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Key words

APOE, p.(Leu167del), familial hypercholesterolemia, lipid-lowering treatment

Abstract

Background and aims: The aim of this work was to compared the effect of lipid lowering drugs among FH subjects with a functional mutation in *LDLR* (*LDLR* FH) and FH with the p.(Leu167del) mutation in *APOE*.

Methods: We retrospectively selected all adults with the p.(Leu167del) mutation on lipid-lowering treatment (n=22) attending the Lipid Unit at the Hospital Miguel Servet. Age and sex matched *LDLR* FH from the same Unit were randomly selected as a control group (n=44).

Results: The mean percentage reduction in LDLc was significantly higher in the p.(Leu167del) carriers (-52.1%) than in the *LDLR* FH (-39.7%) (p = 0.040) when on high intensity statins. Similar differences between groups were observed in non-HDLc -49.4 % and -36.4%, respectively (p = 0.030).

*Conclusions:*Subjects with p.(Leu167del) mutation have a higher lipid-lowering response to statins with or without ezetimibe than *LDLR* FH. This supports the use of genetics for a more efficient management of FH.

Introduction

Autosomal dominant hypercholesterolemias (ADH) are monogenic disorders of lipid metabolism characterized by high plasma levels of low-density lipoprotein (LDL) cholesterol, vertical transmission of the hyperlipidemic phenotype within the family and high risk of premature cardiovascular disease (CVD) [1]. Lipid-lowering treatment, usually potent statins or combination treatment, is required to obtain the recommended (LDL) cholesterol goals in most affected subjects [2].

The most common gene responsible for ADH is the *LDLR* gene, coding for the LDL receptor and causing the disorder of familial hypercholesterolemia (FH) (OMIM: #143890). Two other genes, *APOB* and *PCSK9*, have also been found to cause a similar

FH phenotype [3]. ADH are in most cases responsible for the clinical diagnosis of FH. However, in up to 20-30% of FH cases, a causative mutation in candidate genes is not found, and they probably have a polygenic nature [4], and should not be considered ADH in spite of some familial aggregation [5].

Our group and other authors have recently described a new cause of ADH, the p.(Leu167del) mutation in the *APOE* gene [6–8]. This codon deletion, formerly called Δ L149, is a 3-bp inframe deletion that results in the loss of a leucine at position 167 of the receptor-binding region of apolipoprotein E (apo E), which produces a phenotype indistinguishable from classical FH [6–8]. However, the mechanism of hypercholesterolemia in p.(Leu167del) mutation carriers is different from that in FH. Functional mutations in *LDLR*, *APOB* and *PCSK9* genes induce FH by reducing LDL receptor expression or functionality at the cellular surface or interfering with the recognition of the LDL particles by the LDL receptor [9]. The mechanism by which the p.(Leu167del) mutation in *APOE* gene is associated with FH appears to be different. The *in vitro* cultured cell studies demonstrate that very low-density lipoprotein (VLDL) particles carrying apo E with the p.(Leu167del) mutation have a higher uptake by HepG2 and by THP-1 cells and, subsequently, LDL receptor expression is down-regulated. The decrease of LDL receptor expression at surface membrane of hepatocytes would result in a decrease in LDL internalization, an increase in LDL particles circulating in plasma and therefore an increase in LDL cholesterol levels [10]. Since VLDL contains multiple copies of apo E, this gain-of-function *APOE* mutation binds to the LDL receptors with higher affinity than LDL, which contains only one copy of apo B [10].

Potent statins are the most recommended treatment for adults with FH [2,11]. The effect of statins has been very well demonstrated in heterozygous subjects with

mutations in *LDLR*, *APOB* and *PCSK9* [12,13]. However, whether the lipid-lowering response is similar in subjects with the p.(Leu167del) mutation in *APOE* is still unknown. The different mechanism of LDL cholesterol production could also be associated with a different treatment response. In this retrospective case-control study, we have identified all carriers of the p.(Leu167del) mutation under treatment and we have compared the lipid lowering effects with respect to FH in subjects with a functional mutation in the *LDLR* gene.

