Role of the apolipoprotein A5 gene' natural variants in the development of ischemic stroke

Ph.D. thesis

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Pécs, 2009

LIST OF ABBREVIATIONS

ACE	angiotensin-converting-enzyme
APOA5	apolipoprotein A5
AT1R	angiotensin II receptor
BMI	body mass index
bp	basepair
CT	computed tomography
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
HDL	high density lipoprotein
IMT	intima media thickness
IRE	insulin responsive element
IVS	intronic variance sequence
LPL	lipoprotein lipase enzyme
LPL-HSPG	lipoprotein lipase - heparan sulfate proteoglycans complex
MELAS	mitochondrial encephalopathy, lactic-acidosis, stroke like episodes
MRI	magnetic resonance imaging
MTHFR	methylenetetrahydrofolate reductase
OR	odds ratio
PCR	polymerase chain reaction
PLA	platelet glycoprotein IIb/IIIa membrane receptor
RFLP	restriction fragment length polymorphism
PPRE	peroxisome proliferator responsive element
SEM	standard error of mean
SNP	single nucleotide polymorphism
TOAST	Trial of Org 10172 of Stroke Treatment
UTR	untranslated region
VLDL	very low density lipoprotein
WHO	World Health Organization

1. INTRODUCTION

During the past decade, remarkable progression occurred in the identification of natural genetic variants not only in the background of stroke but in cerebro- and cardiovascular disorders. Studies of these variants provide new perspectives to foster the discovery of pathogenesis of the disorders.

Present work summarizes my research on several genetic variants as possible risk factors in the development of ischemic stroke in cerebrovascular disorders. In my PhD work, the role of natural variants of apolipoprotein A5 gene (APOA5) were under investigation. The protein, encoded by APOA5 gene, is a newly identified member of the apolipoprotein family. Both the apolipoprotein gene and the protein became the center of interest since their discovery, due to their impact on lipid parameters. Changes in lipidparameters – increase in triglyceride and/or total cholesterol levels – are major crucial risk factors for cardio- and cerebrovascular disorders.

High incidence rate of cerebrovascular diseases explains the marked attention, as ischemic stroke is the third leading cause of death after cardiovascular occlusions and tumors in the developing countries. Moreover, number of stroke cases can increase in the future due to the demographic changes and the inadequate treatment of modifiable risk factors.

However, encouraging results could be achieved in the prevention of the disease by combining the efforts of numerous organizations and by the proper education of the general population. Additional risk factors could be discovered with similar analyses, thus, these studies could contribute to the primary (battle against risk factors) and secondary (screening and care of people at risk of developing stroke) stroke prevention.

The polymorphisms in the genes show a pattern specific for each individual. As different genetic variants have been associated with great variety of disorders and with variations in metabolism of certain drugs, thus examination of polymorphisms open up new dimensions in personalized medicine, drawing attention to the importance of population studies.

2. OVERVIEW OF THE LITERATURE

2.1. Stroke and its risk factors

Stroke is a suddenly developing temporary, or in unfavourable cases permanent damage of the brain according to the definition of the World Health Organization. This definition has been used for more than 20 years to describe this remarkably heterogenous condition in clinical practice. This condition can affect both males and females; it can manifest at any time of life; however, it is more prevalent in males and people over 45 years of age.

The disease can be classified into two subgroups according to the manner of development: haemorrhagic and ischemic stroke. Ischemic stroke comprises 80% of cases. The most common cause of its development is atherosclerosis.

The determination of subtypes of ischemic stroke is undoubtedly most problematic from the viewpoint of its etiology. Ischemic stroke can be divided into three subgroups depending on its mechanism of development: thrombotic, embolic and hemodinamic stroke. This classification is based on results received by imaging examinations. Nowadays, it is well known, that these groups overlap and are not necessarily specific enough. However, the exact determination of subgroups cannot be ignored from the view of prevention of subsequent stroke and its treatment. Bamford and colleagues found significant differences in the outcome of condition and probability of development of subsequent stroke between stroke subgroups. A new classification which was built up primarily on the basis of clinical aspect, was initiated in 1991 (Oxford Community Stroke Project). Another classification was introduced in 1993, which was based on the most common pathophysiological mechanisms taking part in the development of stroke (TOAST). The system comprises five subgroups: 1) large-vessel atherosclerotic, 2) small-vessel occlusion type, 3) cardioembolic, 4) stroke with other etiology, 5) stroke with unidentified etiology.

Several risk factors were identified in the development of ischemic stroke, which were categorized as modifiable and non-modifiable risk factors. Age and gender were enrolled into non-modifiable risk factors. Previous stroke in case history, which increases considerably the probability of subsequent cerebrovascular event, can also be enrolled into non-modifiable risk factors.

Hypertonia, diabetes mellitus, obesity, presence of cardiovascular events, elevated triglyceride- and cholesterol levels, smoking, exaggerated alcohol consumption are categorized as among modifiable risk factors in the classification. The factors mentioned above proved to be unambiguously risk factor for the development of disease. Besides, nowadays additional possible risk factors like several natural variants within the genome came into the focus of animal and epidemiological studies.

