3	91, 81, 48 ^c	109, 81, 48	E2S ^{Ch} E3: 109, 91, 81, 48 E2E3 ^{Ch} : 109, 91, 81, 48
E2E4	91, 81, 72, 48	109, 91, 81, 48	E2E4 ^{Ch} : 91, 90, 81, 48 ^C E2 ^{Ch} E4: 109, 81, 72, 48
E3E4	91, 72, 48 ^a	109, 91, 90, 48	E3E4 ^{Ch} : 91, 90, 48 ^b E3 ^{Ch} E4: 109, 72, 48

Only diagnostic bands are included. The numbers indicate the restriction fragment sizes.

^aGenotype call confusion between E4Ch/E4 = E3/E4.

^bGenotype call confusion among E4Ch/E4Ch, E3/E4Ch, and E3/E3.

^cGenotype call confusion between E2/E4Ch and E2/E3.

patients would be classified with an incorrect genotype. This is due to the fact that 91 bp and 90 bp are indistinguishable between them in an agarose gel electrophoresis even in those of high resolution. This means that the following equivalences can be deduced from Table 1: E4Ch/E4Ch = E3/E4Ch = E3/E3; E4Ch/E4 = E3/E4; and E2/E4Ch = E2/E3. However, in *APOE* routine genotyping, an E4Ch allele is indistinguishable from a standard E3. Therefore, E4 alleles might be underrepresented in the results.

Regarding our results, we could identify 12 subjects heterozygous for the *APOE*Ch variant: nine E3/E3Ch, two E3Ch/E4, and one E2/E3Ch or E2Ch/E3. To confirm the Ch variant, firstly, a second PCR was performed: in a final volume of 25 µl, 50–200 ng of genomic DNA was amplified using 0.75 U of Taq DNA polymerase (Roche), 1 mol/l of each primer (Apo-Ch1: 5'-GAGGTGCAGGCCATGCTCGG-3' and Apo-Ch2: 5'-CATCGGCATCGCGGAGGAGC-3'), 0.1 mmol/l of dNTPs, 10% dimetil-sulfoxide, 10 mmol/l Tris-HCl (pH 8.3), 1.5 mmol/l MgCl₂, and 50 mmol/l KCl. DNA amplification was performed in the iCycler (Bio-Rad, Hercules, CA) thermal cycler. The 100-bp length PCR products were digested with 5 U *CfoI* (Roche) and resulting fragment sizes were as follows: (65+35) bp when the Ch variant was present, or (47+35+18) bp when it was not. Afterward, Sanger sequencing was also performed for all positive samples.

The role of RFLP in the detection of Christchurch and other *APOE* variants using *CfoI* was discussed in 1994 (12), but *Hha I* (*CfoI*) digestion has been for years the most employed method for *APOE* genotyping, and indeed many laboratories are still using this method to perform the screening of common variants

Fig. 1. Abnormal 109 base-pair in *APOE* genotyping via PCR-RFLP with *CfoI* when Christchurch is present.

E2, E3, and E4 (1–3). The interest of our work lies on alerting the professionals about the possibility of misclassifying patients when the rare APOE Christchurch variant is present. The relevance of this problem is inversely proportional to the Christchurch frequency, but directly proportional to the number of analysis already done by RFLP and never to be repeated. Nowadays, PCR-RFLP method is being replaced by more practical and faster methods such as real-time PCR with specific probes. In these cases, the misclassification problem is solved but APOE Christchurch is not detected (and no other variant, in fact). Because of this, we recommend exon 4 sequencing to unequivocally detect the common 3 variants and the rare variants as well to find out the real role they play in atherosclerosis, cardiovascular disease, and to estimate its real frequency which is nowadays unclear, in part by the small number of cases identified.

Another important aspect in our work is the finding of an unexpected high frequency of Christchurch and its combination with the classical variants. We have found 12 unrelated patients with the rare variant among a cohort of 2560 patients; they suppose a 0.4% of allelic frequency. To our knowledge, there are no articles in the literature that include such a high number (4–6), and in most cases reported patients are related. While the great majority of authors have found the variant in E2 alleles, we have found the variant in E3 as well.

In summary, we want to remark that sequencing should be the way to avoid missgenotyping, as well as the use of allele-specific methods (like those including specific probes) given that routine APOE genotyping via PCR-RFLP with *CfoI* can induce erroneous results, provided an APOECh variant exists.

REFERENCES

1. de Knijff P, van den Maagdenberg AMJM, Frants RR, Havekes LM. Genetic heterogeneity of apolipoprotein E and its influence on plasma lipid and lipoprotein levels. *Hum Mutat* 1994;4:178–194.
2. Hixson JE, Vernier DT. Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with HhaI. *J Lipid Res* 1990;31:545–548.
3. Breslow JL, Zannis VI, SanGiacomo TR, Third JHLC, Tracy T, Glueck CJ. Studies of familial type III hyperlipoproteinemia using as a genetic marker the apoE phenotype E2/2. *J Lipid Res* 1982;23:1224–1235.
4. Weisgraber KH, Innerarity TL, Mahley RW. Abnormal lipoprotein receptor-binding activity of the human E apoprotein due to cysteine-arginine interchange at a single site. *J Biol Chem* 1982;257:2518–2521.
5. Gregg RE, Zech LA, Brewer HB Jr. Apolipoprotein E alleles in severe Hypertriglyceridaemia. *Lancet* 1983;321:353.
6. Eto M, Watanabe K, Makino I. Increased frequencies of apolipoprotein E2 and E4 alleles in patients with ischemic heart disease. *Clin Genet* 1989;36:183–188.
7. Bonay P, Avila J. Apolipoprotein E4 stimulates sulfation of glycosaminoglycans in neural cells. *Biochim Biophys Acta* 2001;1535:217–220.
8. Bennett DA, Wilson RS, Schneider JA, et al. Apolipoprotein E epsilon-4 allele, AD pathology, and the clinical expression of Alzheimer's disease. *Neurology* 2003;60:246–252.
9. Wardell MR, Brennan SO, Janus ED, Fraser R, Carrell RW. New variant of human apolipoprotein E in a patient with type III hyperlipoproteinemia. *J Clin Invest* 1987;80:483–490.
10. Hubáček JA, Adámková V, Skodová Z. Rare variant of apolipoprotein E (Arg136-Ser) in two normolipidemic individuals. *Physiol Res* 2005;54:573–575.
11. Pocovi M, Cenarro A, Civeira F, et al. Incomplete dominance of type III hyperlipoproteinemia is associated with the rare apolipoprotein E2 (Arg136-Ser) variant in multigenerational pedigree studies. *Atherosclerosis* 1996;122:33–46.
12. Richard P, Thomas G, de Zulueta MP, et al. Common and rare genotypes of human apolipoprotein E determined by specific restriction profiles of polymerase chain reaction-amplified DNA. *Clin Chem* 1994;40:24–29.