Clinical Science (2016) **130**, 2053–2059 doi: 10.1042/CS20160433

Original Paper

APOA5 genetic and epigenetic variability jointly regulate circulating triacylglycerol levels

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Abstract

Apolipoprotein A5 gene (*APOA5*) variability explains part of the individual's predisposition to hypertriacylglycerolaemia (HTG). Such predisposition has an inherited component (polymorphisms) and an acquired component regulated by the environment (epigenetic modifications). We hypothesize that the integrated analysis of both components will improve our capacity to estimate *APOA5* contribution to HTG. We followed a recruit-by-genotype strategy to study a population composed of 44 individuals with high cardiovascular disease risk selected as being carriers of at least one *APOA5* SNP (-1131T>C and/or, S19W and/or 724C>G) compared against 34 individuals wild-type (WT) for these SNPs. DNA methylation patterns of three APOA5 regions [promoter, exon 2 and CpG island (CGI) in exon 3] were evaluated using pyrosequencing technology. Carriers of *APOA5* SNPs had an average of 57.5% higher circulating triacylglycerol (TG) levels ($P = 0.039$). *APOA5* promoter and exon 3 were hypermethylated whereas exon 2 was hypomethylated. Exon 3 methylation positively correlated with TG concentration ($r = 0.359$, $P = 0.003$) and with a lipoprotein profile associated with atherogenic dyslipidaemia. The highest TG concentrations were found in carriers of at least one SNP and with a methylation percentage in exon 3 $\geqslant$82% ($P = 0.009$). In conclusion, CGI methylation in exon 3 of *APOA5* acts, in combination with -1131T>C, S19W and 724C>G polymorphisms, in the individual's predisposition to high circulating TG levels. This serves as an example that combined analysis of SNPs and methylation applied to a larger set of genes would improve our understanding of predisposition to HTG.

Key words: APOA5, DNA methylation, missing heritability, polymorphisms, triacylglycerols.

INTRODUCTION

Epigenetics refers to genomic modifications in response to environmental stimuli that regulate gene expression, without changing the DNA sequence. These epigenetic modifications regulate chromatin structure and DNA accessibility [1]. DNA methylation is one of the most-studied epigenetic markers [2] and results from the covalent union of a methyl group at the fifth carbon of cytosines located upstream of guanines (CpG dinucleotides). DNA methylation is generally associated with a compact chromatin state and inhibition of gene transcription. Mammalian genomes are globally poor in CpG, except at short DNA regions called CpG islands (CGI), which are frequently associated with gene promoters [3]. Cytosine methylation patterns are tissue-specific. Nonetheless, considerable effort has been directed towards validating blood DNA methylation patterns as a reliable disease marker not only in several types of cancer [4] but also in metabolic pathologies such as obesity [5] and diabetes [6].

Hypertriacylglycerolaemia (HTG) is a complex and polygenic pathology highly influenced by environment, and the genetic variability only corresponds to a ~10–20% of the individual's predisposition [7–9]. Within this genetic variability, in the case of HTG, only a 25–30% is explained by known and novel common variations in key genes [10,11]. This is explained, in part by the so-called 'missing' heritability which is thought to correspond to common single nucleotide polymorphisms (SNPs) not yet identified as well as rare variants, structural alterations in genes, epistasis (gene–gene interactions) and epigenetic modifications [12].

Apolipoprotein A5 gene (*APOA5*) has been recognized as one of the main genetic determinants of triacylglycerols (TG) [13,14] and is located in the apolipoprotein A1/C3/A4/A5 gene cluster on chromosome 11q23. Apolipoprotein AV (APOAV) is

Abbreviations: APOA5, apolipoprotein A5 gene; APOAI, apolipoprotein AI; APOAV, apolipoprotein AV; APOB100, apolipoprotein B100; CGI, CpG island; GWAS, genome wide association study; HDLc, HDL cholesterol; HTG, hypertriacylglycerolaemia; LDLc, LDL cholesterol; RLPc, remnant-like particle cholesterol; TC, total cholesterol; TG, triacylglycerol; WT, wild-type.

