Role of the apolipoprotein A5 gene's natural polimorphysms in the development of metabolic syndrome

Ph.D. thesis

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1. LIST OF ABBREVIATIONS

APOA5	apolipoprotein A5 (genen)
APOAV	apolipoprotein AV (protein)
BMI	body mass index
bp	básepair
CVD	cardivascular disease
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
HDL	high density lipoprotein
LDL	low density lipoprotein
LPL-HSPG	lipoprotein-lipase - heparan-sulfate-proteoglicans complex
MS	metabolic syndrome
OR	odds ratio
PCR	polimerase chain reaction
RFLP	restriction fragment length polymorphism
SEM	standard error of mean
SNP	single nucleotide polymorphism
UTR	untranslated region
VLDL	very low density lipoprotein
WHO	World Health Organization

2. INTRODUCTION

The conception of metabolic syndrome (MS) is the X-syndrome described by Reaven in 1988. That is a carbohydrate metabolic disorder characterized by elevated blood pressure, serum triglycerides, low HDL (High Density Lipoprotein)-cholesterol level beside central obesity. Each of these components separately are risk factors of cardio- and cerebrovascular diseases, simultaneous occurrence represents increased risk. MS is typical of the industrial countries, can concern one third of the population. Thus, researching the genetic background of MS has a high relevance beside diabetes type II.

The first complete human genome sequence was published in 2000 within the scope of the Human Genome Project. This step highly accelerated the identification of new genes, such as researching the role of genetic variants and mutations. Besides, technological development created the accessibility of population-wide genetic analyses, therefore hundred thousands of polymorphisms can be analyzed in different groups simultaneously. These made possible the last decade's expressive progression in the identification of genetic variants responsible for diseases involving large populations.

A topic in the focus of research is the genetic alterations influencing lipid parameters. Elevation of serum triglycerides and LDL (Low Density Lipoprotein)-cholesterol levels are main risk factors of cardio- and cerebrovascular diseases. Present work deals with the role of the variants of the apolipoprotein A5 gene (APOA5), one of the genes affecting triglycerides levels. The protein encoded by the gene is the recently described member of the apolipoprotein family. The variants of the gene have strong effect ont he lipid parameters.

3. OVERVIEW OF THE LITERATURE

3.1. Metabolic syndrome

Today every sixth European adult is threatened by the lifestyle disease called MS, which enhances the risk of cardiovascular diseases, such as stroke. MS already threatens Greece and France, where the population was defended against cardiovascular diseases so far, derived from its diet and lifestyle. Essential components of the diagnosis are abdominal obesity, hypertonia, abnormal HDL-cholesterol and triglyceride levels, and morbid fasting glucose level.

People affected by the syndrome have higher cardiovascular morbidity and mortality, including sudden death. Elevated triglyceride- and LDL-cholesterol-level after some time enhances the risk of atherosclerotic plaque formation, which can manifest as atherosclerotic cardiovascular diseases (myocardial infarction, stroke). Insulin resistance and consecutive high insulin and glucose level both enhance the atherosclerotic inflammation and the related oxidative stress. High insulin level contribute enhanced renal sodium retain, which can lead to elevated blood pressure.

According to present theories, the base of the syndrome is the abnormal fatty tissue activity formed on the ground of visceral obesity, which participates in the occurrence of the other abnormities, such as insulin resistance and consecutive hyperinsulinaemia. Low HDL-chlesterol-, high triglyceride level and low density LDL-cholesterol level features the lipid abnormality. Enhanced sympatic activity is also important element in the metabolic syndrome pathogenesis as well as in the formation of complications. Leptin, insulin, free fatty acids, cytokines, and sleep apnoe play cardinal role in the increase of the activity.

Most important cellular manifestations of insulin resistance: insulin-stimulated glucose uptake and glycogen synthesis deficiency in striated muscle, and inappropriate inhibition of hepatic gluconeogenesis and adipocytes' free fatty acid emission. Insulin resistance, leading to the 'overload' of beta-cells, causing apoptosis, can contribute to type II diabetes mellitus formation, when yet relative and later real hypoinsulinaemia exists.

3.2. Definition of MS by WHO

The World Health Organization criteria (1999) require presence of one of:

Diabetes mellitus,

Impaired glucose tolerance,

Impaired fasting glucose or

Insulin resistance;

AND two of the following:

1: blood pressure: ≥ 140/90 mmHg.

2: dyslipidaemia: triglycerides: ≥ 1.695 mmol/L and (HDL-C) ≤ 0.9 mmol/L (male), ≤ 1.0 mmol/L (female).

3: central obesity: waist - hip ratio: > 0.90 (male), > 0.85 (female), and / or BMI > 30 kg/m^2.

4: microalbuminuria: urine albumin void ratio: ≥ 20 mg/min, or albumin / creatinin ratio ≥ 30 mg/g.

