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Short Communication.

The *APOB* polymorphism rs1801701 A/G (p.R3638Q) is an independent risk factor for early-onset coronary artery disease: data from a Spanish cohort

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Abstract.

Background and aims: Apoliprotein B (ApoB) has been associated with hypercholesterolemia and ischemic coronary disease. This study was aimed to determine the effect of two *APOB* gene variants in the risk of developing early-onset coronary artery disease (EO-CAD) in a Spanish population. The association of these polymorphisms with hypercholesterolemia was also analysed.

Methods and results: The study involved a total of 889 healthy population controls (397 male) and 790 EO-CAD cases (636 male; EO-CAD was defined as male <60 years and women <65 years). All the patients had at least one vessel with angiography documented atherosclerotic lesion. Patients and controls were genotyped for the *APOB* variants rs1801701 A/G (p.R3638Q) and rs1367117 C/T (p.T98I). Allele and genotype frequencies were compared between the groups (patients vs. controls, hyper- vs. normo-cholesterolemia) by logistic regression.

The rs1801701 was significantly associated with EO-CAD in male (OR=1.44, 95%CI=1.05-1.99) and female (OR=2.22, 95%CI=1.58-3.14). This SNP was significantly associated with hypercholesterolemia in female, with a trend in male. The association with EO-CAD was independent of hypercholesterolemia (multiple logistic regression)

Conclusion: A common *APOB* polymorphism (rs1801701) was an independent risk factor for EO-CAD in our population. The risk-effect was more significant in female than in male.

1. Introduction.

Coronary artery disease (CAD) is mainly caused by the development of atherosclerotic lesions in coronary vessels. In addition to classical acquired risk factors (smoking, hypertension, hypercholesterolaemia, diabetes), an inherited predisposition contributes to the development of atherosclerosis and CAD (**1,2**). Most of the CAD occurs in individuals >65 years, but approximately 10-15% are early-onset cases (EO-CAD; men <60 years, women <65 years). Due to its greater heritability, EO-CAD is of particular interest to uncover the gene variants associated with coronary atherosclerotic events (**3**). Apoliporotein B gene (*APOB*) variants have been linked to the blood levels of total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C), as well as to the risk of suffering heart and brain ischaemic events (**4-6**). Rare pathogenic *APOB* variants are found in families with hypercholesterolaemia, while common *APOB* polymorphisms have been associated with a higher risk for increased TC and LDL-C in the general population (**7**). Our aim was to determine the association of two common *APOB* missense variants in relation to the risk of developing hypercholesterolemia and EO-CAD in a Spanish population.

2. Methods.

The study involved a total of 889 healthy population controls and 790 EO-CAD cases. They were recruited for a case-control research to characterize the genetic variation that contributes to CAD in the population of Asturias (a Northern Spain region, total population 1 million) (**8, 9**). Early-onset CAD was defined according to the WHO MONICA (Multinational Monitoring of trends and determinants in Cardiovascular disease) in male <60 years and women <65 years (**10**).

The patients underwent coronary angiography that confirmed the presence of at least one atherosclerotic coronary vessel (defined as a luminal narrowing >70%). All the patient´s information was obtained from their clinical records. Controls were eligible residents and blood bank donors, and the only inclusion criteria was the absence of previous coronary or brain ischaemic episodes. This information was obtained during an interview by a qualified physician who explained the study objectives. All the patients and controls were Caucasian and gave their informed consent to participate in the study, approved by the Ethical Committee of Hospital Central Asturias.

Individuals with a clinical history of diabetes mellitus or who had a fasting blood glucose level >125 mg/dL (7.0 mmol/L) were classified as type 2 diabetics (T2DM). Hypertensives were those receiving antihypertensive drugs or showing a systolic or diastolic blood pressure >140 or >90 mm

Hg, respectively. Individuals treated with lipid-lowering drugs or showing Cholesterol>220 mg/dL or LDL-C >150 mg/dL were considered as hypercholesterolaemics.