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synthesized and secreted from the liver and reduces plasma TG but the exact mechanism of action is unclear. Inhibition of very low density lipoprotein (VLDL) hepatic production, stimulation of lipoprotein lipase activity and increase in hepatic receptor-mediated uptake of TG-rich lipoproteins are the most plausible mechanisms [15–17]. Association between *APOA5* SNPs and TG in humans is well documented, and S19W (rs3135506) and -1131T>C (rs662799) define the two main haplotypes that have been associated most closely with TG [18]. In addition, another SNP has been recently identified by genome-wide association studies (GWAS) to be significantly associated with high TG and to be one of the most important signals associated with coronary artery disease, the 724G>C (rs964184) which is located in the *APOA5-ZNF259* region [19–21].

We hypothesize that the integrated analysis of the genetic and the epigenetic modifications will improve our capacity to estimate *APOA5* contribution to HTG.

MATERIALS AND METHODS

Study population

Participants ($n = 78$) for the current study were selected among those subjects attending the Vascular Medicine and Metabolism Unit of the Hospital Universitari Sant Joan (a population around 1000 patients) de Reus in Spain. Participants are middle-aged individuals with high cardiovascular risk, and those presenting any of the following conditions were excluded: prior coronary heart disease; cerebral or peripheral vascular disease; and cancer, hepatic, renal, lung, endocrine or inflammatory chronic diseases. Patients on lipid lowering drugs had a wash-out period of 6 weeks (8 weeks if they were receiving fibrates). Anamnesis and physical examination data were recorded.

Following the latest published data about APOA5 methylation levels, we calculated the optimal sample size to detect methylation differences of 10% with an 8% standard deviation, and a statistical power of 80%. A total of 78 individuals were included following a recruit-by-genotype approach.

The Hospital Ethical Committee approved the study and all patients gave their written consent to participate. Data were coded for anonymity in accordance with current Spanish law on biomedical research.

Blood samples collection and storage

An overnight fasting blood sample was obtained from each individual. The cellular buffy coat was obtained and stored at −80 °C until DNA analyses were performed. DNA was extracted with the DNeasy Blood & Tissue Kit (Qiagen).

Biochemical analyses

Plasma was separated from an overnight fasting blood sample and frozen at −80 °C. Total cholesterol (TC), HDL cholesterol (HDLc), LDL cholesterol (LDLc), TG, apolipoprotein B100 (APOB100), apolipoprotein AI (APOAI) were measured using standard enzymatic and colorimetric techniques adapted to a Cobas Mira autoanalyser (Roche Diagnostics). Remnant-like particle cholesterol (RLPc) was measured in plasma using RLP-Cholesterol Assay Kits (Jimro-II, Japan Immunoresearch Laboratories) according to the manufacturer's instructions [22].

NMR lipoprotein profile

Distribution of lipoprotein plasma subclasses were analysed by NMR in a Vantera clinical spectrometer, produced by LipoScience. The NMR LipoProfile test by LipoScience involves measurement of the 400 MHz proton spectrum of samples and uses the characteristic signal amplitude of the lipid methyl group broadcast by every lipoprotein subfraction as the basis for quantification. NMR using the LipoProfile-3 algorithm was performed to quantify the average particle size and concentrations of VLDL, LDL and HDL. Subparticle concentrations were determined for three VLDL subclasses (large or chylomicrons, medium and small), three LDL subclasses (large, medium and small) and three HDL subclasses (large, medium and small). The NMR was carried out on EDTA plasma stored at −80 °C and thawed just prior to the analysis.

