Original Article

Strong Association between *APOA5* Gene Polymorphisms and Hypertriglyceridaemic Episodes

(triglycerides / apoa5 / gene polymorphism / prediction)

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Abstract. Plasma triglyceride (TG) levels represent a significant risk factor of cardiovascular and total mortality. Concentrations of TG in the plasma depend, to a large extent, on the genetic background, and the apolipoprotein A5 (*APOA5*) gene seems to be one of the most powerful players in the plasma TG metabolism regulation. In total, we analysed three tagging *APOA5* (rs964184 rs662799, rs3135506) SNPs in 209 patients with plasma TG levels over 10 mmol/l (HTG) on at least one occasion and in 379 treatment-naïve controls (NTG) with plasma TG values within the normal range. Minor alleles of all three analysed *APOA5* polymorphisms significantly (all P < 0.0001) increased the risk of hypertriglyceridaemia. The most significant association (P < 0.0000001) was observed for the rs964184 polymorphism, where the minor GG homozygotes had the odds ratio (OR, 95% CI) for hypertriglyceridaemia development 21.30 (8.09-56.07, P < 0.000001) in comparison with the major CC allele homozygotes. Carriers of at least one minor allele at rs3135506 had OR (95% CI) 4.19 (2.75-6.40); (P < 0.000005) for HTG development and similarly, carriers of a minor allele at rs662799 had OR (95% CI) 3.07 (2.00-4.72) (P < 0.0001). The cumulative presence of risk alleles (unweighted gene score) significantly differed between patients with episodes of high TG and controls at P < 0.0000001. There were 73 % of subjects without any of the risk alleles among the controls and 46 % in the patients. In contrast, the controls just included 3 % of subjects with score 3 and more in comparison with 18 % in HTG patients. We conclude that common *APOA5* variants are very important genetic determinants of episodic hypertriglyceridaemia in the Czech population with a high potential to be applied in personalized medicine.

Introduction

Plasma triglycerides (triacylglycerols, TG) have long been debated as a risk factor for cardiovascular disease (Miller et al., 2011) and some other morbidities, but most importantly, also as an independent predictor of all-cause mortality (Liu et al., 2013; Pikhart et al., 2015).

The population variability of the plasma TG levels is relatively high, and 90 % of population exhibit TG values between 0.5 mmol/l and 7 mmol/l. Except for the environmental factors such as smoking, dietary habits, alcohol intake and physical activity, plasma TG concentrations are also determined by non-modifiable factors such as age, gender, and most importantly genetic factors (Johansen and Hegele, 2011; Schwarzova et al., 2015).

Rare mutations (their estimated cumulative prevalence is about 1 : 1,000,000) within some genes (for example, *LPL*, *APOC2*, *LMF1*, and G*PIHBP1*) are associated with extremely increased TG values, but the major part of plasma TG heritability has a polygenic background. The estimated heritability of TG values ranges from 40 % to 60 % (Arsenault et al., 2011) and dozens of genetic variants have been associated with plasma TG values (Teslovich et al., 2010; Vrablik and Hubacek, 2010; Schwarzova et al., 2015). The *APOA5* gene poly-

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Abbreviations: HDL – high-density lipoprotein, HTG – hypertriglyceridaemic, NTG – normoriglyceridaemic, TG – triglyceride, VLDL – very low-density lipoprotein.

morphism has been suggested to play a very important role (Hubacek, 2005, 2016; Guardiola and Ribalta, 2017).

The gene for apolipoprotein A5 (*APOA5*, gene ID 116519, OMIM accession number – 606368) was identified by comparative sequencing of human and mouse DNA as the last member of the *APOA1/APOC3/APOA4/APOA5* gene cluster (Pennacchio et al., 2001). The *APOA5* gene is small and codes for a 366 amino acid protein, which is expressed almost exclusively in the liver of humans. APOA5 associates mostly with chylomicrons, very low-density lipoprotein (VLDL) and high-density lipoprotein (HDL) particles. Plasma APOA5 concentration is very low in comparison with other apolipoproteins (O'Brien et al., 2005), suggesting rather a catalytical (APOA5 has been described to play a role in lipoprotein lipase activation) than structural function (Nilsson et al., 2011).

Almost all *APOA5* mutations (more than 20 described so far) are associated with very high plasma TG values, albeit not with 100 % penetrance (reviewed by Melegh et al., 2012), and in Caucasians, three *APOA5* common variants (rs964184, rs662799 and rs3135506) were described as important determinants of plasma TG (Pennacchio et al., 2002; Talmud et al., 2002). For all three SNPs, the minor alleles are associated with increased TG values. The effect of two of them (rs662799 and rs3135506) has also been confirmed in the Czech population in studies with different designs (Horinek et al., 2003; Vrablik et al., 2003; Hubacek et al., 2004).