The salting-out method was used to obtain the DNA from blood leukocytes (**11**). We designated a PCR-RFLP procedure for genotyping two single-nucleotide polymorphisms (SNPs) in the *APOB* gene that were previously associated with the blood lipid profile in other studies: *APOB*-rs1801701 A/G (p.R3638Q) and *APOB*-rs1367117 C/T (p.T98I). The information about these variants (flanking sequence, population frequencies) was obtained from the *Ensembl* web site (www.ensembl.org). The two SNPs were genotyped by polymerase chain reaction (PCR) amplification of genomic DNA with specific primer-pairs followed by digestion with a restriction enzyme and electrophoresis size-fractioning (RFLP) of the corresponding fragment-alleles (**Suppl. figure**). As a quality control of the PCR-RFLP we confirmed the genotype of several individuals by Sanger sequencing of PCR fragments.

All the participant´s anthropometric, analytical and genetic values were collected in an Excel file. These values (anonymized for the identification of participants) are available upon request to the corresponding author. We confirmed that the genotype frequencies for each polymorphism did not deviate from the Hardy–Weinberg equilibrium (http://www.oege.org/software/hwe-mr-calc.shtml). The statistical analysis was performed with the R-software (http://www.r-project.org). We used a multivariate logistic regression analysis (linear generalized model, LGM) including the genetic variants, smoking, diabetes, hypertension, and hypercholesterolemia to determine the association of these variables with the risk for EO-CAD in male and female.

3. Results.

Table 1 summarizes the main anthropometric and analytic values in male and female CAD-patients and healthy controls. Smoking was the strongest predictor for EO-CAD in our population. There was a significantly higher frequency of hypercholesterolemia and hypertension among the patients. Type 2 diabetes was associated with CAD in the female. For the two *APOB* SNPs the observed genotype frequencies did not deviate from the expected under the Hardy-Weinberg equilibrium. Moreover, allele frequencies in our controls were close to the reported in Europeans (**suppl. figure**). The rs1801701 A frequency was significantly increased in the male and female patients compared to controls. Allele-A carriers (AA+AG) were significantly associated with EO-CAD in male (OR=1.44, 95%CI=1.05-1.99) and female (OR=2.22, 95%CI=1.58-3.14) (**table 1**). The rs1367117 T-allele frequency was non-significantly increased in the patients.

An adjusted multiple logistic regression (LGM; including hypercholesterolemia, type 2 diabetes, hypertension, smoking, and the genotypes as covariables) was performed to obtain the corresponding Odds Ratios after adjusting for each variable. Carriers of rs1801701 A remained significantly associated with EO-CAD in male and female (**Table 2**). Thus, in our population the *APOB* p.3638Gln variant was an independent risk factor for developing early-onset CAD.

The association of the two *APOB* SNPs with hypercholesterolemia in patients and controls showed that rs1801701 A-carriers were significantly more frequent in the hypercholesterolemics in the two groups (**figure 1**). According to the sex, the frequency was higher in all the hypercholesterolic patients and controls, but only significant in the female controls (**suppl. table**).

4. Discussion.

The main finding of our study was a significant association between the *APOB* rs1801701 SNP and EO-CAD. (p.R3638Q). This SNP was associated with ischemic stroke in a meta-analysis (OR=1.72-2.13) (**12**). The pro-atherogenic effect of this (and other) *APOB* variants might be explained by increased cholesterol and LDL-cholesterol in blood of the risk-allele carriers (**5, 13**). In the large scale Copenhagen City Heart Study the rare 3638Gln variant was associated with higher atherogenic lipids (**14**). We also found a significant association of this variant with hypercholesterolemia in our patients and controls. This could explain the higher risk of EO-CAD among Gln carriers, but the association remained significant after correcting by hypercholesterolaemia in male and female (**table 2**). This suggested that the *APOB* variant might have a proatherogenic effect beyond the increased risk of higher TC and LDL-C. Several large-scale studies showed that Apo-B would have a greater predictive value than LDL-C for adverse events in CAD (**15, 16**). ApoB levels strongly correlated with atherosclerotic lesion length and plaque-size (**16**). The Multi-Ethnic Study of Atherosclerosis (MESA) reported a discordant effect of apoB levels relative to LDL-C in the risk of developing subclinical atherosclerotic measured by coronary artery calcium (CAC). This effect was observed after adjustment for metabolic syndrome components (**17**).

The *APOB* rs1367117 C/T (p.T98I) minor allele was associated with significant higher mean cholesterol levels in a cohort from Netherlands (**18**). This allele was increased in our hypercolesterolemics but the difference was non-significant (**suppl. table**). In our population this SNP was not significantly associated with the risk of developing EO-CAD.