SNP selection and genotyping

A recruit-by-genotype approach was followed by screening more than 1000 individuals to select 78 individuals; 44 carriers of at least one of these *APOA5* polymorphisms (S19W, -1131T>C and 724C>G) and 34 individuals wild-type (WT) for these polymorphisms. In that way, we were able to increase the three SNPs frequency in our study population (from 0.05 to 0.13, from 0.02 to 0.15 and from 0.12 to 0.29 respectively) and reducing the necessary sample size. S19W and -1131T>C are the most-studied *APOA5* polymorphisms and both have been strongly associated with TG [18]. In contrast, 724C>G has been recently identified by GWAS as an important determinant of TG [19–21]. See supplementary Figure S1 for a schematic view of the SNP location in *APOA5*. The criterion used to select the individuals was to be carrier of at least one rare allele of these polymorphisms for the Carrier group and not to be carrier of any one for the WT group. Genotyping was performed by CEGEN (Spanish National Genotyping Center) using the Sequenom MassARRAY platform.

Methylation patterns

We used the USCS Server (http://genome.ucsc.edu/) to select regions-of-interest in *APOA5*; the CGI in exon 3, the region in exon 2 likely to be regulating histone methylation and the gene promoter for its central role in genetic transcription control (Supplementary Figure S1).

Bisulfite PCR and sequencing

Approximately 1 μg DNA was subjected to sodium bisulfite treatment and purified using the EZ GOLD methylation kit (ZYMO). Bisulfite specific primers for each region were used with Hotstar Taq polymerase (Qiagen) for 45 PCR cycles. All PCR products were cloned into pGEM-T Easy vector (Promega) with a minimum of 12 clones selected for sequencing. All primers for bisulfite PCR can be found in Supplementary Table S1.

Methylation pyrosequencing

Standard bisulfite PCR was used to amplify regions of interest; the characteristic being that one primer was biotinylated. The entire biotinylated PCR product (diluted to 40 μl) was mixed with 38 μl of binding buffer and 2 μl (10 mg/ml) streptavidin-coated polystyrene beads. Bead-amplicon complexes were captured on a vacuum prep tool (Qiagen) and the PCR products denatured using 0.2 mol/l NaOH. The denatured DNA was re-suspended in 40 pmol of sequencing primer dissolved in 12 μl water and primer annealing was achieved by heating the sample to 80 °C for 2 min before cooling to room temperature. For sequencing, an internal primer was designed to the complementary strand. The pyrosequencing reaction was performed on a PyroMark Q96 instrument. The peak heights at C/T variants of CpG dinucleotides were determined using the pyrosequencing commercial software (Biotage). Primers for methylation pyrosequencing can also be found in Supplementary Table S1.

Statistical analysis

Pyrosequencing analyses are expressed as percentage of methylation ranging from 0 % (unmethylated) to 100 % (fully methylated) at each CpG position. The average methylation was tested for association with the polymorphisms and lipid-related variables. TG-related variables which did not follow a normal distribution (tested by Kolmogorov–Smirnov) were log-transformed before the statistical analyses. Relationships between lipid-related variables and mean methylation values were determined by partial correlations, adjusted for age, gender and BMI. Associations between lipid variables and genotypes were evaluated using ANOVA. Linear regression models were used to evaluate TG variability. Significant differences between models were assessed by changes in R^2 in an enter block model. All statistical analyses were adjusted for age, gender and BMI. The SPSS package version 19.0 (IBM) was used throughout. A value of $P < 0.05$ was considered significant.

RESULTS

Patients

We studied 78 middle-aged dyslipaemic individuals at high cardiovascular disease risk from metabolic alterations such as diabetes (51 %), obesity (40 %), hypertension (33 %) or metabolic syndrome (60 %). There were no significant differences in genotype frequencies among disease types. Baseline data of the study sample are shown in Table 1.

APOA5 polymorphisms and triacylglycerols

Of the 78 individuals, 44 were carriers of *APOA5* SNPs; 21 were carriers of S19W; 21 were carriers of -1131T>C; 42 were carriers of 724C>G. The genotype distributions are presented in Table 2. Almost 80 % of carriers were S19W/724C>G heterozygotes or -1131T>C/724C>G heterozygotes. The WT and the Carrier groups were comparable with respect to age, BMI, gender and all lipids except for TG concentrations (Table 1). *APOA5* Carriers had 57.5 % higher TG on average ($P = 0.039$) than WT.