2. **Materials and methods**

2.1 Subjects

2.1.1 *APOE* p.(Leu167del) carriers

All adult patients with the p.(Leu167del) mutation on lipid-lowering treatment (n=22) attending the Lipid Unit at the Hospital Universitario Miguel Servet, Zaragoza, Spain were retrospectively studied. We routinely screen for this mutation in all patients attending our Unit with a clinical diagnosis of primary hyperlipidemia.

2.1.2 *LDLR* heterozygous carriers

Age and sex matched subjects with genetically defined heterozygous FH from the same Unit were randomly selected as control group (n=44). Two controls were selected for each case. All controls were heterozygous carriers of a functional mutation in *LDLR* and were attending in the same period frame than cases.

2.2 Biochemical analysis

Ethylenediaminetetraacetic acid plasma and serum samples were collected from all participants after at least 10 hours of fasting, after 6 weeks without lipid-lowering drugs, to obtain baseline biochemical characteristics. Annual subsequent lipid analysis with lipid lowering drugs was recorded. In case that a subject had two or more lipid results in

the same year, the last one of that same year was used. Total cholesterol and triglyceride levels were determined by standard enzymatic methods. High-density lipoprotein cholesterol was measured directly by an enzymatic reaction using cholesterol oxidase (UniCel DxC 800; Beckman Coulter Inc., Brea, California, United States). Lipoprotein(a) (Lp(a)), apo A1, apo B, and C-reactive protein (CRP) were determined by IMMAGE kinetic nephelometry (Beckman Coulter Inc.). LDL cholesterol was calculated using the Friedewald's formula.

2.3 Genetic diagnosis

DNA was isolated from EDTA blood samples following standard protocols. *LDLR*, *APOB* and *PCSK9* genes were analyzed for functional mutations with Lipochip® platform (Progenika Grifols, Spain) [14]. Exon 4 of the *APOE* gene was sequenced in all participants, as previously described [15].

2.4 Follow-up

All patients underwent checkups 1 or 2 times a year. Throughout the study, hypercholesterolemia was treated in accordance with the recommendations of the International Panel on Management of FH [16], which established the therapeutic target as a reduction in LDL cholesterol and/or non– high-density lipoprotein cholesterol (non–HDL cholesterol), according to risk factors. Since November 2013, the therapeutic target for patients with CVD or diabetes has been amended to LDL cholesterol < 70 mg/dL. All participants received checkups through a face-to-face interview [17]. Statin use was adjusted to equivalents of rosuvastatin [11]. All participants gave written consent before participating in the protocol, which was approved by the Clinical Research Ethics Committee of Aragón, Spain.

2.5 Statistical analyses

Analyses were performed using SPSS version 20.0 (Chicago, Illinois, United States). The nominal level for significance was $p < 0.05$. Normal distribution of variables was analyzed with the Kolmogorov–Smirnov test. Numerical variables with normal distribution are expressed as mean ± standard deviation and those with skewed distribution are expressed as median [percentile 25-percentile 75]. We used Student's t or Mann-Whitney tests to assess differences among two categories quantitative variables while Chi-squared or Fisher tests were used for categorical variables as appropriate.

3. Results

A total of 66 subjects, 22 carriers of the p.(Leu167del) mutation in *APOE* gene and 44 heterozygous FH carriers of an *LDLR* functional mutation (*LDLR* FH) were included in the study. Most of the clinical, anthropometric and lipid baseline characteristics did not differ between groups (Table 1). Mean age was 43 years in both groups. The distribution of *APOE* common polymorphisms between cases and controls did not differ either. However, all p.(Leu167del) carriers were ε3/ε3, thus this codon deletion in *APOE* was in linkage disequilibrium with the ε3 allele. The p.(Leu167del) carriers presented triglycerides and CRP concentrations significantly higher than *LDLR* FH, ($p < 0.001$, and $p < 0.03$, respectively). Lp(a) was significantly lower in p.(Leu167del) carriers ($p = 0.002$). The rest of the clinical and biochemical parameters did not differ between the two groups (Table 1).