This study has several limitations. It was based on a limited sample size, particularly in the female cohort. This was due to the low frequency of female with EO-CAD, that hampered the recruitment of large sample sizes by a single center. In spite of this limitation, the post hoc power calculation for rs1801701 and EO-CAD was 99.8% in the female group compared to 56% among the male. We did not perform coronary angiography in healthy controls and we could thus not exclude the presence of atherosclerotic vessels in some of them. The non-inclusion of controls with subclinical atherosclerosis would likely result in higher association values for the *APOB* variants and EO-CAD. In conclusion, we report a significant association between the *APOB* rs1801701 SNP and early-onset CAD. The association was independent of the classical risk factors, including hypercholesterolemia, suggesting that ApoB might increase the risk of developing premature CAD through a mechanism beyond higher blood levels of proatherogenic lipid.

Contributorship. All the authors contributed to this work by recruiting the cohorts or performing the genetic and statistical analysis.

Competing interests. None of the authors have competing interests related to this work.

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References.

1. **Myocardial Infarction Genetics Consortium**. Genome-wide association of early-onset myocardial infarction with common single nucleotide polymorphisms, common copy number variants, and rare copy number variants. Nat Genet. 2009; March ; 41(3): 334–341. doi:10.1038/ng.327.

2. Christiansen MK, Nissen L, Winther S, Møller PL, Frost L, Johansen JK, Jensen HK, Guðbjartsson D, Holm H, Stefánsson K, Bøtker HE, Bøttcher M, Nyegaard M.J Genetic Risk of Coronary Artery Disease, Features of Atherosclerosis, and Coronary Plaque Burden. Am Heart Assoc. 2020 Feb 4;9(3):e014795. doi: 10.1161/JAHA.119.014795. Epub 2020 Jan 25.PMID: 31983321

3. Christiansen MK. Early-onset Coronary Artery Disease Clinical and Hereditary Aspects. Dan Med J. 2017 Sep;64(9):B5406.PMID: 28874246

4. Sharifi M, Futema M, Nair D, Humphries SE. Polygenic **Hypercholesterolemia** and Cardiovascular Disease Risk. Curr Cardiol Rep. 2019 Apr 22;21(6):43. doi: 10.1007/s11886-019-1130-z.PMID: 31011892

5. **Au** A, Griffiths LR, Irene L, Kooi CW, Wei LK. The impact of APOA5, APOB, APOC3 and ABCA1 gene polymorphisms on ischemic stroke: Evidence from a meta-analysis. Atherosclerosis. 2017 Oct;265:60-70. doi: 10.1016/j.atherosclerosis.2017.08.003. Epub 2017 Aug 19. Review.
PMID:

6. **Al-Bustan** SA, Alnaqeeb MA, Annice BG, Ebrahim GA, Refai TM. Genetic association of APOB polymorphisms with variation in serum lipid profile among the Kuwait population. Lipids Health Dis. 2014 Oct 8;13:157. doi: 10.1186/1476-511X-13-157.

7. **Sun** YV, Damrauer SM, Hui Q, Assimes TL, Ho YL, Natarajan P, Klarin D, Huang J, Lynch J, DuVall SL, Pyarajan S, Honerlaw JP, Gaziano JM, Cho K, Rader DJ, O'Donnell CJ, Tsao PS, Wilson PWF. Effects of **Genetic** Variants Associated with

Familial **Hypercholesterolemia** on Low-Density Lipoprotein-Cholesterol Levels and Cardiovascular Outcomes in the Million Veteran Program. Circ Genom Precis Med. 2018 Dec;11(12):e002192. doi: 10.1161/CIRCGEN.118.002192.PMID: 31106297

8. Palacín M, Alvarez V, Martín M, Díaz M, Corao AI, Alonso B, Díaz-Molina B, Lozano I, Avanzas P, Morís C, Reguero JR, Rodríguez I, López-Larrea C, Cannata-Andía J, Batalla A, Ruiz-Ortega M, Martínez-Camblor P, Coto E. Mitochondrial DNA and TFAM gene variation in early-onset myocardial infarction: evidence for an association to haplogroup H. Mitochondrion. 2011 Jan;11(1):176-81. doi: 10.1016/j.mito.2010.09.004. Epub 2010 Sep 21.PMID: 20863902