Table 1 Baseline data of subjects and comparisons between WT and carriers groups

Values are expressed as mean (S.D.). *P value is adjusted by age, gender and BMI.

	All, $n = 78$	WT, $n = 34$	Carriers, $n = 44$	P *
Age, years	53.7 (10.8)	55.0 (10.9)	52.0 (10.7)	ns
Gender, % women	35.9	38.0	34.0	ns
BMI, kg/m^2	29.8 (5.9)	29.40 (6.9)	30.2 (5.1)	ns
TG, mmol/l	1.8 (1.5)	1.4 (0.9)	2.2 (1.8)	0.039
TC, mmol/l	5.4 (1.1)	5.4 (1.1)	5.4 (1.1)	ns
HDLc, mmol/l	1.1 (0.3)	1.1 (0.4)	1.1 (0.3)	ns
LDLc, mmol/l	3.5 (0.9)	3.6 (1.0)	3.4 (0.9)	ns
APOAI, mg/dl	135.6 (25.4)	136.8 (28.2)	134.7 (23.4)	ns
APOB100, mg/dl	117.5 (32.8)	118.2 (34.5)	116.9 (31.9)	ns
RLPc, mg/dl	10.75 (8.57)	9.40 (5.84)	11.79 (10.1)	ns

Table 2 Genotype distribution by SNP in carriers, and the corresponding TG levels

TG values are expressed as mean (S.D.)

	Individuals, N	Frequency, %	TG, mmol/l
S19W	21	26.9	1.8 (0.4)
−1131T>C	21	26.9	2.7 (1.7)
724C>G	42	46.2	2.2 (1.8)

Although alleles were not evenly distributed among our 44 carriers, circulating TG levels increased in an allele dose-dependent manner ($P = 0.025$) (Supplementary Figure S2). Analysis of the effect of allele distribution on TG levels showed no statistically significant differences among genotypes in the Carrier group (Supplementary Table S2).

APOA5 methylation and triacylglycerols

A total of 17 CpGs located within three *APOA5* regions were analysed: two CpGs in the promoter region, five in exon 2 and ten in the CGI in exon 3. The global methylation patterns of the three regions and the specific individual CpG methylation percentage are showed in Supplementary Table S3.

The promoter region is hypermethylated with a methylation range between 48.9 and 93.2 % (median: 87.8 %, IQR: 7.4 %). Exon 2 is hypomethylated with a range in the five CpGs of between 4.9 and 13.1 % (median: 7.9 %, IQR: 1.9 %). These regions and their individual CpGs showed no statistically significant correlations with circulating TG levels or any other lipid parameter.

CGI in exon 3 is hypermethylated with a methylation range among its ten CpGs of between 58.0 and 91.6 % (median: 83.3 %, IQR: 11.6 %). This region was positively correlated with plasma TG ($r = 0.359$, $P = 0.003$); circulating RLPc levels ($r = 0.279$, $P = 0.023$); total VLDL particles ($r = 0.263$, $P = 0.044$) and

Table 3 Linear regression models to explain TG variability

Adjusted R^2 denotes the adjusted proportion of the variance explained by the model. *p* denotes the significance of the model; *B* denotes the variable estimate; *P* denotes the significance of the variable.

