Examination of role of triglyceride-level modulating polymorphisms in development of ischemic stroke

Ph.D. thesis

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1. INTRODUCTION

1.1. Stroke

Stroke is a common multifactorial disorder; the pathogenesis is very complex and both environmental and genetic factors contribute to its etiology. A stroke, also known as a cerebrovascular accident (CVA), is a suddenly developing focal or global loss of brain functions due to disturbance in the blood supply to the brain. Rapidly developing symptoms, which takes over 24 hours, or lead to death of patient.

As pathological viewpoint, the disease can be classified into two subgroups according to the manner of development: thrombotic or embolic infarcts, and haemorrhagic stroke. All stroke types comprise 83% ischemic atherothrombosis: 36%, embolism: 24%, lacunar infarcts: 23%; 10% intracerebral haemorhage (brain haemorhage), and not more than 7% subarachnoidal haemorrhage.

The primary (the prevention and screening of risk factors, treatment) and the secondary (prevention of development newer stroke, screening and care of people at risk of development stroke) prevention have an importance in the successful treatment of stroke.

In my PhD work, the associations between gene polymorphisms and circulating triglyceride-levels in Hungarian population were investigated, moreover, its possible effect on the development of stroke.

1.2. Risk factors

Acute ischemic stroke is a multifactorial disorder, in the manifestation modifiable and non-modifiable risk factors also play a role. Into non-modifiable risk factors gender, population origin, age, hereditable factors, and previous cerebrovascular disease in case history are enrolled. Hypertonia, smoking, exaggerated alcohol and drug consumption, presence of diabetes mellitus, cardiovascular diseases, obesity, and unfavourable lipid profile, high cholesterol- and triglyceride levels are categorized as among modifiable risk factors. In the manifestation of ischemic stroke other risk factors can take part: depression, migraine, pregnancy and the postmenopausal interval, physical inactivity, plasma fibrinogen level, obstructive sleep apnea, atrial fibrillation, and different genetical factors impacts.

In several studies increased triglyceride levels were proved as susceptible factor in manifestation of cardio- and cerebrovascular disorders, however the role of triglycerides in the development of disease is still under debate.

1.2.1. APOA5 gene

The *APOA5* gene is located near the *APOAI-APOCIII-APOAIV* gene cluster at 11q23 chromosome as a member of the apolipoprotein family. The gene includes four exons, and encodes a protein containing 366 amino acids. Alternative polyadenylation results in two transcripts, which are 1.3 and 1.9 kb length. APOA5 protein is a 39kDa weight molecule, which is expressed in the liver. Then the protein is secreted into the plasma as the constituent part of VLDL and HDL, where plays a central regulatory role in the triglyceride metabolism.

In recent studies, 40 polymorphisms have been identified in *APOA5* gene, however among these, the most common natural variants, the T-1131C (rs662799), T1259C (rs2266788), C56G (rs3135506) and IVS3+G476A (rs2072560) polymorphisms have been repeatedly reported to associate with elevated triglyceride levels. The T-1131C, as well as IVS3+G476A were found to be independent risk factors for metabolic syndrome, just as hypertriglyceridemia, additionally, numerous cardio- and cerebrovascular disorders. While the C56G and T1259C variants were proved as risk factors for cardio- and cerebrovascular diseases.

1.2.2. GCKR gene

The glucokinase regulatory protein (*GCKR*) gene, located on chromosome 2p23.3-p23.2. *GCKR* is 27 kb length, consists of 19 exons and encodes a protein of 625 amino acids, which is 68 kDa. The glucokinase regulatory protein (GCKR) regulates the mechanism of glucokinase enzyme of the liver. The glucokinase (hexokinase IV) enzyme plays a central role in the glucose homeostasis of the blood. This enzyme is a determinative glucose-phosphorylase of the liver and the pancreatic β-cells.

During the past few years, genome-wide association studies (GWAS) have identified functional variants in *GCKR* gene associated with hypertriglyceridemia. Two most frequent polymorphisms were examined such as the intronic rs780094 variant and the exonic rs1260326, which results a Leu/Pro change at amino acid - position 446. Combining of the *APOA5* and *GCKR* gene risk alleles, an additive effect was proved, and an association between fasting triglyceride levels and hypertriglyceridemia showed.

1.2.3. MLXIPL gene locus

A Max-like-interacting-protein-like (*MLXIPL;* or carbohydrate response element-binding protein, *ChREBP*) gene is 1.5 Mb lengths. *MLXIPL* is located in the WBSCR14 deletion region, at chromosome 7q11.23. The gene is composed of 852 amino acids and encodes a transcriptional factor. The human ChREBP is expressed in multiple tissues, particularly in liver, in adipose tissue, as well as in tissues of the brain and the intestinal tract. In last years, genome-wide association studies (GWAS) have correlated the plasma triglyceride-level changes with *MLXIPL* locus. In several studies, the impacts of triglyceride-level increase of the major alleles of the rs17145738 and rs3812316 variants in *MLXIPL* locus were observed.

1.2.4. GALNT2 gene locus

The UDP-N-acetyl-alpha-D-galactosamine: polypeptide-N-acetyl–galactos-aminyl-transferase 2 (GALNT2) protein was purified from human placenta, which was named as GalNAc-T2. The *GALNT2* gene is located at chromosome 1q41-q42, includes 16 exons, and encodes a protein containing 571 amino acids, which is 64 kDa protein. In recent GWAS studies, the minor G-allele of the rs4846914 intronic variant associated with elevated triglyceride concentrations of the plasma.

1.2.5. ANGPTL3, CILP2 and TRIB1 gene loci

In last years an association has been detected between dyslipidemia and the rs16996148 (near *CILP2*), rs17321515 (near *TRIB1*), rs12130333 (near *ANGPTL3*) variants. Moreover, these loci were correlated with the manifestation of cardiovascular diseases.

ANGPTL3 gene is located at chromosome 1p31. *ANGPTL3* is expressed almost exclusively in liver, and is presumed to function as a circulating inhibitor of lipoprotein lipase, an enzyme regulating the plasma levels of triglycerides and HDLs (high-density lipoproteins).

The *CILP2* gene is located at 19p13.11. The proteins' relation to lipid metabolism is not yet discovered. In a genome-wide association study, investigating Caucausian population, a triglyceride level decreasing function of the rs16996148 polymorphism was described.