9. Coto E, Reguero JR, Avanzas P, Pascual I, Martín M, Hevia S, Morís C, Díaz-Molina B, Lambert JL, Alonso B, Cuesta-LLavona E, Díaz-Corte C, Gómez J. Gene variants in the NF-KB pathway (NFKB1, NFKBIA, NFKBIZ) and risk for early-onset coronary artery disease. Immunol Lett. 2019 Apr;208:39-43. doi: 10.1016/j.imlet.2019.02.007. Epub 2019 Mar 19.PMID: 30902734

10. **Tunstall-Pedoe H**, Kuulasmaa K, Amouyel P, Arveiler D, Rajakangas AM, Pajak A. Myocardial infarction and coronary deaths in the World Health Organization MONICA Project. Registration procedures, event rates, and case-fatality rates in 38 populations from 21 countries in four continents. Circulation. 1994 Jul; 90(1):583-612. doi: 10.1161/01.cir.90.1.583.PMID: 8026046

11. **Miller** SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 1988 Feb 11; 16(3): 1215. doi: 10.1093/nar/16.3.1215 PMCID: PMC334765 PMID: 3344216

12. **Au** A, Griffiths LR, Irene L, Kooi CW, Wei LK. The impact of APOA5, APOB, APOC3 and ABCA1 gene polymorphisms on ischemic stroke: Evidence from a meta-analysis. Atherosclerosis. 2017 Oct;265:60-70. doi: 10.1016/j.atherosclerosis.2017.08.003. Epub 2017 Aug 19.PMID: 28865324

13. **Lu** Y, Feskens EJ, Boer JM, Imholz S, Verschuren WM, Wijmenga C, Vaarhorst A, Slagboom E, Müller M, Dollé ME. Exploring genetic determinants of plasma total cholesterol levels and their predictive value in a longitudinal study. Atherosclerosis. 2010 Nov;213(1):200-5. doi: 10.1016/j.atherosclerosis.2010.08.053. Epub 2010 Aug 19.PMID: 20832063

14. **Benn M**, Stene MC, Nordestgaard BG, Jensen GB, Steffensen R, Tybjaerg-Hansen A. Common and rare alleles in apolipoprotein B contribute to plasma levels of low-density lipoprotein cholesterol in the general population. J Clin Endocrinol Metab. 2008 Mar;93(3):1038-45.

15. **McQueen** MJ, Hawken S, Wang X, Ounpuu S, Sniderman A, Probstfield J, et al. Lipids, lipoproteins, and apolipoproteins as risk markers of myocardial infarction in 52 countries (the INTERHEART study): a case-control study. Lancet. 2008;372(9634):224–233. pmid:18640459

16. **Sierra-Johnson** J, Fisher RM, Romero-Corral A, Somers VK, Lopez-Jimenez F, Ohrvik J, et al. Concentration of apolipoprotein B is comparable with the apolipoprotein B/apolipoprotein A-I ratio and better than routine clinical lipid measurements in predicting coronary heart disease mortality: findings from a multi-ethnic US population. Eur Heart J. 2009;30(6):710–717. pmid:18676970

17. Ohwada T, Sakamoto T, Kanno Y, Yokokawa S, Amami K, Nakazato K, Takeishi Y, Watanabe K. Apolipoprotein B correlates with intra-plaque necrotic core volume in stable coronary artery disease. PLoS One. 2019 Feb 19;14(2):e0212539. doi: 10.1371/journal.pone.0212539. eCollection 2019.PMID: 30779793

18. **Cao** J, Nomura SO, Steffen BT, Guan W, Remaley AT, Karger AB, Ouyang P, Michos ED, Tsai MY. Apolipoprotein B discordance with low-density lipoprotein cholesterol and non-high-density lipoprotein cholesterol in relation to coronary artery calcification in the Multi-Ethnic Study of Atherosclerosis (MESA). J Clin Lipidol. 2020 Jan-Feb;14(1):109-121.e5. doi: 10.1016/j.jacl.2019.11.005. Epub 2019 Nov 29.PMID: 31882375

Table 1. Summary of the analytical and genetic values in male and female controls and EO-CAD patients. AV: adjusting variable.

