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Common functional variants of *APOA5* and *GCKR* accumulate gradually in association with triglyceride increase in metabolic syndrome patients

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Abstract The common functional variants of the apolipoprotein A5 (*APOA5*) and the glucokinase regulatory protein genes (*GCKR*) have been shown to associate with increased fasting triglyceride (TG) levels. Albeit the basic association has been extensively investigated in several populations of different origin, less is known about quantitative traits of them. In our study accumulation rates of four *APOA5* (T-1131, IVS3 + G476A, T1259C and C56G) and two *GCKR* (C1337T and rs780094) functional SNPs were analyzed in patients stratified into four TG quartile groups. Randomly selected 325 metabolic syndrome patients were separated into four quartile (q) groups based on the TG levels as follows q1: TG <1.38 mmol/l; q2: 1.38–1.93 mmol/l; q3: 1.94–2.83 mmol/l; and q4: TG >2.83 mmol/l. We observed significant stepwise increase of prevalence rates of minor allele frequencies in the four plasma TG quartiles for three *APOA5* SNPs: −1131C (q1: 4.94%; q2: 8.64%; q3: 11.6%; q4: 12.3%), IVS3 + 476A (q1: 4.32%; q2: 7.4%; q3: 10.36%; q4: 11.1%), and 1259C (q1: 4.94%; q2: 7.41%; q3: 10.4%; q4: 11.7%). The haplotype analysis revealed, that the frequency of APOA5*2 haplotype gradually increased in q2, q3 and q4 (q1: 9.87%; q2: 14.8%; q3: 18.3%; q4: 21%). The distribution of the homozygotes of the two analyzed *GCKR* variants resembled to the *APOA5* pattern. Contrary to the hypothetically predictable linear association coming from the current knowledge about the *APOA5* and *GCKR* functions, the findings presented here revealed a unique, TG raise dependent gradual accumulation of the functional variants of in MS patients. Thus, the findings of the current study serve indirect evidence for the existence of rare *APOA5* and *GCKR* haplotypes in metabolic syndrome patients with higher TG levels, which contribute to the complex lipid metabolism alteration in this disease.

Keywords Metabolic syndrome · APOA5 · GCKR · Triglycerides

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Introduction

The apolipoprotein A5 (*APOA5*) gene is located on chromosome 11q23 within the *APOA1/C3/A4/A5* gene cluster, and it comprises 3 exons encoding 366 amino acids [1]. Numerous studies confirmed that some common naturally occurring variants of the *APOA5* gene (like −1131C, IVS3 + 476A, 1259C and 56G alleles) associate with elevated triglyceride (TG) concentrations [2–4]. Besides, some of them have been shown to confer risk for the development of cardio-, and cerebrovascular diseases [5–8]. These common functional variants associate with a moderate, even less than 100% TG increase, while pathogenic mutations of the gene result in enormous increases, reaching even the 15–60 mmol/l [9, 10]. As a link between these two closing stages, in a recent study excess of rare variants of *APOA5* gene were identified, also combination of the rare variants with the common functional ones

resulted in special haplotype combinations, and increased the TG levels into an intermediate zone [11–13].

The glucokinase regulatory protein (*GCKR*) gene located on chromosome 2p23.3-p23.2 consists of 19 exons and encodes a protein of 625 amino acids [14, 15]. The gene product functions as a regulatory protein that inhibits glucokinase in liver and pancreatic cells by binding the enzyme non-covalently to form an inactive complex [16, 17]. Functional variants were discovered in a genome-wide association study; and later an inverse relationship of TG and glucose levels had also been reported in relation with *GCKR* functional alleles [18–20]. No information is available about the quantitative traits.

As an important step in the research of the quantitative features, significant stepwise relationships between *APOA5* C56G and T-1131C minor allele carrier frequencies and plasma TG quartiles were found in a group of patients with one of the four specified classes of hyperlipoproteinemia [11]. After this study, our laboratory could detect similar association in average normal population samples with different TG concentrations [21]. Here we report the results of our replication study targeting a cohort of randomly selected patients with different degrees of TG level increase, which study was initiated by the known profound changes of TG metabolism in metabolic syndrome (MS) in mind.