The human tribbles–1 gene is located at chromosome 8q24. The TRIB1 facilitates the proteosome-dependent protein degradation. In an Asian Malay population, the polymorphism adjacent to the *TRIB1* locus (rs17321515) was associated with elevated total cholesterol and LDL-cholesterol and with increased risk of coronary heart disease and cardiovascular disease.

2. AIMS OF THE STUDY

The aims of examinations in Hungarian population with ischemic stroke:

1. Examination of the allelic distribution of variants of the *APOA5*: T-1131C (rs662799), T1259C (rs2266788), C56G (rs3135506) and IVS3+G476A (rs2072560); the *GCKR*: C1337T (rs1260326) and *GALNT2* (rs4846914) genes, as well as the *MLXIPL/TBL2* (rs17145738 and rs3812316) and the *ANGPTL3* (rs12130333), *CILP2* (rs16996148), *TRIB1* (rs17321515) loci.

2. Study of the effect of the possible genetic combination of *APOA5*, *GCKR* genes, on triglyceride-level changes, in patients and control subjects. Moreover, analysing the possible associations with the manifestation of stroke.

3. Evaluation of *GALNT2* (rs4846914) gene variant and the *MLXIPL/TBL2* (rs17145738 and rs3812316) polymorphisms' effect on triglyceride-level alteration, and their possible susceptible role in ischemic stroke.

4. Analysis of *ANGPTL3* (rs12130333), *CILP2* (rs16996148), *TRIB1* (rs17321515) gene loci, and possible association with the development of ischemic stroke.

3. MATERIALS AND METHODS

3.1. Examined patient population

The patients with ischemic stroke and the control samples which enrolled in studies, derived from our Department's Biobank, belonging to the Central National Biobank Network of Hungary (www.biobanks.hu) and the Pan-European Biobanking and Biomolecular Resources Research Infrastructure (BBMRI) program (http://bbmri.eu/bbmri/). The governance, maintenance and management principles of the Biobank had been approved by the national Scientific Research Ethics Committee, Budapest (ETT TUKEB).

All of the stroke patients had a previously defined stroke, or with an acutely developing ischemic stroke were referred to outpatient clinic. All of the patients underwent a detailed clinical scrutiny. The magnetic resonance imaging (MRI) examinations were performed within 2 days after the outbreak of symptoms to define precisely the affected areas.

After extensive neurological and MRI examination, the patients were enrolled into three stroke subgroups (TOAST). First subgroup consists patients who had large-vessel infarcts (large-vessel patients), with cortical or cerebellar lesions and/or brainstem infarcts or subcortical hemispheric infarcts.

The next group created from patients with small-vessel occlusion on MRI, subcortical hemispheric or brainstem infarcts. The third cohort contains all patients with cardioembolic stroke or other non-specified etiologic stroke was detected, and one or more lacunar and large-vessel infarcts were on MRI. The mixed-group classification of the patients was required because of the small sample number from statistical point of view.

The control subjects (as their anamnesis) did not suffer from any stroke events, there were no neurological alterations on MRI and CT images. For the patients, age-matched, healthy control subjects were selected randomly.

3.2. Applied molecular biology methods

3.2.1. PCR reaction

Our examinations were executed on genomic DNA, which was extracted from peripheral EDTA-anticoagulated blood leukocytes, by a standard desalting method. The amplification was performed by DNA-specific synthetic oligonucleotide primers, using polymerase chain reaction (PCR).

3.2.2. RFLP method

After the digestion with restriction endonucleases, the incubation was applied according to the enzymes' temperature requirements. During planning of the methods, all were designed to include an obligate cleavage site on the amplicon in the amplified DNA sequence thus enabling us to monitor the efficacy of the digestion.

3.2.3. Direct sequencing

To detect and define DNA sequences, direct sequencing method was used to confirm our results on ABI Prism 3100 Avant automated sequencer. Winstar genetical program was applied to analyse the data.

3.3. Statistical analysis

All clinical data are represented as mean ± SEM (standard error of the mean). Kolomogorov-Smirnov test was used to determine the distribution of variables. If the variables showed Gaussian distribution, parametric tests were applied. For variables with no Gaussian distribution, nonparametric tests were used. In all statistical analyses, for effective differences among all groups were examined by Kruskal-Wallis test. Pairwise analyses of differences between groups in discreet clinical and laboratory parameters with normal distribution, and to calculate odds ratios (OR) of specific combinations of two genes, also χ^2 tests were used. Continuous variables with normal distribution were analysed with Student's T-tests. For comparison of differences between groups in continuous variables with skewed distribution Mann-Whitney-U tests were applied.

Mann-Whitney-test was used for comparing differences of clinical parameters between group of patients and controls. Correlations were analysed and crude/adjusted odds ratios (OR) were defined using multiple logistic regression model. The confidence intervals and p-values of significance were established 95% and 0.05 for all analyses. All allele distributions were in Hardy-Weinberg-equilibrium both in stroke groups and in controls.

4. RESULTS

4.1. Examination of GCKR and APOA5 genes

We detected a significant increase of allele frequencies in the promoter region of *APOA5* gene T-1131C variant, in the intronic IVS3+G476A variant, and in C56G variant located in the third exon. According to the Odd Ratios, in all variants, in all stroke subgroups, carrying minor alleles are associated with ischemic stroke, except the C56G variant, which was not showed any significant difference in small-vessel subgroup comparing the control subjects. The T1259C polymorphism did not show significant difference compared to control subjects. The rs1260326 (C1337T) variant of *GCKR* gene showed similar allele frequencies both in the stroke and control groups. Both in stroke patients and in controls the level of triglycerides was elevated in all *APOA5* variant -1131C, 1259C, and IVS+G476A minor allele carriers compared to the non-carriers. By contrast, the *GCKR* gene's minor allele 1337T was not associated with triglyceride level changes either in stroke patients or in controls. Odds Ratios were adjusted for age, gender, body mass index, ischemic heart disease, hypertension, diabetes mellitus, smoking- and drinking habits. In our study, the adjusted ORs for carrying -1131C, 56G, and IVS3+476A demonstrated an independent risk for the development of stroke. In contrast, we could not detect any association of the 1259C allele with stroke; in addition, the *GCKR* 1337T allele did not exhibit a risk or protective nature for the disease (Table 1.).