	MALE			FEMALE		
	Controls 397	Patients 636	p-value	Controls 492	Patients 154	p-value
Mean age	52.26±9.08	49.74±7.40	AV	54.29±7.32	58.44±5.48	AV
Age range	50-81	26-60	AV	50-81	47-65	AV
Diabetics	94 (24%)	139 (22%)	0.496	141 (29%)	66 (43%)	<0.001
Hypertension	114 (29%)	258 (41%)	<0.001	171 (35%)	91 (59%)	<0.001
Hypercolest	117 (30%)	244 (38%)	0.004	189 (38%)	83 (54%)	<0.001
Smokers	119 (30%)	475 (35%)	<0.001	97 (20%)	87 (56%)	<0.001
rs1801701 A/G Arg3638Gln						
AA	**3 (1%)**	**12 (2%)**		**8 (2%)**	**4 (3%)**	
AG	**52 (13%)**	**106 (17%)**	0.049	**86 (17%)**	**55 (36%)**	<0.001
GG	342 (86%)	518 (81%)		398 (81%)	95 (62%)	
AA+AG	OR=1.42, 95%CI=1.01-2.02			OR=2.63, 95%CI=1.77-3.90		
MAF, A	0.07	0.10	0.03	0.10	0.20	<0.001
	OR=1.44, 95%CI=1.05-1.99			OR=2.22, 95%CI=1.58-3.14		
rs1367117 T/C Thr98Ile						
TT	**36 (9%)**	**62 (10%)**	0.434	**41 (8%)**	**15 (10%)**	
TC	**148 (37%)**	**259 (41%)**		**220 (45%)**	**78 (51%)**	0.278
CC	213 (54%)	315 (49%)		231 (47%)	61 (39%)	
MAF, T	0.28	0.30	0.24	0.31	0.35	0.15
	OR=1.12, 95%CI=0.92-1.37			0R=1.22, 95%CI=0.93-1.59		

Table 2. Odds Ratios (OR) and 95% confidence intervals for EO-CAD after multiple logistic regression, patients vs. controls in male and female.

	MALE, OR (95%CI)	FEMALE, OR (95%CI)
DIABETES	1.64 (0.67-122)	1.87 (1.28-2.71)
HYPERTENSION	1.69 (1.30-2.22)	2.71 (1.88-3.94)
HYPERCHOLESTEROLEMIA	1.49 (1.14-1.95)	1.87 (1.30-2.71)
SMOKING	6.89 (5.23-9.14)	5.29 (3.59-7.82)
rs1801701 A-carriers AA+AG	1.42 (1.01-2.02)	2.63 (1.77-3.90)