Mean follow-up under lipid-lowering drugs was 4 years in p.(Leu167del) carriers and 3 years in *LDLR* FH, without differences regarding treatment time between groups ($p = 0.330$). Table 2 describes lipid values at the last visit during follow-up. Lipid parameters substantially improved at this visit. All subjects were on stable dose of

statin with or without ezetimibe at follow-up for at least 3 months. They did not differ in any biochemical characteristic including lipid parameters between p.(Leu167del) carriers and *LDLR* FH subjects at the final visit. Both groups obtained substantial reductions in their lipid values without differences between groups. However, the p.(Leu167del) carriers obtain similar LDL cholesterol reductions in spite of significantly lower dose of statins and lower use of ezetimibe than *LDLR* FH ($p = 0.044$ and $p = 0.022$, respectively) (Table 2).

The lipid-lowering effect of the different statins with or without ezetimibe showed important differences between groups. For this analysis, all annual lipid results under different treatments were used, with only one analysis per treatment per subject. Because only two subjects, both p.(Leu167del) carriers, were on low intensity statins, low and moderate intensity statin groups were put together in a single group. A total of 36 results with low/moderate intensity statins, 28 results with high intensity statins and 30 results with combination of high intensity statins and ezetimibe were analyzed (Supplemental table 1).

The mean percentage reduction in LDL cholesterol was significantly higher in the p.(Leu167del) carriers (-52.1%) than in the *LDLR* FH (-39.7%) ($p = 0.040$) when on high intensity statins. Similar differences between groups were observed in non-HDL cholesterol -49.4 % and -36.4%, respectively ($p = 0.030$). We also observed an important significative reduction in triglycerides between groups in patients with low/moderate and high intensity statins (Fig.1). There were not differences in weight, total cholesterol, HDL cholesterol and apo A1 between groups with any treatment group (Supplementary Table 1). To be able to rule out the difference effect is not due to the well-known non-lineal dose response association, we have compared the lipid lowering effect with the same simvastatin and rosuvastatin dose in the p.(Leu167del) carriers and

LDLR FH. The mean percentage reduction in non-HDL cholesterol was significantly higher in the p.(Leu167del) carriers (-52.7%) than in the LDLR FH (-34.1%) (p = 0.048) when on simvastatin 40 mg/dL. Similar differences were observed in triglycerides -43.2 % and -6.63 %, respectively (p = 0.016).

4. Discussion

This study analyzes for the first time the effect of lipid-lowering treatment in subjects with the p.(Leu167del) mutation in *APOE* and shows a different effect with respect to a heterozygous FH population with a functional mutation in *LDLR*. We have analyzed the lipid-lowering response of a treatment based on statins, with or without ezetimibe, the most recommended drug treatment in these subjects [2,11]. The p.(Leu167del) mutation in *APOE* represents the fourth cause of FH together with functional mutations in *LDLR*, *APOB* and *PCSK9* genes. In our population, it is the cause of approximately 3% of clinically defined FH in whom *LDLR*, *APOB*, and *PCSK9* mutations were not found [10]. This prevalence of mutation carriers, although much lower than *LDLR* mutations, is higher than that observed for the *PCSK9* mutation causing FH, and similar to the *APOB* mutations [14].

The therapeutic response in this form of FH shows that these subjects have a lipid-lowering response higher than that observed in the most common FH population with functional mutation in *LDLR*, with an average lowering response in LDL cholesterol 12.4% higher. Consequently, FH carriers of the p.(Leu167del) mutation would needed lower doses of statins for LDL cholesterol control than *LDLR* FH. Interestingly, p.(Leu167del) carriers have higher TG baseline and more intense lipid-lowering drugs response for LDL cholesterol and TG. The more likely explanation of this mechanism is that there is a hypercatabolism of the small and medium VLDL

without modifying or decreasing the catabolism of large VLDL. Actually, apo E has an important lipolytic effect [18] that could be modified in p.(Leu167del) carriers. In addition, statins have key activities involved in apo E-dependent VLDL metabolism, which could be involved in the greater response to treatment [19]. On the other hand, p.(Leu167del) carriers have lower Lp(a) baseline. It has been demonstrated that *APOE* genotype has a strong influence on Lp(a) levels [20]. The p.(Leu167del) mutation seems to stimulate the Lpa particle clarification, which indicates this gain of function mutation is not limited to VLDL and remnants.