Beside single locus genetic analysis, we also tested the effect of the specific combinations of *GCKR* C1337T and *APOA5* polymorphisms. Individual *APOA5* genotypes were studied in combination with *GCKR* C1337T genotypes. We generated four groups for all *APOA5* variants as follows: NC_{C1337T}-$NC_{T\text{-}1131C}$; NC_{C1337T}-$C_{T\text{-}1131C}$; C_{C1337T}-$NC_{T\text{-}1131C}$ and C_{C1337T}-$C_{T\text{-}1131C}$ where "NC" refers to the wild genotype; "C" means carrying the minor allele of the variant indicated.

Compared to individual ORs of the variants the relative risk for ischemic stroke conferred by the presence of their combinations was significantly greater in certain groups. The relative risk for stroke was increased by C_{C1337T}-$C_{T-1131C}$ in the small vessel group, in the large vessel group, in the mixed group, and in the overall cohort; by C_{C1337T}-$C_{IVS+G476A}$ in the small and mixed groups as well as and in the whole stroke cohort; and by C_{C1337T}-C_{C56G} in the large-vessel group, in the mixed group and in the whole group of stroke patients. For C_{C1337T}-$C_{T-1131C}$ lower ORs were seen in overall as well as in large and mixed-vessel groups. For the other combinations we could not detect any changes affecting the risk of the disease. Although, for all the individual *APOA5* variants significant association with elevated non-fasting triglyceride level was found in all stroke subgroups, for the specific genotype combinations only C_{C1337T}-C_{T1259C}, C_{C1337T}-$C_{IVS+G476A}$ (small, overall) and C_{C1337T}-C_{C56G} (overall) showed the same effect (Table 2.).

The C1337T variant of GCKR gene has an effect on triglyceride levels, impaired fasting glycaemia, and have possible a risk of type II diabetes mellitus. Carrying the 1337T allele showed elevated triglyceride levels and higher risk of dyslipidemia, but a lower fasting plasma glucose rates and a lower risk for the susceptibility of hyperglycemia. In our studied population the presence of diabetes mellitus conferred increased risk for stroke in all stroke subgroups (adjusted OR in small vessel: 3.69*; in large vessel: 4.537*; in mixed: 6.157*, in overall: 4.395*). Subsequently, we examined the effect of *GCKR* C1337T variant on stroke patients with diabetes mellitus, the minor allele of *GCKR* (1337T) in homozygous form showed an increased risk for stroke in two subgroups (in small vessel group, in overall). Because of the statistical low number of samples with diabetes mellitus, we are unable to draw a far-reaching conclusions.

4.2 Examination of GALNT2 and MLXIPL gene loci

Both the triglyceride and the total cholesterol levels of each stroke subgroup and the overall stroke group proved to be significantly higher than those of the control group.
The allele frequencies observed among stroke patients did not significantly differ from those of the control group for either polymorphic variant. Also, genotype frequencies were similar to frequencies obtained in other populations and to data available in the International HapMap Project's database (www.hapmap.org) for the Caucasian CEPH population of European origin. neither rs4846914-G, nor rs17145738-C, nor rs3812316-C variants proved to be a risk factor for the development of stroke disease in our population sample. In addition, homozygote status for the risk alleles of any of the three analyzed SNPs did not significantly associate with the risk of stroke either (Table 3.).

For rs17145738, rs3812316 and rs4846914 the mean blood lipid concentrations did not significantly differ in heterozygous and homozygous carriers from those of the non-carriers in either the stratified stroke subgroups, or the overall stroke disease group (Table 4.).

4.3. Examination of ANGPTL3, CILP2 and TRIB1 gene loci

Examining variants, we could not detect any significant differences in allele frequencies comparing the stroke subgroups to the controls, either for rs16996148, rs17321515, or for rs12130333 polymorphisms. We did not find any association between serum triglyceride or total cholesterol levels and carrying the functional variants analysed (Table 5.). Nor did we find a significant change in disease risk in the carriers of rs16996148-T; rs17321515-G, minor alleles after adjusting the multiple regression analysis (Table 6.).

Table 1. The effects on lipid parameters and logistic regression analysis of APOA5 and GCKR gene variants

	Stroke patients								Control (n=172)	
	Small-vessel (n=232)		Large-vessel (n=139)		Mixed (n=142)		Overall (n=513)			
APOA5										
T-1131C	**TT** (n=197)	**TC+CC** (n=30+5)	**TT** (n=118)	**TC+CC** (n=19+2)	**TT** (n=119)	**TC+CC** (n=19+4)	**TT** (n=434)	**TC+CC** (n=68+11)	**TT** (n=159)	**TC+CC** (n=12+1)
Triglyceride (mmol/l)	1.68±0.04	1.97±0.15*	1.73±0.06	2.11±0.20*	1.72±0.06	1.99±0.17*	1.70±0.03	1.92±0.09*	1.51±0.04	1.84±0.12*
OR#	1.937# (1.026 – 4.541)		2.813# (1.114 – 7.104)		3.533# (1.421 – 8.781)		2.929# (1.418 – 6.051)			
T1259C										
	TT (n=190)	**TC+CC** (n=40+2)	**TT** (n=113)	**TC+CC** (n=25+1)	**TT** (n=112)	**TC+CC** (n=28+2)	**TT** (n=415)	**TC+CC** (n=93+5)	**TT** (n=145)	**TC+CC** (n=27+0)
Triglyceride (mmol/l)	1.67±0.04	1.97±0.12*	1.73±0.07	2.00±0.14*	1.71±0.06	1.94±0.14*	1.69±0.03	1.89±0.07*	1.50±0.04	1.69±0.07*
OR#	1.211 (0.616 – 2.381)		1.204 (0.560 – 2.588)		1.952 (0.932 – 4.091)		1.466 (0.838 - 2.566)			
IVS+G476A										
	GG (n=201)	**GA +AA** (n=30+1)	**GG** (n=125)	**GA +AA** (n=14+0)	**GG** (n=121)	**GA +AA** (n=20+1)	**GG** (n=447)	**GA +AA** (n=64+2)	**GG** (n=163)	**GA +AA** (n=9+0)
Triglyceride (mmol/l)	1.67±0.04	2.05±0.16*	1.73±0.06	2.28±0.21*	1.70±0.06	2.09±0.17*	1.69±0.03	2.00±0.09*	1.52±0.04	1.80±0.11*
OR#	2.439# (1.272 – 6.120)		1.888# (1.252 – 5.464)		3.893# (1.445 - 10.489)		3.173# (1.408 – 7.150)			
C56G										
	CC (n=205)	**CG +GG** (n=25+2)	**CC** (n=118)	**CG +GG** (n=21+0)	**CC** (n=121)	**CG +GG** (n=20+1)	**CC** (n=444)	**CG +GG** (n=66+3)	**CC** (n=160)	**CG +GG** (n=12+0)
Triglyceride (mmol/l)	1.64±0.03	1.95±0.13*	1.74±0.06	2.02±0.14*	1.73±0.06	1.91±0.12*	1.69±0.03	1.96±0.08*	1.52±0.04	1.66±0.05*
OR#	2.156 (0.836 – 5.561)		2.873# (1.086 – 7.601)		2.939# (1.079 – 8.010)		2.316# (1.059 – 5.067)			
GCKR										
C1337T	**CC** (n=55)	**CT+TT** (n=125+52)	**CC** (n=34)	**CT+TT** (n=77+28)	**CC** (n=34)	**CT+TT** (n=77+31)	**CC** (n=123)	**CT+TT** (n=279+111)	**CC** (n=48)	**CT+TT** (n=80+44)
Triglyceride (mmol/l)	1.63±0.06	1.68±0.04	1.79±0.12	1.78±0.07	1.94±0.12	1.70±0.06	1.76±0.06	1.72±0.03	1.58±0.09	1.51±0.04
OR#	1.585 (0.867 – 2.899)		1.231 (0.638 – 2.374)		0.976 (0.498 – 1.914)		1.289 (0.790 – 2.104)			