Ischemic stroke is a multifactorial disorder regarding its etiology, several monogenic disorders can stand in the background of stroke; on the other hand, stroke can be a component of multisystemic

disorders like MELAS-syndrome. The chance of developing ischemic stroke may be increased by a single polymorphism of a gene, as well. However, co-occurrence of more polymorphisms of a given gene or polymorphisms of several genes could multiply the risk of stroke development, which could also be influenced by interactions with environmental risk factors. Several genes and variants have been described in the literature in the background of ischemic stroke. Among these, several polymorphisms of genes encoding enzymes involved in the renin-angiotensin system and nitric-oxide production; and of genes influencing hemostasis, homocysteine- and lipid metabolism are shown in *Table 1.*

Table 1.
Genes and their polymorphisms identified in the background of ischemic stroke

Genes	Polymorphisms
hemostasis	
II. factor (prothrombin) *(OMIM+176930)*	G20210A
V. factor (Leiden) *(OMIM*612309)*	Arg506Gln
VII. factor *(OMIM+227500)*	Arg353Gln
XIII. factor *(OMIM+134570)*	Val34Leu
fibrinogen *(OMIM*134830)*	G-455A
renin-angiotensin system	
ACE *(OMIM+106180)*	I/D
AT1R *(OMIM*106165)*	A1166C
nitric-oxide-synthase system	
eNOS *(OMIM+163729)*	Glu298Asp
homocysteine metabolism	
MTHFR *(OMIM*607093)*	C677T
	A1298C
lipidmetabolism	
APOAI *(OMIM*107680)*	C-3031T
	317-321ins
APOE *(OMIM +107741)*	ε4, ε3, ε2
APOCIII *(OMIM*107720)*	T-2854G, C-455T
	C-482T, C3238G
LPL *(OMIM*609708)*	Asn291Ser

Abbreviations: ACE: angiotensin-converting-enzyme encoding gene; APOAI; -E; CIII: apolipoprotein AI; -E; -CIII protein encoding genes; Arg: arginine; Asn: asparagine; AT1R: angiotensin-receptor encoding gene; eNOS: endothelial nitric-oxide synthase encoding gene; Gln: glutamine; Leu: leucine; LPL: lipoprotein-lipase encoding gene; MTHFR: methylenetetrahydrofolate reductase encoding gene; OMIM: http://www.ncbi.nlm.nih.gov/sites/entrez?db=OMIM

2.2. Role of apolipoprotein A5 in lipid metabolism

Lipids play a crucial role in constructing of cell membranes (cholesterol) and the energy storage (triglycerides). Delivery of lipids is carried out by lipoprotein compartments. Lipoproteins are assembled from phospholipids and proteins (apoproteins). The apoproteins on the surface of lipoproteins fulfill structural and catalitical functions. They can activate or inhibit enzymes involved in lipoprotein metabolism, or serve as signals for receptors of liver or other cells, respectively. The apoproteins that are known so far are as follows: AI, AII, AIV, B48, B100, CI-III, E. The newly identified member of the apoprotein family is apolipoprotein A5 (APOA5). The gene encoding APOA5 protein, a member of APOAI-CIII-AIV gene cluster is located at chromosome 11q23. Examination of the orthologous human genomic sequence indicated its 27% sequence homology with APOAIV, which is 27 kb upstream of APOA5. Numerous studies support the concept that members of the apolipoprotein gene family arose by gene duplications, although the exact evolutionary events leading to the present-day cluster are not understood. The APOA5 gene is composed of 366 amino acids, and two transcripts of about 1.3 and 1.9 (kb) in length were identified, which are the result of alternative polyadenylation. The mature human APOA5 is a 39 kDa protein, which is expressed exclusively in the liver. Structural analyses revealed that APOA5 contains 76% α-helical structure presuming high affinity for lipid interfaces, its coiled-coil elements form two domains, and its N-terminal region shows high homology to other apoprotein domains. Concentration of APOA5 is high in the liver, however, after secretion into the plasma, its concentration decreases extremely: to 0.1-0.4 µg/ml.

APOA5 is a complex regulator of triglyceride metabolism. The protein has been identified in VLDL and HDL particles, it is distributed between these particles. Certain studies revealed that APOA5 can facilitate the catabolism of chilomicron and VLDL particles, but it does not influence the production of chilomicrons in the intestine and that of VLDL in the liver. APOA5 contributes to the elimination of triglyceride-rich lipoprotein particles by hydrolysation of plasma triglycerides. The exact mechanism, by which APOA5 decreases triglyceride levels, is not known. Certain *in vitro* studies presumed that APOA5 directly activates proteoglycan-bound lipoprotein lipase (LPL) to enhance intravascular triglyceride hydrolysis. Others found that APOA5 protein indirectly affects LPL activity by promoting its association with cell surface heparan sulfate proteoglycans, and by stabilizing lipoprotein lipase - heparan sulfate proteoglycan complexes (LPL-HSPG). Nevertheless, it can not be excluded, that APOA5 decreases triglyceride levels by modifying function of other apoproteins such as APOCIII.

Around 40 polymorphisms of the APOA5 gene have been identified since its discovery of APOA5 gene. Among these, only a few SNPs are known to have pathological significance. Among the variants causing structural changes in the protein, the C442T (Q148X) alteration was detected in homozygous form in a 9-year-old male first in the literature. This mutation is predicted to result in a

truncated APOA5 of 144 amino acids devoid of key functional domains like hydrophob lipid-binding and heparan-binding domains. Another mutation (Q139X) was identified in heterozygous form in a 63-year-old male. Q139X is predicted to determine a truncation of APOA5 at residue 16 of the mature protein, generating a 15 kDa truncated peptide, which does not contain a lipid-binding domain, thereby the protein cannot ensure the stability of LPL-HSPG complex, triggering plasma triglyceride level increase.. Besides, the IVS3+G3C alteration was found in heterozygous form, which affects the donor splice site of intron 3. The mutation causes exon skipping leading to expression of a protein of only 18 amino acids.

In addition, four frequent naturally occurring polymorphisms were identified in the APOA5 gene. All of these variants have been investigated in relation to their possible modifying effect on lipid metabolism in several studies. The T-1131C, T1259C, C56G, and IVS3+G476A allelic variants are located in the promoter region, in the 3' untranslated region, in the 3rd exon, and in the 3rd intron of the gene, respectively. Only the C56G variant could have functional relevances due to its position. The C56G sequence variant results in a nonconservative change of serine to tryptophan at codon 19. Similarly to other polypeptides that function in plasma, APOA5 is known to contain N-terminal export signal sequences with a cleavage site between amino acids 23 and 24, aiding the transport from the site of production to circulation. The change of a serine to a bulky tryptophan residue at position 19 could therefore reduces the rate of APOA5 export from the liver and results in higher triglycerides in humans.