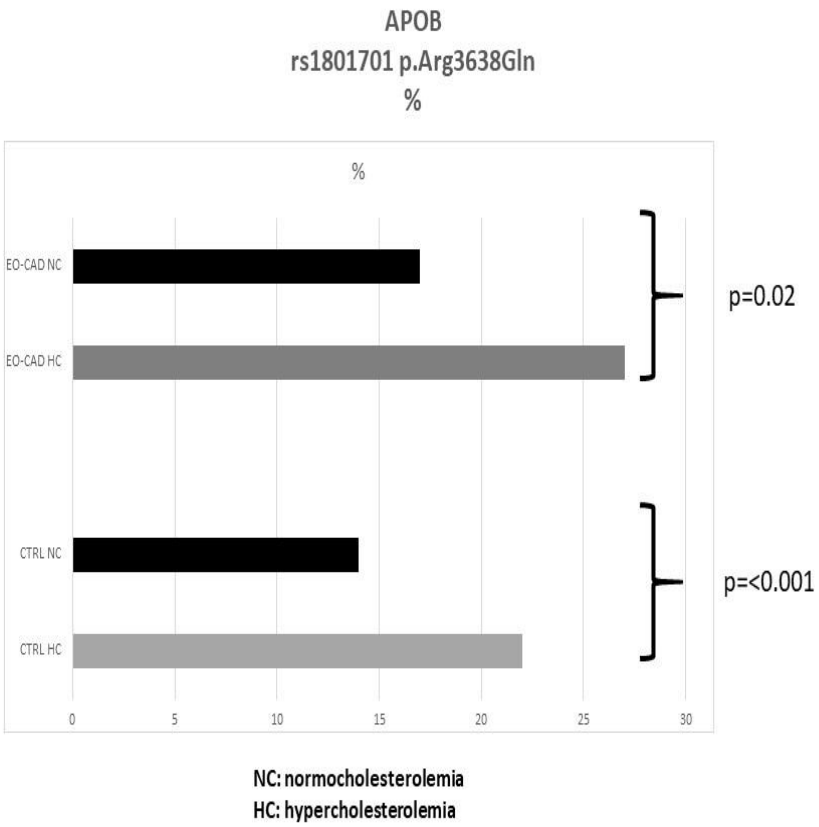
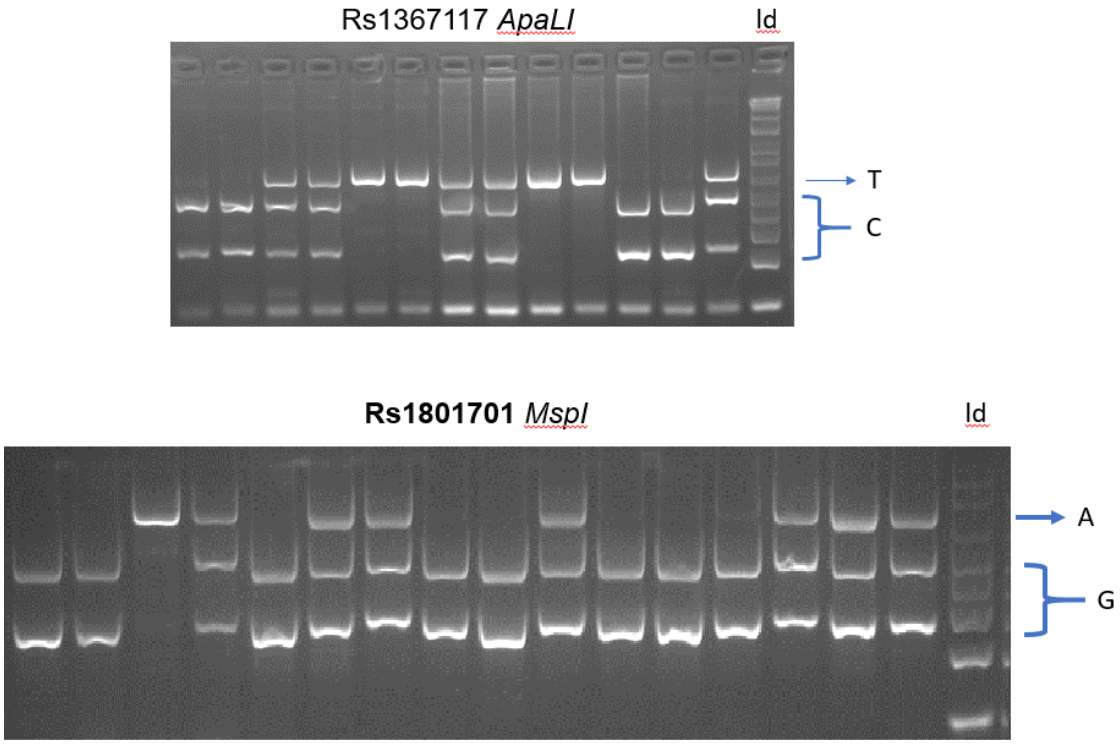


Figure 1. Minor allele frequency of the rs1801701 (3638 Gln) in the hypercholesterolemic (HC) and normocholesterolemic (NC) EO-CAD patients and healthy controls.