Values are means ± SEM. Triglycerides and serum total cholesterol levels are mmol/l. *p<0.05 vs. non-carriers.
Adjusted OR (95% CI) for differences in age, gender, BMI, total serum cholesterol, hypertension, diabetes mellitus, ischemic heart diseases, smoking – and drinking habits.
*p<0.05 vs. controls.

Table 2. The effects on lipid parameters and logistic regression analysis of special genotype combinations of APOA5 and GCKR gene

		***GCKR* C1337T - *APOA5* T-1131C**		***GCKR* C1337T - *APOA5* T1259C**		***GCKR* C1337T - *APOA5* IVS+G476A**		***GCKR* C1337T - *APOA5* C56G**	
		NC_{C1337T}-$NC_{T-1131C}$	**C_{C1337T}-$C_{T-1131C}$**	**NC_{C1337T}-NC_{T1259C}**	**C_{C1337T}-C_{T1259C}**	**NC_{C1337T}-$NC_{IVS+G476A}$**	**C_{C1337T}-$C_{IVS+G476A}$**	**NC_{C1337T}-NC_{C56G}**	**C_{C1337T}-C_{C56G}**
Small-vessel (n=232)		(n=47)	(n=25)	(n=44)	(n=31)	(n=48)	(n=24)	(n=50)	(n=22)
	TG	1.65±0.05	1.79±0.13	1.67±0.06	1.88±0.10*	1.67±0.06	1.92±0.12*	1.60±0.06	1.96±0.16
	OR#	1	2.809* (1.189-6.631)	1	1.483 (0.727-3.028)	1	4.400* (1.545-12.533)	1	2.200 (0.918-5.272)
Large-vessel (n=139)		(n=28)	(n=15)	(n=27)	(n=19)	(n=30)	(n=10)	(n=31)	(n=18)
	TG	1.73±0.13	1.96±0.26	1.70±0.14	1.96±0.18	1.71±0.12	2.22±0.29	1.77±0.12	2.02±0.16
	OR#	1	2.619* (1.010-6.790)	1	1.481 (0.665-3.303)	1	2.993 (0.911-9.447)	1	2.903* (1.155-7.297)
Mixed (n=142)		(n=27)	(n=16)	(n=25)	(n=21)	(n=27)	(n=14)	(n=30)	(n=17)
	TG	1.84±0.13	1.84±0.20	1.83±0.13	1.80±0.16	1.84±0.13	1.98±0.22	1.93±0.13	1.89±0.14
	OR#	1	2.897* (1.124-7.467)	1	1.768 (0.797-3.923)	1	4.563* (1.477-14.096)	1	2.833* (1.117-7.186)
Overall (n=513)		(n=102)	(n=65)	(n=96)	(n=71)	(n=105)	(n=48)	(n=111)	(n=57)
	TG	1.72±0.05	1.86±0.09	1.72±0.06	1.88±0.08*	1.73±0.05	2.00±0.10*	1.74±0.06	1.95±0.09*
	OR#	1	2.780* (1.267-6.102)	1	1.557 (0.832-2.913)	1	4.023* (1.501-10.783)	1	2.568* (1.173-5.622)
Control (n=172)		(n=44)	(n=9)	(n=40)	(n=19)	(n=44)	(n=5)	(n=45)	(n=9)
	TG	1.53±0.09	1.68±0.10	1.54±0.10	1.63±0.08	1.55±0.09	1.70±0.18	1.57±0.09	1.61±0.06

Values are means ± SEM. Triglycerides and serum total cholesterol levels are mmol/l. *p<0.05 vs. non-carriers.
„NC": carrying of the wild genotype; „C": carrying of at least one variant allele of the SNP marked in the subscript.
#OR relative to „NC-NC" genotype, (95% CI). *p<0.05 vs. controls.

Table 3. The logistic regression analysis of variants of GALNT2 and MLXIPL gene loci

	Stroke patients			
	Small-vessel (n=212)	**Large-vessel** (n=127)	**Mixed** (n=128)	**Overall** (n=467)
GALNT2 rs4846914 **OR**				
G carriers	1.021 (0.579-1.802)	1.801 (0.928-3.497)	1.277 (0.667-2.446)	1.318 (0.821-2.116)
GG homozygotes	1.104 (0.548-2.225)	1.202 (0.565-2.560)	1.214 (0.545-2.705)	1.174 (0.653-2.112)
MLXIPL rs17145738 **OR**				
C carriers	3.933 (0.347 – 44.609)	1.238 (0.119 – 12.855)	2.058 (0.146 – 28.985)	1.877 (0.299 – 11.777)
CC homozygotes	1.435 (0.762-2.702)	1.047 (0.524-2.093)	1.264 (0.616-2.593)	1.229 (0.734-2.055)
MLXIPL rs3812316 **OR**				
C carriers	1.015 (0.138-7.459)	1.238 (0.119 – 12.855)	0.226 (0.031-1.654)	0.682 (0.141-3.295)
CC homozygotes	1.825 (0.962-3.597)	1.085 (0.537-2.194)	1.653 (0.771-3.544)	1.354 (0.793-2.313)

Adjusted OR (95% CI) for differences in age, gender, BMI, total serum cholesterol, hypertension, diabetes mellitus, ischemic heart diseases, smoking – and drinking habits.
*p<0.05 vs. controls.