3. AIMS OF THE STUDY

1. Determination of the allelic distribution of common natural variants of APOA5 gene (T-1131C, C56G, IVS3+G476A and T1259C), associated with other disorders like metabolic syndrome, hypertriglyceridemia and ischemic heart disease, in a stroke population divided into three subgroups according to TOAST classification.

2. Study of the effect of APOA5 gene -1131C, 56G, IVS3+476A and 1259C allelic variants on triglyceride- and cholesterol profile in patients and control subjects.

3. Evaluation of a possible susceptibility role of four natural APOA5 gene variants in the development of ischemic stroke.

4. MATERIALS AND METHODS

4.1. Human subjects

The patients and control samples examined were selected for our study form the sample collection of our department's Biobank, which belongs to the Central National Biobank Network of Hungary (www.biobank.hu). During the collection and use of DNA samples, the clinical data guidelines and regulations of the local ethics committee and those of the Helsinki Declaration in 1975 were followed; at the time of blood collection patients gave informed consent for the deposition of their anonymous DNA into Biobank and future use of it. The collection of DNA samples has been in progress since 2001 in collaboration with the Department of Cerebrovascular Diseases at Pándy Kálmán County Hospital.

All of the examined patients suffered from acutely developing ischemic stroke or were referred to the outpatient clinic because of a previously diagnosed stroke. All of the patients underwent extended clinical assessment, including general physical and laboratory examinations, cardiological and neurological examinations, electrocardiography, extracranial and transcranial Doppler sonography of brain-supplying arteries, transthoracic and/or transesophageal echocardiography, where appropriate, and past medical and familial anamnesis and evaluation of possible risk factor. Precise mapping of affected areas was performed with magnetic resonance imaging (MRI) examinations within 2 days of the first observation of the symptoms. An experienced investigator without knowledge of the clinical and laboratory data read all scans. Subjects, whose MRIs could not be recorded or whose examined clinical parameters and risk factors could not be obtained with certainty as a consequence of some technical cause or death were excluded from the study groups. After receiving MRI and clinical neurological results the patients were allocated to one of three stroke subgroups, which basically correspond to TOAST classification. The patients categorized as first (small-vessel) group had small-vessel infarcts (one or more subcortical hemispheric or brainstem infarcts with a diameter <1.5 cm on MRI, with one of the features of the traditional clinical lacunar syndrome and without cerebral cortical dysfunction). The patients in large-vessel group had cortical or cerebellar lesions and/or brainstem infarcts or subcortical hemispheric infarcts greater than 1.5 cm in diameter on MRI, with or without cerebral cortical impairment or brainstem or cerebellar dysfunction. The third cohort, the mixed group, contains all the patients, who had stroke with cardioembolism, or other non-specified etiology according to TOAST classification; and one or more lacunar and large-vessel infarcts on MRI, respectively. Such ordering of the patients into the mixed group was required because of the small number of patients from statistical point of view.

The age- and sex-matched control subjects were selected randomly for the study. Only unrelated Caucasian individuals free from neuro-imaging alterations and clinical history of stroke events were enrolled into the control group. Other parameters, such as the high-density lipoprotein-cholesterol levels of the subjects were not available for the study.

4.2. Applied methods

4.2.1. Polymerase chain reaction (PCR)

The genomic DNA was obtained from peripheral blood leucocytes using a standard salting out method. The examined parts of the available DNAs were amplified with polymerase chain reactions. The

GenBank reference sequence signed by AY422949 accession number was applied for the study design. The amplifications in all molecular analyses were carried out in a final volume of 50 μl containing 200 μM of each dNTP, 0.2 mM of each primer, 5 μl of reaction buffer (containing 500 mM KCl, 10 mM Tris-HCl, 14 mmol/L MgCl$_2$, pH 9.0), 1U of Taq polymerase (10 U/μl) and 1 μg extracted DNA as template. The sequences of oligonucleotide primers applied and circumstances of PCR reactions were reviewed in *Table 1*.

4.2.2. Restriction fragment length polymorphism (RFLP)

For RFLP assays 10-15 microlitres of PCR products were digested with 1U of appropriate restriction endonuclease with 10x enzyme buffer incubating on the appropriate temperature. The primers were designed to create obligatory cleavage sites of the proper restriction enzymes in the amplicons independently of the genotype to control the accuracy of the digestion. The applied restriction endonucleases and their recognition and cleavage sites were reviewed in *Table 2*. The bands received were electrophoresed through an ethidium-bromide-stained 3% agarose gel and were analysed with UVIdoc geldocumentation system.

4.3. Bidirectional DNA sequencing and analyses

To validate our genotyping results bidirectional sequencing was performed for some samples. The examinations were carried out using ABI Prism 3100 Avant Genetic Analyser. The sequence alingments were made using Winstar genetic program.

4.4. Statistical evaluation

All clinical data are expressed as mean±SEM. The distributions of the variables were examined using the Kolmogorov-Smirnov test. If the variables showed Gaussian distribution, we applied parametric tests. For variables with no Gaussian distribution, we applied nonparametric tests. In all statistical analyses, we checked for possible differences among all groups and subgroups using the Kruskal-Wallis test. Pairwise analyses of differences between groups in discreet clinical and laboratory parameters with normal distribution χ^2 tests were used. Continuous variables with normal distribution were analysed with Student't t-tests. For comparison of differences between groups in continuous variables with skewed distribution Mann-Whitney U tests were applied. Correlations were analysed and crude/adjusted odds ratios (OR) were ascertained using multiple logistic regression model. The confidence intervals and p-values of significance were established 95% and 0.05 for all analyses. All statistical analyses were performed using MS Excel, SPSS 11.5 és SAS packages.