Materials and methods

Study population

DNA and dataset of total of 325 MS patients (141 males and 184 females; mean age: 60.5 ± 10.08 years; range: 23–74 years), diagnosed according to the criteria of the modified Adult Treatment Panel III of the National Cholesterol Education Program, were selected for the study from our biobanks. The diagnostic criteria applied require the simultaneous presence of at least 3 of the following factors at the time of the diagnosis: body mass index (BMI) >25 kg/m^2; TG ≥1.70 mmol/l; serum HDL-cholesterol <0.9/1.1 mmol/l (male/female); systolic blood pressure ≥130 mmHg and diastolic blood pressure ≥85 mmHg; and fasting plasma glucose level >5.60 mmol/l.

According to the available triglyceride values, MS patients were stratified into four quartile groups (q) as follows. The q1 represented TG <1.38 mmol/l; q2 ranged between 1.38 and 1.93 mmol/l; q3 between 1.94 and 2.83 mmol/l; while q4 represented TG >2.83 mmol/l.

The DNA with the clinical and laboratory dataset were deposited to the local biobank, part of the Hungarian National Biobank Network (www.biobanks.hu), which was established with the endorsement and approval of the National Ethics Committee, Budapest (ETT TUKEB). Patients involved in the biobanking procedure gave their informed consent for the future use of their samples for genetic tests and research upon deposition of their samples into the biobank.

Genetic analysis

DNA was isolated from peripheral blood leukocytes by a standard salting method. *T-1131C*, *GCKR* C1337T and the intronic polymorphism of the *GCKR* gene (rs780094) alleles were determined as previously described [5, 22]. For all SNPs we considered the principle to design primers creating an obligatory cleaving site in the PCR product, which enabled the monitoring of the digestion efficacy.

To test the IVS3 + G476A alteration the following oligonucleotides were used for amplification: 5′-CTC AAG GCT GTC TTC AG-3′ and 5′-CCT TTG ATT CTG GGG ACTG G-3′ (antisense). The PCR product (15 µl) was digested with 1 U of *Mnl*I (Fermentas, Burlington, ON, Canada) restriction endonuclease at 37°C overnight. Restriction fragments were analysed using 3% agarose gel stained with ethidium bromide, and visualized with UV transilluminator. With GG genotype the digestion resulted 25, 114, and 141 bp fragments; while in homozygous samples 25, 41, 73 and 141 bp long products were detected. The T1259C polymorphism was detected using the primers 5′-TCA GTC CTT GAA AGT GGC CT-3′ and 5′-ATG TAG TGG CAC AGG CTT CC-3′ (antisense). The PCR product was digested with 1 U of *BseG*I (Fermantas, Burlington, ON, Canada) restriction endonuclease at 55°C overnight. After the digestion, the normal (TT) genotype gave fragments of 122 and 165 bp, whereas the homozygous form (CC) resulted 35, 87 and 165 bp fragments. The *C56G* polymorphism site was amplified with 5′-AGA GCT AGC ACC GCT CCT TT and 5′-TAG TCC CTC TCC ACA GCG TT primers. The 256 bp amplicon was digested with *Cfr*13I enzyme (Fermantas, Burlington, ON, Canada). After digestion 79 and 177 bp fragments were detected in the samples with CC genotype; while in homozygous GG samples 26, 79 and 151 bp products were detected.

PTC-200 PCR (Bio-Rad, Hercules, CA, USA) equipments were used for amplification. The conditions were similar for all polymorphisms: a 2 min initial denaturation at 96°C was followed by 35 cycles of 20 s at 96°C; 20 s at 60°C; and 20 s at 72°C; the final extension at 72°C was 5 min long. The amplification was carried out in a final volume of 50 µl containing: 5 µl reaction buffer (500 mM KCl, 14 mM $MgCl_2$, 10 mM Tris–HCl, pH 9.0), 1 µl 50 mM $MgCl_2$, 0.2 mM of each dNTP, 1 U of *Taq* polymerase, 0.2 mM of each reaction specific primers and 100 ng DNA.