Table 4. The effect on lipid parameters of polymorphisms of GALNT2 and MLXIPL gene loci

		GALNT2 rs4846914	Triglyceride	Total cholesterol	*MLXIPL* rs17145738	Triglyceride	Total cholesterol	*MLXIPL* rs3812316	Triglyceride	Total cholesterol
Stroke patients	**Small-vessel** (n=212)	**AA n=70**	1.69±0.06	5.74±0.13	**TT n=4**	1.73±0.06	5.78±0.88	**GG n=5**	1.6±0.10	5.24±0.23
		AG+GG n=96+46	1.68±0.04	5.94±0.09	**TC+CC n=54+154**	1.68±0.03	5.87±0.08	**GC+CC n=44+163**	1.68±0.03	5.89±0.08
	Large-vessel (n=127)	**AA n=36**	1.64±0.09	6.08±0.21	**TT n=2**	1.05±0.15	6.15±0.75	**GG n=2**	1.05±0.15	6.15±0.75
		AG+GG n=64+27	1.87±0.08	5.88±0.13	**TC+CC n=28+97**	1.81±0.06	5.93±0.11	**GC+CC n=25+100**	1.82±0.06	5.93±0.11
	Mixed (n=128)	**AA n=49**	1.77±0.09	6.05±0.24	**TT n=2**	1.50±0.10	8.55±0.05	**GG n=4**	2.1±0.24	7.40±1.4
		AG+GG n=57+22	1.76±0.08	5.77±0.14	**TC+CC n=23+103**	1.77±0.06	5.83±0.12	**GC+CC n=18+106**	1.76±0.06	5.83±0.12
	Overall (n=467)	**AA n=155**	1.71±0.04	5.92±0.11	**TT n=8**	1.50±0.11	6.56±0.61	**GG n=11**	1.7±0.18	6.19±0.57
		AG+GG n=217+95	1.76±0.04	5.88±0.07	**TC+CC n=105+354**	1.74±0.03	5.88±0.06	**GC+CC n=87+369**	1.74±0.03	5.88±0.06
Control (n=156)		**AA n=55**	1.63±0.08	5.31±0.12	**TT n=3**	1.47±0.15	4.97±0.98	**GG n=3**	1.47±0.15	4.97±0.98
		AG+GG n=76+25	1.51±0.04	5.32±0.09	**TC+CC n=40+113**	1.55±0.04	5.32± 0.07	**GC+CC n=34+119**	1.55±0.04	5.32±0.07

Values are means ± SEM. *p<0.05 vs. non-carriers. Triglycerides and serum total cholesterol levels are mmol/l.

Table 5. Analysis of ANGPTL3, CILP2 and TRIB1 gene loci

	Stroke patients								Control (n=168)	
	Small-vessel (n=183)		Large-vessel (n=135)		Mixed (n=141)		Overall (n=459)			
CILP2 rs16996148	**GG** n=152	**TG+TT** n=29+2	**GG** n=117	**TG+TT** n=18+0	**GG** n=124	**TG+TT** n=17+0	**GG** n=393	**TG+TT** n=64+2	**GG** n=145	**TG+TT** n=21+2
Triglyceride (mmol/l)	1.68±0.04	1.66±0.10	1.77±0.06	1.89±0.19	1.79±0.06	1.56±0.15	1.74±0.03	1.70±0.08	1.55±0.04	1.43±0.08
Total cholesterol (mmol/l)	5.87±0.09	5.80±0.21	5.99±0.12	5.58±0.31	5.86±0.12	6.18±0.36	5.90±0.06	5.84±0.16	5.38±0.08	5.30±0.20
TRIB1 rs17321515	**AA** n=46	**GA+GG** n=98+39	**AA** n=34	**GA+GG** n=79+22	**AA** n=34	**GA+GG** n=65+42	**AA** n=114	**GA+GG** n=242+103	**AA** n=54	**GA+GG** n=75+39
Triglyceride (mmol/l)	1.75±0.08	1.65±0.04	1.77±0.10	1.79±0.07	1.70±0.11	1.78±0.06	1.74±0.06	1.73±0.03	1.51±0.05	1.54±0.05
Total cholesterol (mmol/l)	5.73±0.19	5.90±0.09	5.99±0.18	5.91±0.13	5.65±0.26	5.98±0.13	5.79±0.12	5.93±0.07	5.31±0.13	5.40±0.09
ANGPTL3 rs12130333	**CC** n=118	**TC+TT** n=59+6	**CC** n=92	**TC+TT** n=39+4	**CC** n=91	**TC+TT** n=45+5	**CC** n=301	**TC+TT** n=143+15	**CC** n=114	**TC+TT** n=44+10
Triglyceride (mmol/l)	1.70±0.05	1.63±0.06	1.79±0.08	1.78±0.10	1.78±0.07	1.73±0.08	1.75±0.04	1.70±0.05	1.51±0.05	1.58±0.06
Total cholesterol (mmol/l)	5.90±0.12	5.78±0.12	5.98±0.13	5.84±0.19	5.76±0.15	6.15±0.19	5.88±0.08	5.91±0.09	5.38±0.09	5.35±0.13

Values are means ± SEM. *p<0.05 vs. non-carriers. Triglycerides and serum total cholesterol levels are mmol/l.