Table 2.
PCR-RFLP assays for polymorphisms investigated in the study

Polymorphism	Oligonucleotide primers	Length of amplicons (bp)	Restriction endonuclease		Length of received fragment according to genotypes (bp)
			Name	Recognition sequence	
APOA5					
T-1131C *(rs662799)*	f: 5'-CCCCAGGAACTGGAGCGACCTT-3' r: 5'-TTCAAGCAGAGGGAAGCCTGTA-3'	398	*Mse*I	5'-T^TAA-3' 3'-AAT^T-5'	homozygous normal (TT): 22, 109, 267 heterozygous (TC): 22, 109, 267, 289 homozygous mutant (CC): 109, 289
T1259C *(rs2266788)*	f: 5'-TCAGTCCTTGAAAGTGGCCT-3' r: 5'-ATGTAGTGGCACAGGCTTCC-3'	287	*Bse*GI	5'-GGATGNN^-3' 3'-CCTAC^NN-5'	homozygous normal (TT): 122, 165 heterozygous (TC): 35, 87, 122, 165 homozygous mutant (CC): 35, 87, 165
C56G *(rs3135506)*	f: 5'-AGAGCTAGCACCGCTCCTTT-3' r: 5'-TAGTCCCTCTCCACAGCGTT-3'	256	*Cfr*13I	5'-G^GNCC-3' 3'-CCNG^G-5'	homozygous normal (CC): 79, 177 heterozygous (CG): 26, 79, 151, 177 homozygous mutant (GG): 26, 79, 151
IVS3+G476A *(rs2072560)*	f: 5'-CTCAAGGCTGTCTTCAG-3' r: 5'-CCTTTGATTCTGGGGACTGG-3'	280	*Mnl*I	5'-CCTC(N)$_7$^-3' 3'-GGAG(N)$_6$^-5'	homozygous normal (GG): 25, 114, 141 heterozygous (GA): 25, 41, 73, 114, 141 homozygous mutant (AA): 25, 41, 73, 141

5. RESULTS AND DISCUSSION

5.1. The role of APOA5 gene T-1131C variant

The T-1131C variant of the promoter region is the most intensively studied among naturally occurring variants of APOA5 gene. The alteration was found in 6% of the healthy European population. The frequency of the variant is very different among other population: 35% of the Japanese, 29% the Chinese and 20% of the Indian population carried the mutant allele. Our examinations revealed a 5% allele frequency in the controls representing the healthy population (*Table 4.*). The results correspond to findings reported by others on allele frequencies of European population. Besides, we found that the -1131C allele was at least two-fold more frequent in all stroke subgroups than in the controls. The allele distribution was in Hardy-Weinberg equilibrium in stroke subgroups and in controls.

Plasma lipid levels are considered to be important determining factors of cardio- and cerebrovascular disorders. One mmol/l elevation in the triglyceride level can increase risk of coronary artery disease with 14% in males and with 37% in females. The possible role of lipids is controversial. The contradictions could be explained by the heterogenity of the study populations and by the fact that several genetic factors influence triglyceride levels. In our study, triglyceride and serum total cholesterol profiles were examined in stroke groups and in controls in relation to the APOA5 −1131 genotypes, our results are summarized in *Table 5*. The triglyceride levels were significantly elevated in subjects carrying the mutant −1131C allele in homo- or heterozygous form, compared with non-carriers both in the overall group of stroke patients and in the controls. The serum total cholesterol levels did not show allele specific divergence in any groups. The impact of −1131C allelic variant on lipid parameters was intensively studied in adult and pediatric samples. The results regarding its effect on triglyceride levels are consistent, and our results are in agreement with these. Therefore we can conclude that carrying the −1131C mutant allelic variant elevates triglyceride levels independently of the population. The mechanism of action, by which −1131C allele increases triglyceride levels, is not clarified. As a hypothesis, it was assumed that promoter region alteration can have an impact on the transcription of APOA5 gene. However, Talmud et al could not verify this assumption. Vu-Dac et al identified a peroxisome proliferator responsive element (PPRE) in the promoter region (-272, -260) using bioinformatic tools, which regulates APOA5 expression. Carriage of −1131C allele could change the affinity to the regulatory elements decreasing APOA5 expression and as a consequence increasing triglyceride levels.

Another explanation comes from the possible linkage between the T-1131C variant and other genes. APOA5 could exert its impact on triglyceride levels through such an association. Complete linkage was found between APOA5 T-1131C and A-3G variants. The APOA5 A-3G variant was found to be in the Kozak consensus sequence (GACACCATGG), 3 bp upstream from the start codon. The basepair substitution in this position leads to a decrease in APOA5 mRNA translational rate, lower plasma APOA5

level and as a result to an increase of triglceride levels. Besides, strong linkage disequilibrium was observed not only between T–1131C and APOA5 polymorphisms but the variants of other genes in the apolipoprotein gene cluster like C-482T or T-455C polymorphisms in the insulin responsible element (IRE) of the APOCIII gene. This IRE element was identified in the promoter region of APOACIII, which is the key component in the regulation of APOCIII by insulin. Polymorphisms in the promoter region could alter the important regulatory element, therefore abolish *in vitro* insulin regulation of APOCIII gene expression, and increase the APOCIII as well as the triglyceride levels.

Since its discovery the APOA5 T-1131C variant has been intensively examined in the background of several conditions in different populations. The T-1131C natural variant was unambigously identified as a susceptibility factor for familiar hyperlipidemia and hypertriglyceridemia in Dutch, British, Spanish and Irish populations. The possible susceptibility role of T-1131C variant in the development of metabolic syndrome has been investigated by Japanese and Hungarian researchers. The variants have been found to confer risk for this condition.