Statistical analysis

Results are expressed as mean±SEM. Statistical significance was assessed by the Mann-Whitney U-test to compare the differences between groups. We used HAPSTAT 3.0 (http://www.bios.unc.edu/~lin/hapstat/) for haplotype assignment. Chi-square tests were used to compare qualitative data. A value of $P \leq 0.05$ was considered to indicate statistical significance. All statistical analyses were performed using SPSS 17.0 software (SPSS Inc. Chicago. IL, USA).

Results

Clinical characteristics of the four quartile groups are shown in Table 1. Both the serum total cholesterol and TG were gradually increased in q2, q3, and q4 compared to q1. The ages of the subjects did not differ in the quartiles.

Table 2 shows the *APOA5* genotypes and allele frequencies in the four groups. The frequency of the minor variants of three *APOA5* alleles (−1131C, IVS3 + 476A, 1259C) are higher in q3 and q4 compared to q1. While allele frequency of 56G does not increase unambiguously with higher average TG levels, −1131C, IVS3 + 476A, and 1259C variants demonstrate an increase in prevalence of over 100% in quartiles 3 and 4 compared to quartile 1. Even though the risk alleles show a tendency of towards a higher allele frequency in quartile 2, this difference is not significant for any of these variants. It is important to note that despite the significant alterations of allele frequencies, no significant differences could be observed at the genotype levels, as the prevalence of risk allele carriers did not prove to be significantly higher in any of the quartiles compared to q1. The allele frequencies of all *APOA5* variants studied were in Hardy–Weinberg equilibrium in each group.

The frequencies of *APOA5* haplotypes in the quartiles are summarized in Table 3. The frequency of *APOA5*2* haplotype is higher in q2, q3 and q4. On the other hand, no significant increase in *APOA5*3* haplotype frequency could be observed with elevating average TG levels, which may be due to either the relatively small number of carriers or that this combination of APOA5 alleles may not be associated with elevating TG levels in our cohort.

The frequency of the homozygote minor form of the two *GCKR* variants analyzed are higher in q4, but no further significant differences were present at the level of allele frequencies. The allele frequencies of all *GCKR* variants studied were consistent with Hardy–Weinberg equilibrium expectation in every group (Table 4).

Discussion

The ApoAV has special coexisting roles in the complex regulation of the circulating TG in humans. First, ApoAV interacts with lipoprotein lipase, the central enzyme involved in the regulation of circulating TG; and thereby it is an activator of the intravascular triglyceride hydrolysis.

Table 1 Major clinical parameters of the four quartile groups

	Quartiles of plasma triglycerides			
	<1.38 $n = 81$	1.38–1.93 $n = 81$	1.94–2.83 $n = 82$	>2.83 $n = 81$
Males/females	41/40	33/48	27/55	40/41
Age (years)	61.5 ± 1.00	61.2 ± 1.31	63.5 ± 1.06	58.5 ± 1.33
BMI (kg/m^2)	32.6 ± 0.54	33.6 ± 0.65	33.6 ± 0.72	33.9 ± 0.62
Triglycerides (mmol/l)	1.07 ± 0.03	1.63 ± 0.02*	2.36 ± 0.03*	5.22 ± 0.44*
Total cholesterol (mmol/l)	4.90 ± 0.12	5.05 ± 0.12	5.44 ± 0.11*	6.26 ± 0.23*
HDL cholesterol (mmol/l)	1.34 ± 0.04	1.22 ± 0.03*	1.20 ± 0.03*	1.20 ± 0.05*
LDL cholesterol (mmol/l)	2.80 ± 0.11	2.78 ± 0.09	3.01 ± 0.14	3.05 ± 0.14
Systolic blood pressure(mmHg)	137 ± 1.81	143 ± 2.10	141 ± 3.02	141 ± 2.45
Diastolic blood pressure (mmHg)	83.6 ± 1.10	86.6 ± 1.37	83.2 ± 1.37	84.2 ± 1.35
Fasting Glucose (mmol/l)	9.02 ± 0.34	11.2 ± 1.80*	10.9 ± 0.57*	10.7 ± 0.55*
RR systole (mmHg)	135 ± 2.12	136 ± 1.11	136 ± 1.04	138 ± 1.32
RR diastole (mmHg)	82.7 ± 1.17	82.2 ± 0.67	82.3 ± 0.97	83.3 ± 0.52