		Variable	R^2	*p*	*B*	*P*
Model 1			0.278	<0.001		
		Age			0.007	0.027
		Gender			0.113	NS
		BMI			0.020	0.001
Model 2			0.319	<0.001		
	Model 1 +	WT compared with SNP			0.134	0.039
Model 3			0.347	<0.001		
	Model 1 +	Exon 3 methylation			0.016	0.003
Model 4			0.353	<0.001		
	Model 2 +	Exon 3 methylation			0.015	0.008

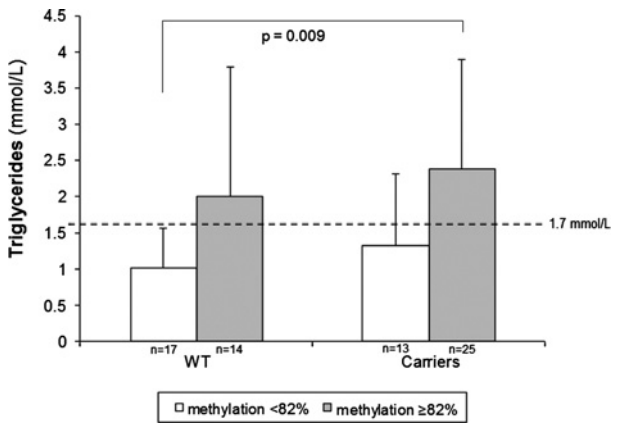

Figure 1 Circulating TG levels in relation to *APOA5* genotype and percentage methylation in exon 3

Dashed line indicates the value at which TG levels are considered of pathological significance. Standard deviation bars are included.

its largest subclasses [large and medium VLDL ($r = 0.312$, $P = 0.016$)]; and total LDL particles ($r = 0.261$, $P = 0.045$) and its smallest subclasses [medium LDL ($r = 0.628$, $P = 0.04$), small LDL ($r = 0.307$, $P = 0.018$)], and with small HDL ($r = 0.272$, $P = 0.037$). This region also shows an inverse correlation with large HDL ($r = -0.315$, $P = 0.015$).

We also checked for correlations between methylation, BMI and waist circumference. A positive correlation was observed between CGI methylation and BMI ($r = 0.416$, $P < 0.001$) and waist circumference ($r = 0.256$, $P = 0.038$).

Combined effect of *APOA5* polymorphisms and methylation patterns

Whether there was an additive effect of *APOA5* methylation and polymorphisms on TG plasma levels was assessed. Methylation at the promoter and exon 2 was comparable between WT and Carrier but, nonetheless, we observed a higher degree of methylation in exon 3 in Carrier group compared with WT (WT mean: 78.8%, IQR: 11.7% compared with Carrier mean: 85.3%, IQR: 10.12%; $P = 0.027$).

The highest TG concentrations were found in Carriers of *APOA5* SNPs with ⩾82% methylation in exon 3; the TG in such group being 2.35-fold higher than in WT with <82% methylation in exon 3 ($P = 0.009$) (Figure 1).

We generated four models to explain TG variability (Table 3). Gender, BMI and age explained 27.8% of TG variability. However, adding *APOA5* polymorphisms to the first model increased the prediction value by 4%. Adding CGI methylation to the first model increased the prediction value by 7%. Adding CGI methylation and *APOA5* polymorphisms together (third model) increased the TG prediction value by 7.5%, relative to the model involving gender, BMI and age alone (Figure 2).

DISCUSSION

Unlike rare familial syndromes associated with severe HTG that are caused by mutations with a large effect of a few specific genes, most cases of HTG are regulated by a complex interaction of multiple known and unknown genes in concert with environmental factors. The known common genetic variants have a limited role in the overall genetic variation in HTG, suggesting that there are other factors that would help reveal the true genetic contributions.

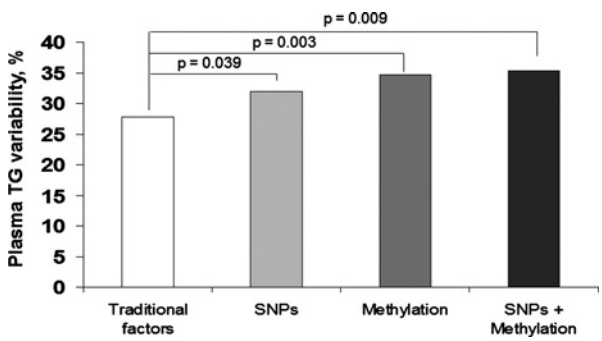

Figure 2 Percentage of plasma TG variability explained in four regression models: using traditional factors (gender, BMI and age) and with the addition of the contributions of SNPs and/or methylation in the CGI