Several important considerations can be drawn from our work. First, it points out the importance of the *APOE* gene in the therapeutic response to statins. It is well known and confirmed by several studies and meta-analysis that part of the interindividual variability found in response to statins is explained by the *APOE* genotype. In this way, subjects carrying the *APOE* ε4 allele obtain lower and carriers of the ε2 alleles obtain greater LDL cholesterol reductions with statins in comparison with the ε3 carriers [21]. The mechanism of this difference is not well established but it has been speculated that *APOE* genotypes affect lipid-lowering response because of the differences in binding of apo E isoforms to lipoprotein receptors [22]. We speculate that the higher lipid-lowering response in p.(Leu167del) carriers is due to the fact that this mutant *APOE* has a markedly increased binding affinity as compared with apo E3, and statin treatment reduces the synthesis of VLDL particles and increase the uptake of LDL by increasing the expression of receptors [23], hence reducing circulating lipoproteins. Subjects with the p.(Leu167del) mutation have normal *LDLR* and *APOB* alleles, so an increase in LDL receptors would have a greater impact than in *LDLR* FH patients. In addition, the reduction of VLDL particle synthesis with statins could have a greater effect on subjects

with the p.(Leu167del) mutation. The reduction of triglycerides is more intense in FH subjects with p.(Leu167del) mutation than with functional mutations in *LDLR*.

Secondly, our results provide another argument for the indication of genetic studies in familial hypercholesterolemia. The knowledge of the genetic bases does not only confirm the diagnosis and facilitates the identification of affected relatives, but it could modulate treatment. The European and American guidelines [2,11] indicate high doses of high potency statins in suspected genetic hypercholesterolemia with LDL cholesterol values >190 mg/dL. Our results would support that in the presence of the p.(Leu167del) mutation, a less intensive treatment from the beginning and escalating doses according to response could be reasonable, since the patients obtain a mean 50% reduction in LDL cholesterol with moderate intensity statins.

Our study has some limitations: it is a retrospective study and not all subjects had the same statin treatment. On the other hand, the number of subjects recruited in this study is not very high, however, subjects with p.(Leu167del) mutation are approximately only 3% of clinically defined FH without mutations in candidate genes, which limits the availability of thesubjects. To overcome this limitation, two LDLR FH were selected for each case matched by sex and age, which increased the statistical power of the study.

In summary, subjects with p.(Leu167del) mutation, a rare form of FH, seem to have a higher lipid-lowering response to statins with or without ezetimibe than FH subjects with a functional mutation in *LDLR*. Our results postulate the importance of *APOE* in response to statins, the different effect of statins depends on the mechanism of production of dyslipidemia, and indicates the use of genetics tools for a more efficient management of subjects with FH. New studies along this line are mandatory to clarify the results on *APOE* in lipids metabolism.

Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

AMB, ILM, VMB, SPC, RMG, CM and EJ this conducted research. AMB, ILM and RMG analyzed data. AMB and FC contributed to the writing of the article. AMB, FC and AC contributed to the research design and had the primary responsibility for final content. All authors participated in acquisition, analysis and interpretation of the data, in the drafting of the article or critical revision, and in the final approval of the version to be published.

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Fig. 1. Variation (%) in LDL cholesterol, non-HDL cholesterol, triglycerides and apolipoprotein B in p.(Leu167del) mutation carriers and heterozygous FH subjects with mutation in *LDLR* divided by lipid-lowering treatment intensity.

Quantitative variables with normal distribution are expressed as mean ± standard deviation. The *p* value was calculated by Student's t test.

Table 1. Baseline clinical and biochemical characteristics of study subjects with the *APOE* p.(Leu167del) mutation and matched FH with *LDLR* functional mutation.