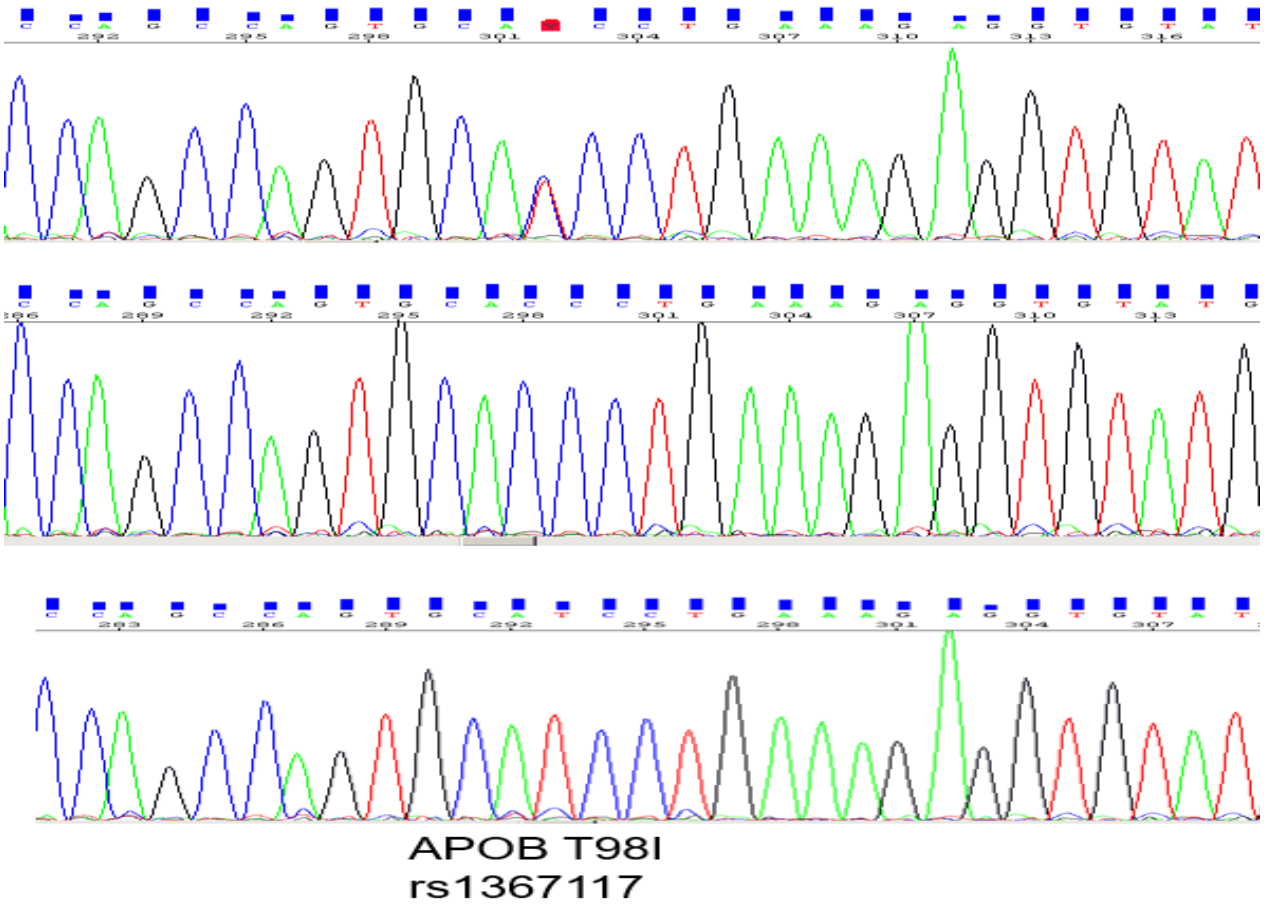
Suppl table.

rs1801701 A/G, p.Arg3638Gln									
	MALE					FEMALE			
	CTRL, 397		PATS, 635			CTRL, 492		PATS, 154	
	HC	NC	HC	NC		HC	NC	HC	NC
	117	280	244	392		189	303	83	71
AA	2	1	8	4		4	4	2	2
	(2)	(1)	(4)	(1)		(2)	(2)	(3)	(3)
AG	17	35	45	61		43	43	35	20
	(15)	(13)	(18)	(16)		(23)	(14)	(42)	(28)
GG	98	244	191	327		142	256	46	49
	(83)	(87)	(78)	(83)		(75)	(84)	(55)	(69)
AA+AG									
p-value	**0.37**		**0.10**			**0.01**		**0.08**	
OR	**OR=1.31**		**1.40**			**1.74**		**1.79**	
95%CI	**0.72-2.40**		**0.93-2.09**			**1.11-2.74**		**0.92-3.98**	

rs1367117 T/C, p.Thr98Ile									
	MALE					FEMALE			
	CTRL, 397		PATS, 635			CTRL, 492		PATS, 154	
	HC	NC	HC	NC		HC	NC	HC	NC
	117	280	244	392		189	303	83	71
CC	145	299	118	197		79	152	34	27
	(47)	(51)	(48)	(50)		(42)	(50)	(41)	(38)
CT	134	234	96	163		95	125	44	34
	(44)	(40)	(39)	(42)		(50)	(41)	(53)	(48)
TT	27	50	30	31		15	26	5	10
	(9)	(9)	(12)	(8)		(8)	(9)	(6)	(14)
TT+CT									
p-value	**0.27**		**0.62**			**0.07**		**0.71**	
OR	**1.17**		**1.08**			**1.40**		**0.88**	
95%CI	**0.89-1.54**		**0.79-1.49**			**0.97-2.02**		**0.46-1.69**	



Suppl. Figure. PCR-RFLP.