Of the *APOA5* variants, the rs662799 (T-1131C) is the most intensively analysed, and a meta-analysis of 101 studies (including app. 300,000 control subjects with no history of CVD and almost 13,000 CVD cases) (Triglyceride Coronary Disease Genetics Consortium, 2010) confirmed that the presence of the each minor *APOA5* allele –1131C increases plasma TG by 0.25 mmol/l and the risk of CVD with the odds ratio of 1.18.

The importance of *APOA5* polymorphisms for plasma TG levels has later also been confirmed by genome-wide association studies (Kathiresan et al., 2009; Teslovich et al., 2010), which pointed out the importance of the third SNP of interest, namely rs964184.

The first aim of our study was to confirm the impact of the rs964184 *APOA5* variant on TG values in the Czech population. The second focus was to analyse the simultaneous additive effects of the three *APOA5* variants in the development of severe hypertriglyceridaemia defined as TG levels over 10 mmol/l in the fasting state on at least one occasion.

Material and Methods

Patients

Two hundred nine adult subjects (HTG group) aged 25–74 years having experienced at least one episode of the very high (10 mmol/l or more) plasma TG values were enrolled in the study. The subjects were treatment-naïve before the episode and examined at the 3rd Department of Internal Medicine of the First Faculty of Medicine, Charles University (Czech Republic).

Controls

Normotriglyceridaemic controls (NTG) (N = 379; aged 28–65 years) represent a preselected subsample of the Czech post-MONICA study (Cifkova et al., 2010; Hubacek et al., 2012, 2017a,b). Controls were examined three times between 1998 and 2007, and only individuals with treatment-naïve TG values below 1.8 mmol/l on all three occasions were selected.

All subjects were unrelated self-reported Caucasian and voluntarily signed informed consent with their participation in the study. All performed analyses were in accordance with the ethical standards of the institutional and national ethics committees and with the 1964 Declaration of Helsinki and its later amendments.

DNA analysis

DNA was isolated from frozen EDTA blood samples using the standard "salting-out" method (Miller et al., 1988).

Genetic variants were analysed with PCR-RFLP (Hubacek et al. 2015) in a PCR device DYAD PTC-220 (MJ Research, Reno, NV). Oligo sequences, PCR conditions, and restriction enzymes used are summarized in Table 1. Restriction fragments were separated in 12% polyacrylamide gel using the MADGE technique (Day and Humphries, 1994) in Tris-EDTA buffer.

Statistical analysis

HW equilibrium was analysed as described in www.dr-petrek.eu/documents/HWE.xls for the control group only, as the HTG patient group presents an extreme part of the population and deviations from the expected HW equilibrium could be expected.

Chi square (χ^2) (http://www.physics.csbsju.edu/cgi-bin/stats/contingency_form.sh?nrow=2&ncolumn=3)

Table 1. Genotyping details for analysis of APOA5 SNPs

SNP	Primer sequences	PCR product	Enzyme	Size of restriction fragments (bp)	Allele
rs964184	5′ ttt aca ttc ctc cat gac act aat c 5′ ttg ggg att gca gct ggc att taa ttc	195 bp	Bsp143I	195 147 + 48	C G
rs662799	5′ gat tga ttc aag atg cat tta gga c 5′ ccc cag gaa ctg agc gaa a***t***	187 bp	MseI	187 167 + 22	C T
rs3135506	5′ tgc tca cct ggg ctc tgg ctc ttc 5′ cca gaa gcc ttt ccg tgc ctg ggc ggc	178 bp	Eco52I	178 151 + 27	C G

Nucleotides in bold italics are mismatched in order to create an artificial restriction site.

and OR (95% CI) (http://www.hutchon.net/ConfidOR.htm) values were calculated.

For the cumulative unweighted gene score analysis, every individual obtained one point for each TG-increasing allele yielding possible values between 0 and 6. Only subjects with all three genotypes successfully genotyped were included in the score analysis and comparison (92 % among the controls and 92 % within the patient group). Due to the low number of subjects in some subgroups, the differences were assessed after regrouping subjects into three groups (0 risk point vs. 1 + 2 risk points vs. 3 and more risk points).

Due to the large number of performed analyses, P value below 0.005 was considered significant.

Results

Population characteristics

The call rates of the analysed polymorphisms were between 95 % and 100 %. Genotype frequencies of all examined SNPs were within the HW equilibrium in the control group (P = 0.05 for rs3135506). Individual allelic frequencies were similar to other Caucasian populations (when compared to the PubMed SNP database; www.ncbi.nlm.nih.gov/snp/). General characteristics of the examined subjects are summarized in Table 2.