The carriage of -1131C mutant allele was considered as risk factor for cardiovascular disease according to genotypic and statistical data of community-based international cooperation (Framingham Heart Study), but at the same time the role of T-1131C variant in coronary diseases is controversial. Carrying the -1131C mutant allele was established to confer increased risk for coronary arthery disease in Hungarian and in Chinese populations, on the contrary, this association could not be confirmed in Tunisian and Italian population. These results led us to evaluate the possible susceptibility role of T-1131C variant in stroke patients, especially as it has not been examined in ischemic stroke at all. Odds ratios (ORs) calculated using multivariate regression analyses are summarized in *Table 6*. In all statistical analyses, odds ratios were adjusted for differences in age, gender, BMI, serum total cholesterol, ischemic heart disease, hypertension, diabetes mellitus, smoking- and drinking habits between groups. We could confirm that carriage of -1131C mutant allele confers increased risk for the development of ischemic stroke. The presence of APOA5 T-1131C variant results in an increase in triglyceride levels leading to endothel dysfunctions, abnormal lipid deposition in the endothelial cells and formation of atherosclerotic plaques as well as development of occlusions in the cardiovascular system, which could cause ischemic stroke.

Table 3.
Major clinical data and laboratory results of patients with stroke event and control subjects

T-1131C	Patients with stroke			Controls (n=289)
	Large-vessel (n=149)	Small-vessel (n=85)	Mixed (n=68)	
Gender (male/female)	70/79	40/45	32/36	132/157
Age (years)	62.5 ± 10.1*	65.3 ± 11.6*	60.2 ± 15.1*	54.6 ± 13.3
BMI (kg/m^2)	25.8 ± 2.13*	26.7 ± 3.22*	25.9 ± 3.83*	23.0 ± 2.74
Total cholesterol (mmol/l)	6.43 ± 1.76*	6.64 ± 2.43*	6.89 ± 1.74*	4.89 ±0.74
Triglycerides (mmol/l)	1.82 ± 0.53*	1.72 ± 0.63*	2.34 ± 0.79*	1.29 ± 0.64
Hypertension	47.0%*	55.3%*	55.9%*	13.8%
Diabetes mellitus	32.9%*	43.5%*	29.4%*	5.88%
Smokers	28.9%*	31.8%*	33.8%*	13.8%
Drinkers	20.1%*	21.2%*	17.6%*	4.84%
Ischemic heart disease	17.5%*	16.5%*	19.1%*	5.88%

Values are as mean ± SEM, *p<0.05

Table 4.
Distribution of APOA5 -1131 genotypes amongst the different stroke subtypes and the controls.

T-1131C	Patients with stroke				Controls
	Large-vessel n=149	Small-vessel n=85	Mixed n=68	Overall n=302	n=289
TT	117 (78.5%)	67 (78.8%)	53 (77.9%)	237 (78.5%)	261 (90.3%)
TC+CC	30+2 (21.5%)	16+2 (21.2%)	14+1 (22.1%)	60+5 (21.5%)	28 (9.7%)
C allele frequency	11%*	12%*	12%*	12%*	5%

*p<0.05 vs. controls

Table 5.
Serum triglyceride and total cholesterol levels in stroke patients and controls
according to the APOA5 -1131 genotypes

T-1131C	Patients with stroke n=302		Controls n=289	
	TT n=237	TC+CC n=65	TT n=261	TC+CC n=28
Triglycerides (mmol/l)	1.81 ± 0.62	2.21 ± 0.61*	1.48 ± 0.05	2.00 ± 0.30*
Total cholesterol (mmol/l)	6.61 ± 1.67	6.52 ± 1.89	5.03 ± 1.78	4.89 ± 1.56

Values are as mean ± SEM, *p<0.05 vs. non-carriers (TT)

Table 6.
Logistic regression analysis for the association between -1131C allelic variant and risk for stroke

T-1131C	Large-vessel n=149	Small-vessel n=85	Mixed n=68	Overall stroke n=302
Adjusted ORs [#]	1.9* (1.1-5.9)	2.3* (1.3-4.9)	2.2* (1.2-5.1)	2.1* (1.3-4.7)

*p<0.05 vs. controls; [#]Adjusted for differences in age, gender, BMI, serum total cholesterol, ischemic heart disease, hypertension, diabetes mellitus, smoking-, and drinking habits

5.2. The role of C56G natural variant

The C56G variant in the third exon of APOA5 gene is one of the most intensively studied naturally occurring variants of the gene. The C56G alteration causes a hydrophilic serine to hydrophobic tryptophan amino acid change at codon 19 (S19W). Its prevalence was considered to be different among populations. The alteration was found in less than 0.1% of the Japanese and Chinese populations. Additionally, 3, 4.8, 4.8 and 15% of the Indian, Afro-American, French and Spanish population carry the C56G variants, respectively. Our data showed a 5.6% mutant allele frequency in the healthy control subjects, which corresponds to findings in European populations. The distribution of C56G alleles are demonstrated *in Table 8*. The allele distribution was in Hardy-Weinberg equilibrium both in stroke subgroups and in controls. Significant accumulation of 56G mutant allele was observed within the group

with large-vessel infarcts compared to the controls. There were no significant differences observed in small-vessel and mixed groups compared to controls.