Serum triglyceride levels, HDL cholesterol levels and fasting glucose concentration show a significant increase in quartiles 2, 3 and 4 compared to quartile 1, while average total cholesterol levels are only significantly different in quartiles 3 and 4 compared to quartile 1

* $P \leq 0.05$ vs. TG <1.38

Table 2 APOA5 genotypes and allele frequencies in the quartiles

	<1.38 $n = 81$	1.38–1.93 $n = 81$	1.94–2.83 $n = 82$	>2.83 $n = 81$
T-1131C				
TT	74 (89.0%)	67 (82.7%)	66 (80.5%)	62 (76.5%)
TC + CC	6 + 1 (11.0%)	12 + 2 (17.3%)	13 + 3 (19.5%)	18 + 1 (23.5%)
C allele frequency (%)	4.94	8.64	11.6*	12.3*
C56G				
CC	69 (82.9%)	70 (86.4%)	74 (90.2%)	69 (85.2%)
CG + GG	12 + 0 (17.1%)	11 + 0 (13.6%)	7 + 1 (9.80%)	9 + 3 (14.8%)
G allele frequency (%)	7.41	6.79	5.48	9.26
T1259C				
TT	73 (89.0%)	69 (85.2%)	67 (80.5%)	62(76.5%)
TC + CC	8 + 0 (11.0%)	12 + 0 (14.8%)	13 + 2 (19.5%)	19 + 0 (23.5%)
C allele frequency (%)	4.94	7.41	10.4*	11.7*
IVS3 + G476A				
GG	74 (87.8%)	69 (85.2%)	66(80.5%)	64(79.0%)
GA + AA	7 + 0 (12.2%)	12 + 0 (14.8%)	13 + 2(19.5%)	16 + 1(21.0%)
A allele frequency (%)	4.32	7.4	10.36*	11.1*

All but one minor allele frequencies shows a significant increase in quartiles 3 and 4 compared to quartile 1

* $P \leq 0.05$ vs. TG <1.38

Table 3 Frequencies of APOA5 haplotypes in the quartiles A significant increase of *APOA5*2* haplotype carriers in quartiles 2, 3 and 4 is observed compared to quartile 1

	<1.38 mmol/l $n = 81$	1.38–1.93 mmol/l $n = 81$	1.94–2.83 mmol/l $n = 82$	>2.83 mmol/l $n = 81$
*APOA5*1/1*	60 (74.07%)	56 (69.13%)	58 (70.73%)	48 (59.25%)
*APOA5*1/2–2/2*	8 (9.87%)	12 (14.8%)*	15 (18.3%)*	17 (21%)*
*APOA5*1/3–3/3*	12 (14.8%)	11 (13.6%)	8 (9.80%)	12 (14.7%)
Other haplotype variants	1 (1.23%)	2 (2.46%)	1 (1.23%)	4 (4.96%)

* $P \leq 0.05$ vs. TG <1.38

Table 4 GCKR genotypes and allele frequencies in the triglyceride quartiles

	<1.38 mmol/l $n = 81$	1.38–1.93 mmol/l $n = 81$	1.94–2.83 mmol/l $n = 82$	>2.83 mmol/l $n = 81$
GCKR C1337T				
CC	27 (33.4%)	32 (39.5%)	23 (28.0%)	17 (21.0%)
CT	40 (49.3%)	37 (45.7%)	44 (53.7%)	43 (53.1%)
TT	14 (17.3%)	12 (14.8%)	15 (18.3%)	21 (25.9%)*
T allele frequency (%)	41.9	37.7	45.2	52.5
GCKR rs780094				
GG	24 (29.6%)	35 (43.2%)	26 (31.7%)	14 (17.3%)
GA	43 (53.1%)	34 (42.0%)	39 (47.6%)	43 (53.1%)
AA	14 (17.3%)	12 (14.8%)	17 (20.7%)	24 (29.5%)*
A allele frequency (%)	43.9	35.8	44.5	56.05

In case of both GCKR variants studied, a significantly elevated prevalence of risk allele homozygotes could be observed in quartile 4 compared to quartile 1

* $P \leq 0.05$ vs. TG <1.38

This interaction represents the major mechanism by which ApoAV exerts its modifier activity [1, 9] (Fig. 1.).