Table 6. Logistic regression analysis of ANGPTL3, CILP2 and TRIB1 gene loci

		Stroke patients			
		Small-vessel (n=183)	**Large-vessel** (n=135)	**Mixed** (n=141)	**Overall** (n=459)
CILP2 rs16996148	Adjusted OR[#]	1.641 (0.762 - 3.534)	0.824 (0.341 - 1.993)	0.598 (0.230 - 1.551)	1.050 (0.556 - 1.983)
TRIB1 rs17321515	Adjusted OR[#]	1.640 (0.885 - 3.036)	1.718 (0.876 - 3.370)	1.656 (0.859 - 3.195)	1.563 (0.965 - 2.533)
ANGPTL3 rs12130333	Adjusted OR[#]	0.927 (0.512 - 1.677)	1.260 (0.681 - 2.334)	0.941 (0.490 - 1.805)	1.078 (0.669 - 1.737)

Adjusted OR (95% CI) for differences in age, gender, BMI, total serum cholesterol, hypertension, diabetes mellitus, ischemic heart diseases, smoking – and drinking habits.
*$p<0.05$ vs. controls.

5. DISCUSSION OF RESULTS AND CONCLUSIONS

5.1. Individual and combined role of APOA5 and GCKR genes

The role of triglycerides in different occlusive vascular diseases, including ischemic stroke, has been under investigation for a long while. Results are still controversial, and as the delineation of the mechanism of triglyceride elevation has already begun, there might be a chance for verification of the possible roles of *APOA5* and *GCKR* genes. In the postgenomic era several new genes affecting the triglyceride metabolism had already been verified, like the *APOA5* variants. These polymorphisms can influence the function of the protein transcript, which can modify secondarily the interaction of *APOA5* with the lipoprotein lipase and eventually lead to increased circulating triglyceride levels. In the present study we could confirm the previous findings regarding associations with triglyceride levels and stroke susceptibility.

In the Diabetes Genetics Initiative (DGI) genome-wide association study the *GCKR* gene (rs780094) showed a trend toward association with lower fasting glycaemia, less insulin resistance, and lower chance for the development of type II diabetes had been verified. The rs1260326 (Leu446Pro) is in connection with increased plasma triglyceride level, could protect against fasting glycaemia and insulinemia and the minor allele (T) of rs1260326 variant in the *GCKR* gene could protect against diabetes mellitus type II.

The T-1131C, IVS+G476A and C56G variants of *APOA5* gene had a significantly increased allele frequency, and were associated with significantly elevated triglyceride levels in stroke patients collated with controls, suggesting an association with the development of ischemic stroke disease. By contrast, in case of *GCKR* gene (rs1260326) we could not detect any difference in allele frequencies compared with controls; and also found a non-significant triglyceride level rise and no increased risk for ischemic stroke disease. Additionally, we observed that in all stroke subgroups diabetes mellitus was significantly associated with the development of stroke disease, despite the small number of cases.

In summary, in agreement with our previous results, the frequencies of *APOA5* minor alleles' are higher in stroke patients than in controls, and we can conclude, there is a relationship between the triglyceride-increase and the variants studied. We could detect a possible risk for the development of stroke in connection with the *APOA5* polymorphisms. However, we did not find any association between *GCKR* variant and either the triglyceride levels or the susceptibility for the disease, thus in the Hungarian population the *GCKR* gene polymorphism did not prove to be an independent risk factor. Examining the combinations of four *APOA5* polymorphisms with the *GCKR* variant, we found, that in certain subgroups, the gene combinations showed significant correlation with the changes of triglyceride levels and increased the risk for the development of stroke.

5.2. Role of GALNT2 and MLXIPL gene loci

Although several studies already demonstrated that, the examined rs17145738 and rs3812316 of the *MLXIPL* locus, and rs4846914 variant of *GALNT2* polymorphisms cause elevated triglyceride levels which constitute an independent risk factor for cardiovascular diseases and it has relevance in atherosclerosis. *MLXIPL*-related variant rs17145738 has been found to contribute to elevated triglyceride levels in a multiethnic population (European, South-Asian and Chinese ancestry and in an Italian cohort), although its correlation with HDL-cholesterol, as it was suggested by Kathiresan and colleagues was not confirmed. Our data did not reveal any association between rs17143758 and triglyceride- or cholesterol levels, nor did we find an increased risk of stroke development in our study population in the presence of the risk allele.

Findings reported on the second *MLXIPL*-related polymorphism rs3812316 are similarly not consistent. The correlation between triglyceride-level increase and *MLXIPL* rs3812316 in a Japanese cohort could be confirmed, however, these results could not be replicated in their study conducted in a Central European population. Here, in the Hungarian patient group we also were not able to demonstrate a correlation between rs3812316 and blood triglyceride and total cholesterol values, or with the risk of ischemic stroke.

The rs3812316 SNP, located in an evolutionary conserved domain of *MLXIPL* falls within a region of high linkage disequilibrium that includes rs17145738 as well. We believe that the observed lack of association with triglyceride levels and stroke for both *MLXIPL* SNPs validates our data as a result of their linkage disequilibrium.

The third variant, rs4846914 of *GALNT2* was initially described to exert an effect on both triglyceride level increase and HDL-cholesterol decrease, however, the contribution of this polymorphism to changes in triglyceride levels was not supported by additional reports.

We also could not find an association between rs4846914 and plasma triglyceride or total cholesterol levels, either. In addition, we could not detect a correlation of stroke risk with this possible susceptibility factor. Taken together, our results suggest that in stroke patients the polymorphisms rs17145738, rs3812316 and rs4846914 are not associated with altered triglyceride and cholesterol levels, and do not contribute to the risk of ischemic stroke.

5.3. Role of ANGPTL3, CILP2 and TRIB1 gene loci

The examined polymorphisms rs16996148 (near *CILP2*), rs17321515 (near *TRIB1*), and rs12130333 (near *ANGPTL3*) polymorphisms were reportedly associated with decreased triglyceride levels and dyslipidemia. Willer and colleagues published their results linking these loci with coronary artery disease risk. The rs12130333 polymorphism is intergenic, however it has high linkage to *ANGPTL3* gene showing triglyceride decrease, we could not detect any decrease in triglyceride levels.

In a genome-wide association study, the rs16996148 polymorphism near the *CILP2* locus had a triglyceride level decreasing function, however, triglyceride lowering association could not be replicated either on Japanese population, or in our examined Hungarian population.

After analyzing the human tribbles-1 gene locus we did not detect an association of the rs17321515 variant with changes in triglyceride levels or development of stroke. In summary, in our survey, we did not find a significant correlation between the polymorphisms analysed and triglyceride level alteration in the Hungarian population with ischemic stroke.