In this study we were able to examine the impact of C56G variant on lipid parameters. The triglyceride levels presented were elevated in patients carrying the G allele in homo- or heterozygous form compared to non-carriers in all stroke subgroups as well as in the controls. The serum total cholesterol levels did not differ considerably in the groups. The lipid parameters of the patients and controls in correlation to the C56G genotypes are shown in *Table 9*. Based on our results we can conclude that carriage of 56G mutant allele results in a 16-28% elevation in triglyceride levels, which is in agreement with the findings of other research groups. Talmud et al observed a 8-16% increase in triglyceride levels in a Caucasian population, while this increase was higher (18-26%) in a Turkish population. The impact of the variant on lipid parameters and its mechanism by which it can increase triglyceride levels became the focus of several studies. The C56G variant is the only functional variant, which causes increase in triglyceride levels directly, without association with other SNPs. The C to G alteration at nucleotide position 56 results in serine to tryptophan amino acid change in the hydrophil domain of APOA5 signal sequence. This change could influence the transportation through the endoplasmic reticulum negatively, which could decrease the amount of APOA5 protein secreted and increase triglyceride levels. However, synergistic effect of other polymorphisms on the development of diseases can not be excluded. Schaefer et al genotyped a total of 170 patients with elevated fasting triglyceride concentrations for APOE and APOA5 alteration. Almost all of the hypertriglyceridemic individuals with APOE 2/2 genotype had an additional 56G allele. However, they failed to identify APOE 2/2 in combination with APOA5 C56G polymorphism in normolipidemic patients. They therefore hypothesized that APOA5 C56G is an important cofactor for hyperlipidemia in individuals with APOE 2/2 phenotype. This association has not been confirmed in larger population samples yet. The C56G alteration has been examined in several studies because of its drastic impact on triglyceride levels. Carriage of this APOA5 variant confers elevated risk for development of myocardial infacts, coronary artery disease and metabolic syndrome according to the results of population studies. Talmud et al showed faster atherogenesis in the presence of 56G mutant allele. The determination of the carotid intima-media thickness (IMT) is widely accepted for prediction of the degree of vascular occlusions. IMT was considered to be in direct proportion to the probability of the development of stroke or myocardial infarct.

In our study, odds ratios calculated by logistic regression analyses were adjusted for differences in age, gender, BMI, serum total cholesterol, ischemic heart disease, hypertension, diabetes mellitus, smoking- and drinking habits between groups (*Table 10.*). A strong association was found between the presence of the 56G allele and the development of large-vessel associated ischemic stroke; however, carriage of the mutant allele does not confer risk for small-vessel and mixed etiology strokes.

These observations were confirmed by the results of Framingham Offspring Study, which showed that the 56G allele associates with increased common internal carotid artery IMT.

Table 7.
The major clinical and laboratory parameters of the stroke patients and controls

C56G	Patients with stroke			Controls (n=171)
	Large-vessel (n=124)	Small-vessel (n=180)	Mixed (n=99)	
Gender (male/female)	46/78	70/110	43/56	58/113
Age (years)	65.2 ± 1.28	66.7 ± 1.14	64.7 ± 1.44	57.7 ± 1.33
BMI (kg/m^2)	25.0 ± 0.22	25.5 ± 0.14	24.7 ± 0.16	24.5 ± 0.22
Triglycerides (mmol/l)	1.75 ± 0.06*	1.77 ± 0.05*	1.70 ± 0.07*	1.55 ± 0.04
Total cholesterol (mmol/l)	5.81 ± 0.11*	5.74 ± 0.09*	5.67 ± 0.11*	5.20 ± 0.08

#p<0.001 vs. controls; *p<0.05 vs. Controls

Table 8.
Distributions of the APOA5 C56G allele in each stroke group and in controls

C56G	Patients with stroke			Controls (n=171)
	Large-vessel (n=124)	Small-vessel (n=180)	Mixed (n=99)	
CC	98 (79.0%)	163 (90.6%)	84 (84.9%)	153 (89.5%)
CG+GG	25+1 (21.0%)*	16+1 (9.44%)	14+1 (15.1%)	17+1 (10.5%)
G allele frequency	10.9*	5	8.1	5.6

*p<0.05 vs. controls

Table 9.
The triglycerides and serum total cholesterol levels between carriers and non-carriers in each stroke subgroup and in controls

C56G	Patients with stroke						Controls (n=171)	
	Large-vessel (n=124)		Small-vessel (n=180)		Mixed (n=99)			
	CC n=98	CG+GG n=26	CC n=163	CG+GG n=17	CC n=84	CG+GG n=15	CC n=153	CG+GG n=18
Triglycerides (mmol/l)	1.70 ± 0.06	1.97 ± 0.15*	1.72 ± 0.05	2.21 ± 0.18*	1.73 ± 0.08	2.05 ± 0.23*	1.56 ± 0.05	1.71 ± 0.19*
Total cholesterol (mmol/l)	5.79 ± 0.12	5.92 ± 0.28	5.79 ± 0.09	5.30 ± 0.26	5.65 ± 0.12	5.80 ± 0.25	5.18 ± 0.08	5.35 ± 0.22

*p<0.05 vs. non-carriers (CC)

Table 10.
Multivariate logistic regression analyses of C56G variant

C56G	Patients with stroke		
	Large-vessel (n=124)	Small-vessel (n=180)	Mixed (n=99)
Crude OR	2.122* (1.258-3.580) p=0.005	0.615 (0.348-1.089) p=0.095	1.250 (0.678-2.304) p=0.474
[#]Adjusted OR	2.132* (1.184-3.840) p=0.012	0.650 (0.334-1.263) p=0.203	1.315 (0.676-2.558) p=0.421

*p<0.05 vs. controls; [#] Adjusted for differences in age, gender, BMI, serum total cholesterol, ischemic heart disease, hypertension, diabetes mellitus, smoking-, and drinking habits

5.3. The role of APOA5 gene T1259C and IVS3+G476A natural variants

Relatively small amount of knowledge is available about the variant in intronic and in 3'-untranslated (3'-UTR) region of APOA5 gene compared to the variants detailed above. Our research revealed that the IVS3+476A mutant allele was more frequent in all stroke subgroups compared to controls. By contrast, none of the 1259T or C allelic variants showed significant accumulation in any of the stroke subtypes or in controls (*Table 12.*). The allele distributions were in Hardy-Weinberg equilibrium.