APOA5 was identified as part of the *APOA1/C3/A4/A5* gene cluster on 11q23 [1]. Several SNPs in this gene cluster have been reported to affect TG metabolism [23, 24], including some variants of *APOA5*, like the T-1131, IVS3 + G476A, T1259C and C56G, which represent the most common variants. These naturally occurring variants of the *APOA5* gene have been widely studied in the past few years in several populations and in numerous diseases [3, 7, 8, 25–27]. As these natural genetic variants have effect on the activity of their protein transcripts, these alleles have been reported to associate with elevated fasting or postprandial circulating TG levels [2–4]. The increase rate can be moderate, resulting in even less than twofold increase of the normal value. By contrast, pathologic mutations of the gene are associated with an enormous increase of circulating TG concentrations, reaching the 15–60 mmol/l range [9, 10].

As a special approach, Wang et al. found a higher frequency of carriers of *APOA5* variants in lipid clinic patients than in controls, a significant stepwise relationship between *APOA5* minor allele carrier frequencies and plasma TG quartiles, and higher *APOA5* S19W and *APOA5*–T1131C allele and carrier frequencies in lipid clinic patients than in controls for hyperlipoproteinemia types 2B, 3, 4 and 5. These findings indicate that *APOA5* variants C56G and T-1131C are strongly and specifically associated with hypertriglyceridaemia in lipid clinic patients and with several hyperlipoproteinemia phenotypes defined by elevated plasma TG concentration. Hyperlipoproteinemia type 2A, which is not characterized by elevated TG, was not associated with *APOA5* minor alleles

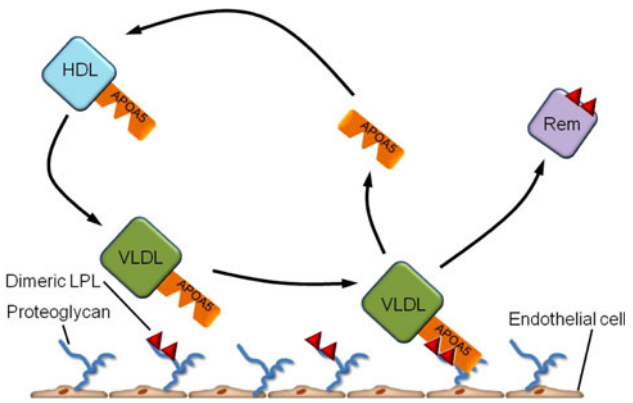

Fig. 1 Triglyceride-lowering effect of APOA5. Triglyceride-rich lipoproteins such as VLDL are hydrolyzed by the lipolytic action of dimeric LPL. APOA5 targets VLDL bind to proteoglycans, placing VLDL in close proximity to LPL. APOA5 may activate proteoglycan-bound LPL by stabilizing the dimerized conformation or by binding to an LPL allosteric site. After hydrolysis, remnant particles (Rem) are released into the circulation

[11]. As a further step, in our laboratory we found a similar association of randomly selected subjects with elevated TG levels [21]. In the current study our findings were similar for metabolic syndrome patients; suggesting that the quantitative traits described by Wang and colleagues and by us is not necessarily restricted to patients with specific types of hyperlipoproteinemia phenotypes, and even the association might reflect specific haplotype distribution [11]. In the current study, we observed that frequencies of *APOA5*2* haplotype were gradually elevated in the TG groups. Although, the haplotype-tagging SNPs used enabled us to stratify the patients to the known major haplogroups, creation of further minor subgroups with this approach was not possible. However, these are still not enough to explain the observed distribution. Again, taking into consideration that the common functional alleles are associated only with moderate TG increases, which in itself cannot explain their accumulation in higher TG quartiles. The most likely explanation comes from the recent observation of Johansen and colleagues, who found an excess of rare variants of *APOA5*, *GCKR*, *LPL* and *APOB* [26]. Albeit we did not resequence the patients with higher TG levels, this explanation looks plausible in case of our patients.