6. CONCLUSION

I. Examining the natural variants of the *APOA5* gene, in all stroke groups and control subjects showed significantly increased plasma triglyceride-levels carrying 1131C, 56G, IVS3+476A and 1259C alleles.

II. Carrying the 1337T allele of *GCKR* gene, there was no plasma triglyceride alteration in any stroke groups, moreover, the alleles of *GCKR* debased the triglyceride level increasing effect of *APOA5* gene polymorphisms' in many cases.

III. Carrying the -1131C and IVS3+476A alleles of *APOA5* gene showed as independent risk factor in all stroke groups. We observed the same in the case of C56G variant, exception of small-vessel group. The T1259C polymorphism of *APOA5* gene and the C1337T variant of *GCKR* gene did not verify as susceptible factor in the development of ischemic stroke.

IV. Carrying the natural genotype combinations of minor alleles of the *APOA5* and *GCKR* genes conferred risk for the development of ischemic stroke in different stroke groups, in the following comnbinations: *GCKR* C1337T - *APOA5* T-1131C; *GCKR* C1337T - *APOA5* IVS+G476A ; *GCKR* C1337T - *APOA5* C56G. The genotype combination of *GCKR* C1337T - *APOA5* T1259C did not prove as an independent risk factor.

V. We ascertained that in all patients with ischemic stroke, the polymorphisms rs17145738 and rs3812316 in *MLXIPL* gene locus, and the rs4846914 in *GALNT2* gene locus, did not show any association with increased plasma triglyceride concentration, and did not confer risk for the development of ischemic stroke.

VI. The variants rs12130333 in *ANGPTL3* locus, rs16996148 in *CILP2* locus and rs17321515 in *TRIB1* locus did not show any significant association either with triglyceride level changes, or with the susceptibility for the development of ischemic stroke in Hungarian population.

7. PUBLICATION LIST

7.1 The thesis based on the following publications

1. Járomi L, Csöngei V, Polgár N, Szolnoki Z, Maász A, Horvatovich K, Faragó B, Sipeky C, Sáfrány E, Magyari L, Kisfali P, Mohás M, Janicsek I, Lakner L, Melegh B. Functional variants of glucokinase regulatory protein and apolipoprotein A5 genes in ischemic stroke. J Mol Neurosci. 2010, Oct 22.
   IF: 2.922 (2010)

2. Polgár N, Járomi L, Csöngei V, Maász A, Horvatovich K, Faragó B, Sipeky C, Sáfrány E, Melegh B. Triglyceride level modifying functional variants of GALTN2 and MLXIPL in ischemic stroke patients. Eur J Neurol. 2010, Febr 10.
   IF: 3.765 (2010)

3. Járomi L, Csöngei V, Polgár N, Rappai G, Szolnoki Z, Maász A, Horvatovich K, Sáfrány E, Sipeky C, Magyari L, Melegh B. Triglyceride level-influencing functional variants of the ANGPTL3, CILP2 and TRIB1 loci in ischemic stroke. J NeuroMol. Med. 2011, Jun 21.
   IF: 4.657 (2010)

7.2. Other articles

I. Accepted articles in international journals

J1. *Kocsis, M.,* ***Járomi, L.,*** *Putnoky, P., Kozma P., Borhidi, A.,* Genetic diversity among twelve grape cultivars indigenous to the Carpathian Basin revealed by RAPD markers, *Vitis, 2005, 44(2):87-91.* IF:0.897 (2005)

J2. *Magyari, L, Bene, J., Komlósi, K, Talián, G, Faragó, B, Csöngei, V,* ***Járomi, L,*** *Sáfrány, E, Sipeky, C., Lakner L, Varga M, Gasztonyi B, Melegh B.,* Prevalence of SLC22A4 1672T and SLC22A5 -207C Combination Defined TC Haplotype in Hungarian Ulcerative Colitis Patients, *Pathology Oncology Research, 2007, 13(1):53-56.* IF:1.272 (2007)

J3. *Maász, A., Kisfali, P., Horvatovich, K., Mohás, M., Markó, L., Csöngei, V., Faragó, B.,* ***Járomi, L.,*** *Magyari, L., Sáfrány, E., Sipeky, Cs., Wittman, I., Melegh, B.,* Apolipoprotein A5 T-1131C variant confers risk for metabolic syndrome. *Journal of Pathology Oncology Research, . 2007, 13(3):243-7.* IF:1.272 (2007)

J4. *Faragó, B., Magyari, L., Sáfrány, E., Csöngei, V.,* ***Járomi, L.,*** *Horvatovich, K., Sipeky, Cs., Maász, A., Radics, J., Gyetvai, Á., Szekanecz Z., Czirják, L., Melegh, B.,* Functional variants of interleukin-23 receptor gene confer risk for rheumatoid arthritis but not for systemic sclerosis. *Ann Rheum Dis Published, doi:10.1136/ard.2007.072819; 2008 Feb;67(2):248-50.* IF:7.188 (2008)

J5. *Maasz, A., Kisfali, P.,* ***Járomi L.,*** *Horvatovich, K., Szolnoki, Z., Csöngei, V., Sáfrány, E., Sipeky, C., Hadarits, F., Melegh, B.,* Apolipoprotein A5 gene IVS3+G476A allelic variant confers susceptibility for development of ischemic stroke. *Circulation Journal, 2008, 72(7):1065-70.* IF:2.387 (2008)

J6. *Lakner, L., Csöngei, V., Sarlos, P.,* ***Járomi, L.,*** *Sáfrány, E., Varga, M., Orosz, P., Magyari, L., Bene, J., Miheller, P., Tulassay, Z., Melegh, B.* IGR2096a_1 T and IGR2198a_1 C alleles on IBD5 locus of chromosome 5q31 region confer risk for Crohn's disease in Hungarian patients. *Int.J.Colorectal Dis., 2009, 24(5):503-507.* IF:2.102 (2009)

J7. *Sipeky, Cs., Csöngei, V.,* ***Járomi, L.,*** *Sáfrány, E., Polgar, N., Lakner, L., Szabó, M., Takács, I., Melegh, B.* Vitamin K epoxide reductase complex 1 (VKORC1) haplotypes in average Hungarian and in Roma population samples. *Pharmacogenomics, 2009, Jun; 10(6):1025-32.* IF:3.893 (2009)

J8. *Sáfrány, E., Pazár, B., Csöngei, V.,* ***Járomi, L.,*** *Polgar, N., Sipeky, Cs., Horváth, F. I., Zeher, M., Poór, Gy., Melegh, B.* Variants of the IL23R gene are associated with ankylosing spondylitis but not with Sjögren syndrome in Hungarian population samples. *Scandinavian J. of Immunology, 2009, Jul;70(1):68-74.* IF:2.108 (2009).