Examinations directed at the impact of APOA5 on lipid parameters revealed that the triglyceride levels were significantly elevated in subjects carrying 1259C and IVS3+476A alleles, compared to non-carriers in both overall stroke patients group and in the controls. (*Table 13.*), which results correspond with data of studies carried out on other European populations. However, in a Costa Rican population no elevation of triglyceride levels was detected in the groups carrying the mutant alleles. The exact mechanism, by which APOA5 variants can influence triglyceride levels, is unknown yet; presumably there is linkage disequilibrium between APOA5 polymorphisms and other variants, which can play a crucial role. In the European populations, a strong linkage disequilibrium was ascertained between APOA5 variants, however, the linkage of the variants in the Costa Rican population was only partial, which could explain why there were no association between carriership of the mutant alleles and triglyceride levels.

Analysing the odds ratios obtained by logistic regression analyses, we can conclude that, in spite of the triglyceride-raising effect of variant T1259C, carrying the mutant C allele does not confer independent risk for ischemic stroke. In contrary, carrying IVS3+476A mutant allele itself or together with other factors can increase the chance of the development of stroke 3-4-fold (*Table 14.*). To understand the exact mechanism, additional haplotype-analyses are needed, by which co-occurrence and impact of certain SNPs can be observed.

5.4. The possible role of the most common haplotypes of APOA5

Pennacchio et al confirmed that the most common alterations in the APOA5 gene are in strong linkage disequilibrium and constitute two major haplotype variant: APOA5*2 (-1131C, 1259C, IVS3+476A) and APOA5*3 (56G). These two haplotypes together with wild type haplotype (APOA5*1,*2,*3) account for approximately 98% of all haplotypes in average populations. The remaining 2% includes rare haplotype variants like APOA5*4 (-1131C) and APOA5*5 (1259C) belong. The number of patients and controls collected for the study is proved to be too small to examine and analyse statistically the role of APOA5 haplotype variants. Haplotype analyses is not the subject of this dissertation; however, it can be stated according to our preliminary results that carrying the APOA5*2

haplotype is strongly associated with increased triglyceride levels and confers susceptibility for ischemic stroke in all stroke subgroups. These findings are in agreement with observations of other research groups in this field. According to our future plans, distribution of all haplotypes determined by APOA5 naturally occurring variants, their impact on lipid parameters and their role in the development of ischemic stroke could be examined following the expansion of our sample pool.

Table 11.
Most relevant clinical and laboratory parameters in both patients with stroke and the controls

	Patients with stroke				Controls n=131
	Large-vessel n=122	Small-vessel n=176	Mixed n=80	Overall stroke n=378	
Gender (male/female)	50/72	67/109	33/47	150/228	41/90
Age (years)	67.6 ± 1.30	66.6 ± 1.17	63.0 ± 1.35	66.1 ± 0.74	58.1 ± 1.51
BMI (kg/m^2)	25.2 ± 0.16	25.5 ± 0.15	24.9 ± 0.18	25.2 ± 0.09	24.0 ± 0.17
Triglycerides (mmol/l)	1.71 ± 0.06*	1.76 ± 0.04[#]	1.83 ± 0.08*	1.76 ± 0.03[#]	1.53 ± 0.05
Total cholesterol (mmol/l)	5.90 ± 0.11[#]	5.75 ± 0.09[#]	5.67 ± 0.13[#]	5.78 ± 0.06[#]	5.18 ± 0.09

Values are as mean ± SEM *p<0.05 vs. controls; [#]p<0.001 vs. controls

Table 12.
Distribution of genotypes and allele frequencies for apolipoprotein A5 (APOA5) T1259C and IVS3+G476A in both patients with stroke and the controls

		Patients with stroke				Controls
		Large-vessel n=122	Small-vessel n=176	Mixed n=80	Overall stroke n=378	n=131
T1259C	TT	95 (77.9%)	137 (77.8%)	60 (75.0%)	292 (77.2%)	103 (78.6%)
	TC+CC	27 (22.1%)	39 (22.2%)	20 (25.0%)	86 (22.8%)	28 (21.4%)
	C allele frequency	11.5%	11.6%	13.1%	11.9%	11.1%
IVS3+G476A	GG	104 (85.2%)	147 (83.5%)	68 (85.0%)	319 (84.4%)	123 (93.9%)
	GA+AA	18 (14.8%)	29 (16.5%)	12 (15.0%)	59 (15.6%)	8 (6.10%)
	A allele frequency	7.40%*	8.52%*	7.50%*	7.94%*	3.05%

* p<0.05 vs. Controls

Table 13.

Triglyceride and serum total cholesterol profiles in certain stroke groups and in controls according to the APOA5 genotypes