Variable	**p.(Leu167del) FH n = 22**	***LDLR* FH n = 44**	***p***
Age, years	43.0 ± 14.1	43.0 ±14.3	0.942
Men, n (%)	9 (40.9)	18 (40.9)	1.000
Smoker, n (%)	5 (22.7)	11 (25.0)	0.943
Non smoker, n (%)	12 (54.5)	21 (47.7)	
Former smoker, n (%)	5 (22.7)	12 (27.3)	
Previous cardiovascular disease, %	2 (9.10)	3 (7.00)	0.754
Type 2 diabetes, n (%)	2 (9.10)	1 (2.30)	0.256
Hypertension, n (%)	4 (18.2)	4 (9.10)	0.425
Corneal arcus, n (%)	8 (36.4)	20 (45.5)	0.481
Tendon xanthoma, n (%)	0	7 (15.9)	0.086
Body mass index, kg/m^2	26.0 ± 5.07	24.3 ± 4.25	0.159
Total cholesterol, mg/dL	345 ± 85.0	346 ± 70.1	0.959
LDL cholesterol, mg/dL	255 ± 75.4	267 ± 65.6	0.539
HDL cholesterol, mg/dL	62.0 ± 22.4	58.0 ± 12.8	0.422
Non-HDL cholesterol, mg/dL	283 ± 72.4	287 ± 73.7	0.875
Triglycerides, mg/dL	146 (119 -282)	101 (73.3 – 126)	<0.001
Apolipoprotein A1, mg/dL	284 ± 72.4	287 ± 74.0	0.074
Apolipoprotein B, mg/dL	169 ± 31.4	154 ± 28.3	0.506
Lipoprotein (a), mg/dL	7.41 (1.50 – 29.0)	38.0 (11.0 – 69.2)	0.002
Glucose, mg/dL	85.0 (80.0 – 94.0)	88.0 (81.0 – 94.0)	0.543

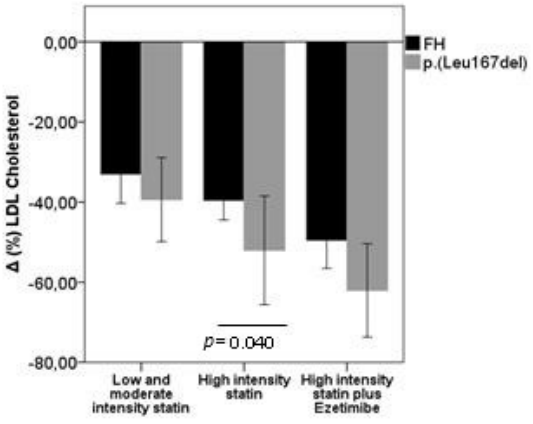
C reactive protein, g/L		2.10 (1.25 – 2.90)	1.05 (0.40 – 3.00)	0.030
APOE genotype, n (%)	ε3/3	22 (100)	31 (72.1)	0.090
	ε3/2	0	4 (9.30)	
	ε3/4	0	6 (14.0)	
	ε4/4	0	1 (2.30)	
	ε2/4	0	1 (2.30)	

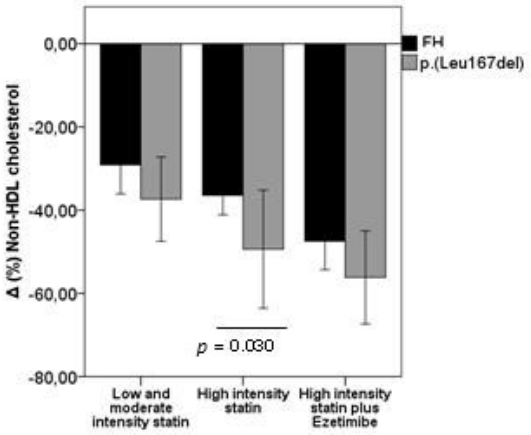
Quantitative variables are expressed as mean ± standard deviation, except for variables not following the normal distribution, expressed as median (interquartile range). Qualitative variables are expressed as n (%). The *p* value was calculated by Student's t test, Mann-Whitney U and Chi - square as appropriate.