The aim of our study was to demonstrate that, in the genetic control of TG levels, there is an additive effect resulting from the degree of DNA methylation and genetic polymorphisms. Hence, we studied a key gene in the regulation of plasma TG, the *APOA5* gene. We report here that CGI methylation in exon 3 of *APOA5* acts, in combination with -1131T>C, S19W and 724C>G polymorphisms, in contributing to the individual's predisposition to high levels of circulating TG.

We had earlier reported that the effect of *APOA5* depends, largely, on the metabolic background of the subject [23]. Here, we report on a group of patients with altered lipid metabolism, patients with type 2 diabetes and metabolic syndrome. To obtain sufficient statistical power, we also followed a strategy of genotype-driven recruitment. Thus, the patients were selected for their genotype of the most relevant *APOA5* polymorphisms: -1131T>C, S19W or 724C>G. Briefly, -1131T>C gene variant is located in the *APOA5* promoter region and has been shown, repeatedly, to be associated with increased TG levels due to a cooperation with two other polymorphisms in *APOA5* (-3A>G and 1891T>C) [24]. The second genotype chosen was S19W in exon 2 which induces an amino acid change in the N-terminal extreme of APOAV that leads to reduced apolipoprotein activity [25]. Finally, the 724C>G gene variant was chosen which, despite little being known on its functionality, has been strongly linked with HTG levels in a GWAS aimed at analysing genetic variants influencing plasma lipid concentrations in a study involving almost twelve thousand subjects [8,26].

As expected, and in concordance with other studies [18,27,28], our results showed that being a carrier of at least one of the *APOA5* polymorphisms studied, increases 1.57-fold the TG levels; a confirmation that *APOA5* variants influence circulating TG levels even though APOAV presence in plasma is very low compared with other apolipoproteins [29]. We also described that circulating TG levels increased in an allele dose-dependent manner, regardless of the polymorphism. Most of our studied subjects presented more than one *APOA5* polymorphism and, since almost 80% of Carriers presented rare alleles of 724C>G plus -1131T>C or S19W, the uneven distribution of the *APOA5* SNPs could be considered as a potential confounder. However, there were no significant differences in TG with respect to the different combinations of SNPs.

As mentioned earlier, it is well documented that genetic variants are insufficient to clarify the whole variability of observed plasma TG distributions. This led us to consider that DNA methylation could be a major factor worth exploring in our analysis because of its role in regulating gene transcription, and because it is influenced by environmental stimuli. Over the past decade some evidence has demonstrated that epigenetics is a key element in the regulation of lipid metabolism. Guay et al. showed that methylation variability on *CETP* and *LPL* promoter loci are associated with blood lipid concentrations, and that *ABCA1* promoter DNA methylation is associated with HDL particle size and composition profiles [30,31]. Pfeiffer et al. analysed a genome-wide DNA methylation pattern and identified CpG sites located in genes such as *ABCG1*, *MIR33B/SREBBF1* and *TNIP1* which were associated with altered lipid parameters [32]. Our search of the current literature was unable to confirm whether there are specific methylation sites in *APOA5* that are involved in the regulation of circulating TG levels. Nonetheless, we recently identified methylation profiles in the *APOA1/C3/A4/A5* cluster that may be directly involved in the transcriptional regulation of this cluster [33].