	Patients with stroke								Controls n=131	
	Large-vessel n=122		Small-vessel n=176		Mixed n=80		Overall stroke n=378			
T1259C	TT	TC+CC	TT	TC+CC	TT	TC+CC	TT	TC+CC	TT	TC+CC
	n=95	n=27	n=137	n=39	n=60	n=20	n=292	n=86	n=103	n=28
Triglycerides (mmol/l)	1.64 ± 0.06	2.01 ± 0.15*	1.67 ± 0.04	2.08 ± 0.14*	1.67 ± 0.07	2.33 ± 0.24*	1.66 ± 0.03	2.12 ± 0.10*	1.45 ± 0.05	1.86 ± 0.17*
Total cholesterol (mmol/l)	5.90 ± 0.13	5.86 ± 0.24	5.71 ± 0.11	5.90 ± 0.19	5.57 ± 0.12	5.95 ± 0.34	5.75 ± 0.07	5.90 ± 0.14	5.25 ± 0.09	4.95 ± 0.20
IVS3+G476A	GG	GA+AA	GG	GA+AA	GG	GA+AA	GG	GA+AA	GG	GA+AA
	n=105	n=17	n=147	n=29	n=89	n=12	n=342	n=57	n=123	n=10
Triglycerides (mmol/l)	1.65 ± 0.06	2.17 ± 0.22*	1.67 ± 0.03	2.22 ± 0.18*	1.70 ± 0.07	2.63 ± 0.36*	1.67 ± 0.03	2.29 ± 0.13*	1.49 ± 0.05	2.08 ± 0.37*
Total cholesterol (mmol/l)	5.87 ± 0.12	6.04 ± 0.334	5.79 ± 0.10	5.60 ± 0.21	5.66 ± 0.13	5.73 ± 0.45	5.79 ± 0.07	5.75 ± 0.17	5.23 ± 0.09	4.54 ± 0.31

Values are as mean ± SEM; *p<0.05 vs. non-carriers

Table 14.

Multiple logistic regression analysis for the association between carrying IVS3+476A and 1259C allelic variants and risk for stroke

		Patients with stroke			
		Large-vessel n=122	Small-vessel n=176	Mixed n=80	Overall stroke n=378
T1259C	Crude OR	1.045 (0.575 - 1.901)	1.047 (0.605 - 1.813)	1.226 (0.636 – 2.363)	1.083 (0.669 - 1.754)
	Adjusted OR[#]	1.054 (0.454 – 2.451)	1.477 (0.668 - 3.267)	1.391 (0.631 – 3.066)	1.400 (0.751 - 2.609)
IVS3+G476A	Crude OR	2.661* (1.112 – 6.369)	3.033* (1.338 – 6.877)	2.713* (1.057 – 6.962)	2.844* (1.320 – 6.124)
	Adjusted OR[#]	3.905* (1.355 – 11.253)	4.748* (1.540 - 14.640)	2.926* (1.021 – 8.384)	3.644* (1.452 – 9.144)

*p<0.05 vs. controls; [#]Adjusted for differences in age, gender, BMI, serum total cholesterol, ischemic heart disease, hypertension, diabetes mellitus, smoking-, and drinking habits

6. SUMMARY

The following observations were made in our study:

1. The examination of the impact of natural APOA5 variants on lipid parameters revealed that the presence of −1131C, 56G, IVS3+476A and 1259C allelic variants resulted in statistically significant elevation in the concentration of triglycerides in all stroke subgroups and control subjects.

2. Analysing the effect of carrying APOA5 variants on cholesterol levels, no significant difference was observed in cholesterol levels in either stroke patients or control subjects.

3. The molecular genetic analysis of allele distribution showed significant accumulation of −1131C, IVS3+476A alleles in all stroke subgroups compared to the controls. In contrary, the 56G allele showed significant accumulation only in large-vessel group in comparison with the controls.

4. No significant differences were found between the APOA5 T1259C allelic distributions in either stroke subgroups or controls, in spite of the fact that, there are significant increase in triglyceride levels caused by the carriage of 1259C allelic variant, similarly to the other three APOA5 variants.

5. Logistic regression analyses revealed that carriage of -1131C and IVS3+476A allelic variants confer independent risk factors for the development of ischemic stroke in all stroke subgroups (large-, small-vessel, mixed). In case of C56G variant, positive association was verifiable only between carrying 56G allelic variants and large-vessel stroke. After regression analyses it can be ascertained that presence of neither the normal nor the mutant allele of T1259C variant confer increased risk for the development of ischemic stroke.

8. LIST OF PUBLICATIONS

The thesis is based on the following publications

1. Havasi V, Szolnoki Z, Talian GC, Bene J, Komlosi K, *Maasz A*, Somogyvari F, Kondacs A, Szabo M, Fodor L, Bodor A, Melegh B. Apolipoprotein A5 gene promóter region T-1131C polymorphism associates with elevated circulating triglyceride levels and confers susceptibility for development of ischemic stroke. J Mol Neurosci. 2006;29(2):177-183.

2. Szolnoki Z, *Maasz A*, Magyari L, Horvatovich K, Farago B, Somogyvari F, Kondacs A, Szabo M, Fodor L, Bodor A, Hadarits F, Melegh B. Coexistence of angiotensin II type 1 receptor A1166C and angiotensin-converting enzyme D/D polymorphism represents susceptibility for small vessel associated ischaemic stroke. Neuromolecular Med. 2006;8(3):353-360.

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4. *Maasz A*, Kisfali P, Horvatovich K, Szolnoki Z, Csongei V, Jaromi L, Safrany E, Sipeky C, Hadarits F, Melegh B. Apolipoprotein A5 gene IVS3+G476A allelic variant confers susceptibility for development of ischemic stroke. Circ J. 2008;72(7):1065-70.

Other publications

1. Putnoky P, Deak V, Bekasi K, Palvolgyi A, *Maasz A*, Palagyi Z, Hoffmann G, Kerepesi I. H protein of bacteriophage 16-3 and RkpM protein of Sinorhizobium *meliloti* 41 are involved in phage adsorption. J Bacteriol. 2004,186(6):1591-1597.

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6. Szolnoki Z, *Maasz A*, Magyari L, Horvatovich K, Farago B, Somogyvari F, Kondacs A, Szabo M, Bodor A, Hadarits F, Melegh B. The combination of homozygous MTHFR 677T and angiotensin II

type-1 receptor 1166C variants confers the risk of small-vessel-associated ischemic stroke. J Mol Neurosci. 2007;31(3):201-207.

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Cumulative impact factor (without citable abstract): 45,112

ACKNOWLEDGEMENTS