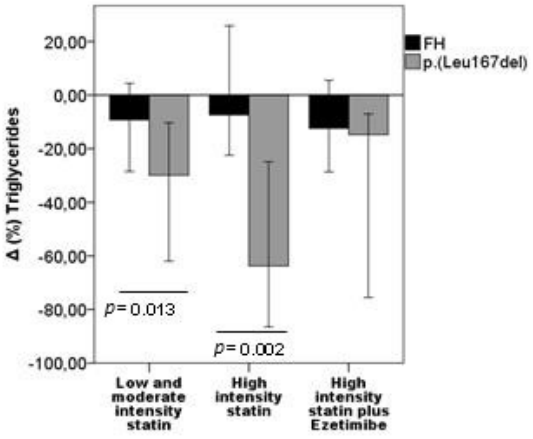
Table 2. Clinical and biochemical characteristics of study subjects with the *APOE* p.(Leu167del) mutation and matched *LDLR* FH at the final visit.

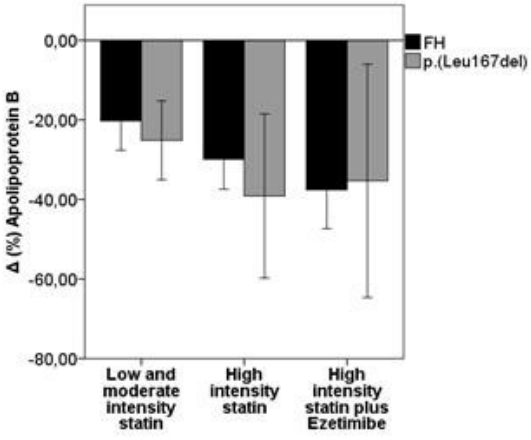
Variable	**p.(Leu167del) carriers n= 22**	***LDLR* FH n= 44**	***p***
Weight, kg	74.1 ± 10.5	67.9 ± 13.1	0.119
Total cholesterol, mg/dL	215 ± 51.8	215 ± 34.6	0.997
LDL cholesterol, mg/dL	127 ± 39.1	137 ± 31.0	0.285
HDL cholesterol, mg/dL	69.1 ± 20.5	59.3 ± 14.3	0.065
Non-HDL cholesterol, mg/dL	146 ± 40.6	156 ± 33.3	0.325
Triglycerides, mg/dL	96.1 ± 31.0	96.4 ± 37.7	0.976
Apolipoprotein A1, mg/dL	175 ± 50.0	162 ± 33.5	0.345
Apolipoprotein B, mg/dL	108 ± 24.0	111 ± 25.9	0.738
Follow-up, years	4 (2.0-5.25)	3 (2.0-4.0)	0.330
Rosuvastatin equivalent dose, mg/day	5.00 (5.00-10.0)	10.0 (5.00-20.0)	0.044
Ezetimibe use, n (%)	7 (31.8)	27 (61.4)	0.022

Quantitative variables are expressed as mean ± standard deviation, except for variables not following the normal distribution, expressed as median (interquartile range). Qualitative variables are expressed as %. The *p* value was calculated by Student's t test, Mann-Whitney U and Chi - square as appropriate.


0,00
-20,00
-40,00
-60,00
-80,00
Δ (%) LDL Cholesterol
FH
p.(Leu167del)
p= 0.040
Low and moderate intensity statin
High intensity statin
High intensity statin plus Ezetimibe


0,00
-20,00
-40,00
-60,00
-80,00
Δ (%) Non-HDL cholesterol
FH
p.(Leu167del)
p = 0.030
Low and moderate intensity statin
High intensity statin
High intensity statin plus Ezetimibe


20,00
0,00
-20,00
-40,00
-60,00
-80,00
-100,00
Δ (%) Triglycerides
FH
p.(Leu167del)
p= 0.013
p= 0.002
Low and moderate intensity statin
High intensity statin
High intensity statin plus Ezetimibe


0,00
-20,00
-40,00
-60,00
-80,00
Δ (%) Apolipoprotein B
FH
p.(Leu167del)
Low and moderate intensity statin
High intensity statin
High intensity statin plus Ezetimibe