Table 2. Characteristics of study subjects

	Patients	Controls
N (% of males)	209 (76 %)	379 (52 %)
Age (years)	57.0 ± 11.6	46.2 ± 9.8
Current smoking (%)	43	18
Hypertension (%)	73	21
Diabetes mellitus (%)	78	4.8
BMI (kg/m^2)	29.0 ± 4.7	25.4 ± 3.3
Total cholesterol (mmol/l)	9.89 ± 6.32	5.26 ± 1.01
Triglycerides (mmol/l)	18.30 ± 25.11	0.87 ± 0.31

Values are presented as mean ± S.D.

Single SNPs

Genotype frequencies of all three *APOA5* SNPs were significantly different (Table 3) between the HTG patients and controls.

The largest difference in genotype frequencies between the patients and controls was detected for rs964184 SNP. Heterozygotes had almost three times increased the odds ratio for the development of a hypertriglyceridaemic episode (OR, 95% CI; 2.90, 1.95–4.30; P < 0.0001), while for minor homozygotes, we demonstrated more than 20 times increased risk (OR, 95% CI; 21.30, 8.09–56.07; P < 0.0000001).

In the case of rs662799, the carriers of minor alleles had increased risk of HTG at P < 0.0001 with OR (95% CI) equal to 3.07 (2.00–4.72).

Finally, for the last gene polymorphism (rs3135506), the carriers of at least one minor allele had OR (95% CI) for the development of HTG of 4.19 (2.75–6.40); (P < 0.000005).

Cumulative frequency of APOA5 alleles – unweighted gene score

The distributions of *APOA5* gene score within the HTG cases and controls is presented in Fig. 1 and differed significantly between the groups (P < 0.0000001).

Among the controls, a vast majority of subjects did not have any TG-increasing allele (73 %) in comparison with only 46 % of such subjects among the patients with the HTG episode. In contrast, within the control group, there were almost no subjects with three and more risk

Table 3. Differences of individual SNPs between the hypertriglyceridaemic patients (HTG) and low triglyceridaemic controls (NTG)

SNP	**NTG**		**HTG**		**P***	**OR (95% CI)**	**P**
rs964184	N	%	N	%			
CC	285	77.0	91	45.7	0.000001§	1.00	
CG	80	21.6	74	37.2	0.0000001†	2.90 (1.95–4.30)	0.0001
GG	5	1.4	34	17.1	0.000001‡	21.3 (8.1–56.1)	0.0000001
rs662799							
TT	320	87.7	146	69.9	0.00001§	1.00	
TC	41	11.2	58	27.8	0.0001†	3.10 (1.99–4.84)	0.0001
CC	4	1.1	5	2.4	0.29‡	2.73 (0.72–10.35	0.12
rs3135506							
GG	314	87.5	128	62.4	0.000005§	1.00	
GC	45	12.5	72	35.1	n.a.†	4.19 (2.75–6.40)	0.000005**
CC	0	0.0	5	2.5	n.a.‡	n.a	

*P values for dominant§, co-dominant† and recessive‡ model
**calculated for GG vs C allele carriers

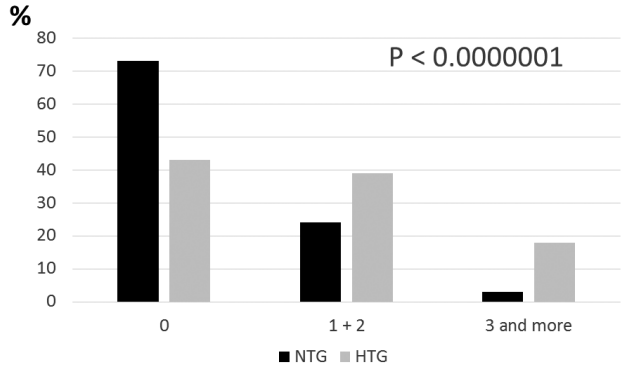

Fig. 1. Distribution of unweighted gene score between the control subjects and subjects with episodes of hypertriglyceridaemia. The distribution is significantly different at 0.00001.

alleles (3 %). The observed frequency of such a score among the patients with episodic HTG was 18 %. No subject carried all six risk alleles.

Discussion

In our study, we confirmed that three common *APOA5* polymorphisms (rs964184, rs662799 and rs3135506) are each highly significantly associated with an increased risk of HTG episodes in the Czech population. Further, we were the first to document that the patients with a history of HTG episodes have about 6-times greater prevalence of carriers of at least three risk alleles of the *APOA5* gene than the normotriglyceridaemic controls.

Our results are in agreement with previous studies, which associated individual minor *APOA5* alleles with an increased risk of high plasma triglyceride levels.