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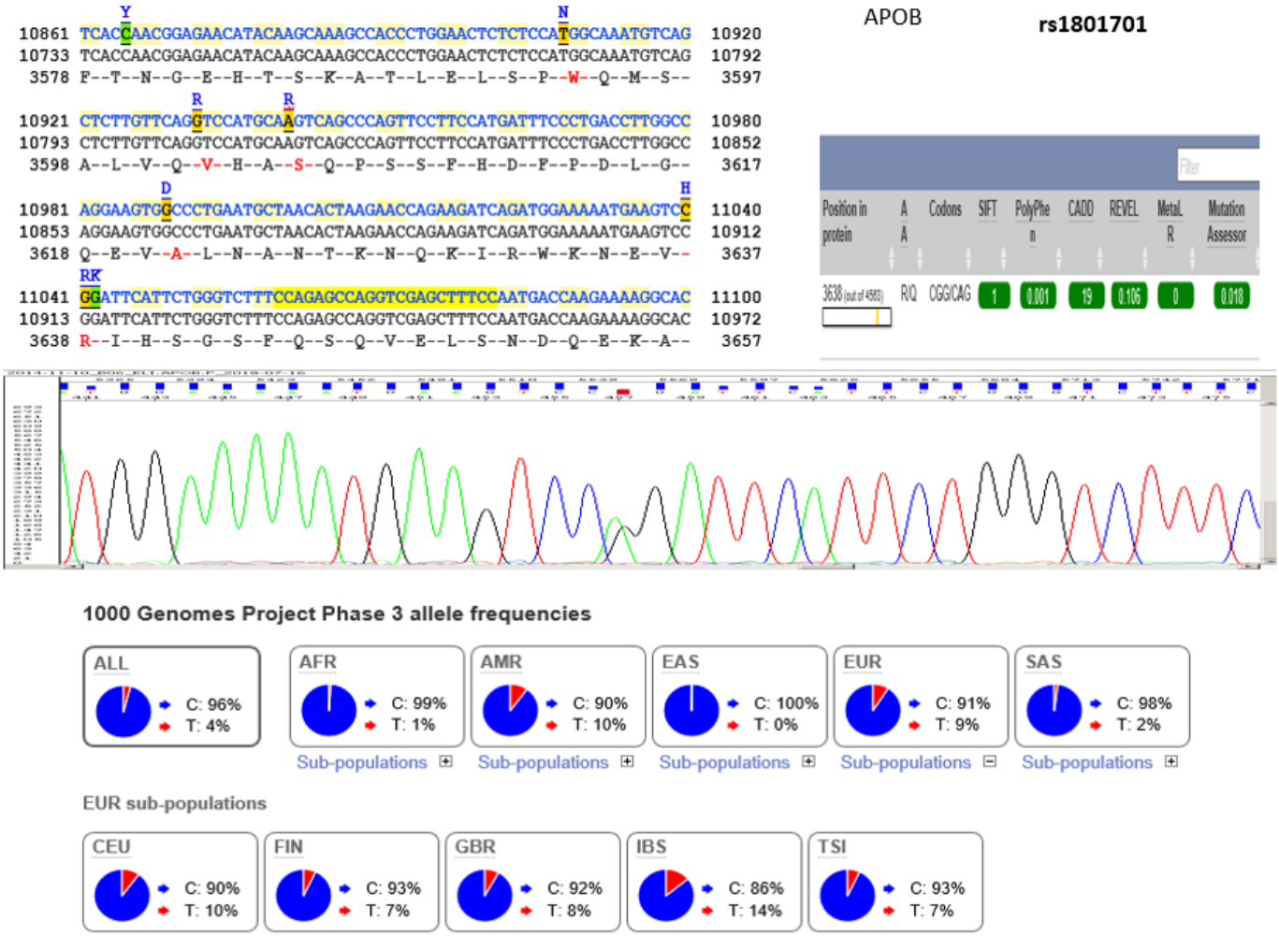

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