We studied the methylation pattern of three regions in *APOA5* and reported that high methylation at the CGI in exon 3 is positively correlated with high plasma TG levels and high large VLDL, small LDL, small HDL particles and high RLPc levels. This pro-atherogenic lipoprotein profile defines the so-called atherogenic dyslipidaemia. We also observed a positive correlation between CGI methylation in *APOA5*, BMI and waist circumference. All these correlations could be explained by the physiopathology of our patients. Obesity is associated with dysfunctional adipose tissue which, in subjects with type 2 diabetes and metabolic syndrome, induces an increased fatty acid flux from visceral fat to the liver, resulting in an increased synthesis and secretion of TG. Thus, fat accumulation in the liver induces not only insulin resistance but also contributes to the development of atherogenic dyslipidaemia [34].

It is not clear whether the altered metabolism in these patients induces changes in DNA methylation, or whether the genetic and epigenetic predisposition causes the lipid alterations. Nevertheless, there is evidence that associates HTG risk factors with gene methylation variability [35,36], and which suggests that the variability in methylation may be secondary to disease processes and can highlight environmental factors such as diet and smoking which can influence gene expression [37].

Although methylation patterns are tissue-specific, we used DNA from circulating cells which would reflect specific methylation variations from populations of blood cells [38,39]. However we contemplate that methylation variations in blood cells would be somehow representative of the methylation pattern occurring in hard-to-access tissues such as the liver, and this is becoming a topic of study since some studies have already described correlations between methylation patterns in DNA from colorectal adenocarcinoma or adipose tissue and that from blood cells [5,40].

Another example of interaction between genetic variability and epigenetic mechanism in *APOA5* regulation has been described recently. Apart from DNA methylation, there is another epigenetic mechanism that influences *APOA5* regulation. Cui et al. recently demonstrated in Chinese patients that the rare allele in 1259T>C (rs2266788) located in the *APOA5* 3'UTR region (which is in linkage disequilibrium with -1131T>C and 724C>G) destroys the microRNA-3201 binding site; the outcome being an increase in gene translation and, subsequently, in high levels of plasma APOAV and TG [41]. In this sense, it would be of considerable value to analyse all epigenetic factors that involve gene regulation. These would include, for example, histone modifications, miRNAs binding sites and epistasis.

Finally, our novel finding of a relationship between plasma TG, *APOA5* SNPs and methylation is of note since the addition of methylation data could explain, at least in part, the missing heritability in the genetic predisposition to HTG, which remains unexplained to-date. The sum of inherited genetic information (SNPs) and acquired genetic information (DNA methylation, together with other epigenetic markers) from other key genes

involved in the control of TG, could help explain the observed variability of plasma TG.

CLINICAL PERSPECTIVES

- CGI in exon 3 of *APOA5* is hypermethylated in blood and correlates with plasma TG levels and with a pro-atherogenic lipoprotein subclasses profile.
- CGI methylation and polymorphisms of *APOA5* act in combination in the individual's predisposition to high circulating TG levels.
- To sum up, *APOA5* methylation and polymorphisms information increase a 7.5% of the gene predictive capacity to HTG compared with traditional risk factors prediction.

AUTHOR CONTRIBUTION

Josep Ribalta, Montse Guardiola, David Monk and Iris Oliva conceived and designed the study. Daiana Ibarretxe, Núria Plana and Lluís Masana participated in patient recruitment and selection. Montse Guardiola, David Monk and Iris Oliva acquired the data. Josep Ribalta, Montse Guardiola, Joan-Carles Vallvé and Iris Oliva interpreted the data. Montse Guardiola and Iris Oliva drafted the manuscript. Josep Ribalta, Montse Guardiola, David Monk and Joan-Carles Vallvé provided critical review of the manuscript. All authors revised the article and approved the final version to be published. Josep Ribalta is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

FUNDING

This work was supported by the Spanish Ministerio de Economía y Competitividad [grant number PI12/01766 (to M.G.)]; and the CIBERDEM (CIBER de Diabetes y Enfermedades Metabólicas Asociadas) [grant number CB07/08/0028], which is an initiative of ISCIII (Instituto de Salud Carlos III).

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Received 9 June 2016/21 July 2016; accepted 9 September 2016

Accepted Manuscript online 9 September 2016, doi: 10.1042/CS20160433