J9. *Polgár N, **Járomi L,** Csöngei V, Maász A, Horvatovich K, Faragó B, Sipeky C, Sáfrány E, Melegh B.* Triglyceride level modifying functional variants of GALTN2 and MLXIPL in ischemic stroke patients. *Eur J Neurol.* 2010 Aug;17(8):1033-9. Epub 2010 Feb 10.. IF: 3.765 (2010)

J10. *Sáfrány, E., Hobor, R ., Jakab, L., Tarr, T., Csöngei, V., **Járomi, L.,** Sipeky, Cs., Valasek, A., Zeher, M., Fust, G., Czirjak, L., Melegh, B.* Interleukin-23 receptor gene variants in Hungarian systemic lupus erythematosus patients. *Inflamm Res., 2010 Feb;59(2):159-64.* IF: 2.004 (2010)

J11. *Csöngei, V., **Járomi, L. ,** Sáfrány, E., Sipeky, C., Magyari, L., Faragó, B., Bene, J., Polgar, N., Lakner, L., Sarlos, P., Varga, M., Melegh, B. Interaction of the major inflammatory bowel disease susceptibility alleles in Crohn's disease patients. World J. Gastroenterol., 2010, 16(2):176-83.* IF: 2.240 (2010)

J12. ***Járomi, L.,** Csöngei, V., Polgár, N., Szolnoki, Z., Maász, A., Horvatovich, K., Faragó, B., Sipeky, Cs., Sáfrány, E., Magyari, L., Kisfali, P., Mohás, M., Janicsek, I., Lakner, L., Melegh, B.* Functional variants of glucokinase regulatory protein and apolipoprotein A5 genes in ischemic stroke. *J. Mol. Neurosci.* 2010 May;41(1):121-8. Epub 2009 Oct 22. . IF: 2.922 (2010)

J13. *Safrany E, Szell M, Csongei V, **Jaromi L,** Sipeky C, Szabo T, Kemeny L, Nagy J, Melegh B.,* Polymorphisms of the IL23R Gene Are Associated with Psoriasis but not with Immunoglobulin A Nephropathy in a Hungarian Population. Inflammation. 2010 Oct 27. [Epub ahead of print]PMID: 20978829 IF: 1.777 (2010)

J14. *Horvatovich K, Bokor S., Baráth Á., Kisfali, P., **Járomi L,** Répásy, J., Endreffy, E., Molnár, D., Melegh B.* Haplotype analyses of the apolipoprtotein A5 gene in obese pediatric patients. Int J Pediatr Obes. 2011 Jun;6(2-2):e318-25. Epub 2010 Sep 30. . IF: 2.654 (2010)

J15. *Mohás M, Kisfali P, **Járomi L,** Maász A, Fehér E, Csöngei V, Polgár N, Sáfrány E, Cseh J, Sümegi K, Hetyésy K, Wittmann I, Melegh B.* GCKR gene functional variants in type 2 diabetes and metabolic syndrome: do the rare variants associate with increased carotid intima-media thickness? Cardiovasc Diabetol. 2010 Nov 29;9:79. IF: 2.72 (2010)

J16. *Sipeky C, Csongei V, **Jaromi L,** Safrany E, Maasz A, Takacs I, Beres J, Fodor L, Szabo M, Melegh B.* Genetic variability and haplotype profile of MDR1 (ABCB1) gene in Roma and Hungarian population samples with a review of the literature. Drug Metab Pharmacokinet. 2011;26(2):206-15. Epub 2010 Dec 17. IF: 2.558 (2010)

J17. ***Járomi, L.,** Csöngei, V., Polgár, N., Rappai, G., Szolnoki, Z., Maász, A., Horvatovich, K., Sáfrány, E., Sipeky, Cs., Magyari, L., Melegh, B.* Triglyceride level-influencing functional variants of the ANGPTL3, CILP2 and TRIB1 loci in ischemic stroke. *J. NeuroMol. Med.* 2011;13(3):179-86. IF: 4.657 (2010)

J18. *Csöngei V, **Járomi L**, Sáfrány E, Sipeky C, Magyari L, Polgár N, Bene J, Sarlós P, Lakner L, Baricza E, Szabó M, Rappai G, Melegh B.* Interaction between CTLA4 gene and IBD5 locus in Hungarian Crohn's disease patients. *Int J Colorectal Dis. 2011 Sep;26(9):1119-25. Epub 2011 Apr 26*. IF: 2.645 (2010)

II. Accepted articles in domestic journals

N1. *Sáfrány, E., Csöngei, V., **Járomi, L.,** Maász, A., Magyari, L., Sipeky, Cs., Melegh, B.,* Mitochondrial DNA and its mutations: novel fields in a new era (in Hungarian: A mitokondriális DNS és mutációi: újabb ismeretek egy új területen), *Hungarian Medical Journal (in Hungarian: Orvosi Hetilap),* 2007, 148(21):971-978.

N2. *Lakner, L., Csöngei, V., Magyari, L., Varga, M., Miheller, P., Sarlós, P., Orosz, P., Bári, Z., Takács, I., **Járomi, L.,** Sáfrány, E., Sipeky, C., Bene, J., Tulassay, Z., Döbrönte, Z., Melegh, B.,* Possible role of selected IGR and SLC22A4/SLC22A5 loci in development of inflammatory bowel diseases. *Hungarian Medical Journal (in Hungarian: Orvosi Hetilap),* 2008, 149(7):325-8.

N3. *Horvatovich, K., Orkenyi, M., Bíró, E., Pongrácz, K., Kisfali, P., Talián, G., Csöngei, V., **Járomi L.,** Sáfrány, E., Harangi, F., Sulyok, E., Melegh, B.,* Pseudo-Bartter syndrome in a case of cystic fibrosis caused by C1529G and G3978A compound heterozygosity, *Hungarian Medical Journal (in Hungarian: Orvosi Hetilap),* 2008, 149(7):325-8.

Cumulative impact factor: 49.061