The mutations in *GCKR* gene resulting in the synthesis of proteins with increased inhibitory activity might be diabetogenic, likely reflecting elevated sensitivity to fructose-6-phosphate or reduced susceptibility to antagonism by fructose-1-phosphate [28]. Probably glucokinase has a role in the development of type 2 diabetes mellitus (T2DM) [20, 29]. The Diabetes Genetics Initiative genome-wide association study for T2DM and quantitative metabolic traits described rs780094, that showed a trend towards association with lower fasting glycaemia, decreased insulin resistance, and lower chance for the development of T2DM. Previously, it was proved that the rs780094 is in strong linkage disequilibrium with the other non-synonymous *GCKR* variant [30]. The *GCKR* C1337T is in connection with plasma TG, impaired fasting glycaemia, and might represent a risk for T2DM. The 1337T variant in the *GCKR* gene could protect against T2DM. Although, the minor T-allele of C1337T was associated with increased TG and higher risk of dyslipidemia; it showed lower fasting plasma glucose rates and decreased risk of hyperglycaemia [29, 31].

The 1337T allele is associated with higher absolute plasma postprandial triglycerides and incremental TG concentrations, as well as postprandial VLDL-cholesterol levels (the TG rich particle of liver origin). The mechanism through which GCKR influences variation in fast and postprandial lipid response remains to be elucidated. This effect could be attributed to variability in VLDL production in the liver. Indeed, GCKR expression is the highest in

human liver. Elevated TG levels may be secondary to increased glucose metabolism caused by overexpression or increased activity of GCKR, which in turn would be expected to increase GK activity. In the liver, increased glycolytic flux as a consequence of increased GK activity would be expected to increase levels of glycerol-3-phosphate and malonyl CoA. Malonyl CoA functions as a physiological inhibitor of carnitine-palmitoyl transferase I, the rate limiting enzyme for beta-oxidation, as well as an intermediate for de novo lipogenesis. Thus, increase in levels of malonyl CoA would inhibit fatty acid oxidation and drive fatty acyl-CoA into TG and VLDL synthesis [14–16, 32] (Fig. 2.)

As a summary, here we found a significant stepwise relationship between *APOA5* minor allele carrier and *APOA*2* haplotype frequencies and serum triglyceride quartiles, and the *GCKR* minor homozygous genotypes exhibited also an accumulation. The role of specific haplotypes with rare SNPs in this quantitative trait looks likely, however, resequencing of patients might be necessary.

Taken together, the current study provides indirect evidence for the existence of haplotypes having rare variants which further alter the TG level modifying effects of common variants. Thus, association of such variants in patients with higher TG levels can even represent a diagnostic challenge, and therefore next generation sequencing of the *APOA1-C3-A4* gene cluster may be considered on the future palette of the extended examinations of such patients.

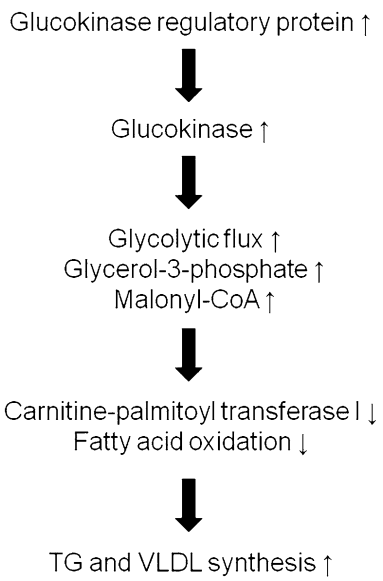

Fig. 2 Role of GCKR in triglyceride metabolism

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