There are several explanations for the APOA5 effect on plasma TG concentrations (summarized by Nilsson et al., 2011 and Guardiola and Ribalta, 2017). Probably the most important is the stimulation of lipoprotein lipase-mediated triglyceride hydrolysis. Further, APOA5 interacts with members of the LDL-receptor family, and thus it can stimulate the removal of lipoproteins from the circulation. Nevertheless, the plasma concentration of APOA5 is very low (probably, there is just one APOA5 molecule per 20–25 lipoprotein particles) (Merkel and Heeren, 2005), suggesting that just a minor proportion of APOA5 acts directly in the plasmatic compartment. Finally, some effect on the secretion of VLDL particles has been suggested.

As in all association studies, in our study variants within the *APOA5* gene were not able to completely discriminate between the patients (with episodic HTG) and healthy controls. On the other hand, despite the relatively low number of subjects examined, the differences observed in our study reached unusually high odds ratios and statistical significance. This can be explained by the fact that our patients, enrolled on the basis of having very high TG values, represent an extreme phenotype with a presumed (and finally confirmed) strong genetic background. Interestingly, when we compare the Czech population frequencies (Hubacek et al., 2004, 2014) of two *APOA5* SNPs (rs662799 and rs3135506), they are almost identical with the ones found among our low TG controls.

It is likely that the impact of *APOA5* variants on the plasma lipids is further influenced by both environmental and other genetic factors.

For example, the effect of *APOA5* polymorphisms on plasma triglycerides is mediated by dietary habits of the examined subjects (Sánchez-Moreno et al., 2011; Zlatohlavek et al., 2012; Weber et al., 2016). Interestingly, an interaction between total energy intake, *APOA5* haplotypes and plasma cholesterol values has also been described (Hubacek et al., 2014). Additional factors, such as physical activity (Suchanek et al., 2008; Liu et al., 2018), alcohol intake (Son et al., 2015) or vitamin D levels (Shirts et al., 2012) interact with *APOA5* variants and impact their TG-modulating effect.

The impact of *APOA5* genetic variability on plasma lipids is also significantly influenced by variants within other genes (gene-gene interactions). Such interactions have been shown for polymorphisms within genes *APOE* (Hubacek et al., 2008; Sousa et al., 2008), *LEPR* (Domínguez-Reyes et al., 2015), *COLEC12* (Lin et al., 2017), or *CETP* and *LIPA* (Lin et al., 2016).

Finally, not only the sequence variability, but also epigenetic mechanisms could add a significant piece to the puzzle (reviewed by Guardiola and Ribalta, 2017). Epigenetics is defined as heritable markers potentially significantly affecting the gene function and, at the same time, not based on changes within the genetic sequence (Ladd-Acosta and Fallin, 2016; Ganesan, 2018). Epigenetic studies are focused mainly on analyses of DNA methylation, micro-RNA and histone modifications (Moore et al., 2013; Dlouha and Hubacek, 2017). Recently, it has been reported that distinct methylation within the *APOA5* exon 3 significantly improves the TG value prediction in subjects with high cardiovascular risk (Oliva et al., 2016). Further, the epigenome-wide study (Lai et al., 2016) has pointed out the importance of cytosine cg12556569 (which is interestingly strongly associated with the rs964184 polymorphism) methylation as an epigenetic determinator of plasma TG values.

In our study, we focused not only on simple analysis of the effects of individual gene polymorphisms on TG levels, but we also created a gene score that combines the effect of all three polymorphisms studied. This approach seems to be the next step in the analysis of genetic predisposition to various diseases (Maher et al., 2015; Smith et al., 2015; Talmud et al., 2015; Hubacek, 2018). We opted for use of a simple unweighted gene score in our study. The weighted gene score, also taking into account the odds associated with each SNP, might seem more accurate; however, in fact it could be misleading for an exact disease prediction in time. In studies in elderly populations, typically used for risk assessment

studies, the genetic component of the risk is confounded by conventional environmental risk factors. Adjustment cannot eliminate the risk of false negative or false positive results. Thus, we suggest the unweighted gene score as more feasible, having sufficient power for the initial genetic risk screening among young asymptomatic subjects.

The results of our study further suggest that genetic variants (as disease predictors) could have comparable effects as the traditional risk factors (Hubacek et al., 2017b), and their inclusion into risk assessment tools in the future would play an important role in personalised medicine (Visvikis-Siest et al., 2017).

In conclusion, the common variants within the *APOA5* gene are strong genetic determinants of elevated plasma TG levels. The unweighted gene score is an important tool and should be used in personalized risk estimations.

Conflict of interest statement

Authors declare no conflict of interest.

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