3.3. Role of apolipoprotein AV protein in lipid metabolism

APOAV protein is expressed in the liver. It is a 39 kDa molecular weight protein with 76% α-helical structure (presuming higher affinity to lipid surfaces), the elements of the coiled-coil form two domains, while its N-terminal region shows strong homology to other apolipoprotein domains. The protein has high concentration in the liver; exported HDL- and VLDL-bound to the plasma, where its concentration is extremely low: 0.1-0.4 μg/ml. This value is 2000 times lower than plasma concentration

of APOAI or APOCIII. Presumably, this low plasma concentration is the reason why APOAV protein was discovered only in the recent past, compared to other members of the protein family.

The studied enzyme is a regulator of triglyceride metabolism. The protein has been identified in VLDL and HDL particles, in between which it is transported during metabolism. According to some studies APOAV facilitates the catabolism of chilomicron and VLDL, but it does not affect the VLDL production of the intestine and the liver. It contributes to the displacement of triglyceride-rich lipoproteins through hydrolysis of the plasma triglycerides. The exact mechanism, through which APOAV can decrease the lipid level, is not known. Some *in vitro* analyses assume direct, others suppose indirect connection between APOAV protein and LPL. One of the suppositions suggests that APOAV exerts its activity through activating proteoglycane-connected LPL. Others presume it stabilizes the lipoprotein-lipase - heparan-sulphate-proteoglycane (LPL-HSPG) complex. It's not excluded that APOAV reduces triglyceride level by modifying other apolipoproteines (APOCIII) function.

3.4. The APOA5 gene

The gene was identified by two independent groups: van der Vliet and colleagues while examining the factors playing role in the liver regeneration, and Pennacchio and colleagues during researching the possible regulating genes of lipid-metabolism.

The 3 exons of APOA5 gene encode 366 amino acids, alternative poliadenilation results in two transcripts (1.3 and 1.9 kb long) with yet unknown functional relations. *APOA5* gene is polimorf charactered, since its discovery 40 SNPs have been identified in its sequence.

At the time of *APOA5* gene discovery 4 frequent polymorphisms have been identified, all associated to elevated triglyceride level. Since these have been objects of several studies: T-1131C in the promoter region; T1259C in the 3' untranslated region (UTR); C56G in the 1. exon and IVS3+G476A in the 3. intron. Only the C56G variant could have functional relevance due to its position – it results in a serine-to-tryptophan change of at codon 19. Similarly to other polypeptides, APOAV is known to contain N-terminal export signal sequences, aiding the transport from the site of production to circulation. In the case of APOAV, this sequence refers to amino acids 1-23. During the amino acid change at position 19 there is a bulky amino acid built in, thus it can directly affect the export, the APOAV plasma concentration can decrease, elevating plasma triglyceride level.

4. AIMS OF THE STUDY

Our research on MS patients had the following aims:

1. We searched the international literature for the allele distribution of the *APOA5* gene's frequent natural variants (T-1131C, C56G, IVS3+G476A and T1259C) connected with the formation of other diseases like hypertriglyceridaemia, stroke and ischemic heart disease.

2. We studied the influence of alleles of *APOA5* gene (-1131C, 56G, IVS3+476A and 1259C) on the triglyceride- and cholesterol values of the patients and control subjects.

3. Our studies also oriented to analyze the minor alleles of the four variants in the *APOA5* gene to explore those possible predisposing role in the formation of MS.

4. Besides, we examined the effects of the haplotypes defined by the variants on the triglyceride- and cholesterol values, such as their role in MS occurrence.

5. MATERIALS AND METHODS

5.1. Human subjects

The DNA samples were from the central Biobank of the University of Pecs, that is part of the National Biobank Network of Hungary (www.biobank.hu), a participant of the pan-European Biobanking and Biomolecular Resources Research Infrastructure (BBMRI) preparatory phase (http://bbmri.eu/bbmri/). The governance, maintenance and management principles of the biobank had been approved by the national Research Ethics Committee, Budapest (ETT TUKEB).

A total of 343 MS patients (158 males and 185 females, mean age: 60.5 ± 10.08 years, range: 23-74 years) were selected according to the criteria of the modified Adult Treatment Panel III of National Cholesterol Education Program. This requires the simultaneous presence of at least 3 of the following factors at the time of the diagnosis: body mass index (BMI) > 25 kg/m2, serum triglycerides ≥ 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 with fasting plasma glucose levels of > 5.60 mmol/l [22,23]. A total of 284 healthy subjects (124 males and 160 females, mean age: 58.8 ± 15.2 years, range: 44-80 years) without known disease served as controls, they were free from any clinical or laboratory sign of MS; their history was also free from any systemic, metabolic or organ-specific disease. Controls, such as patients of traumatology units, blood donors, and medical students, were recruited from the same geographic area as the MS patients.

5.2. Applied methods

5.2.1. 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*.

5.2.2. Restriction fragment length polymorphism (RFLP)

For RFLP assays 10-15 µl 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 electrophoresed through an ethidium-bromide-stained 3% agarose gel and were analysed with UVIdoc geldocumentation system.

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

5.3. 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 alignments were made using Winstar genetic program.

5.4. 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. Pair wise 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 analyzed 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 analyzed and crude/adjusted odds ratios (OR) were ascertained using multiple logistic regression models. 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.

6. RESULTS

The individual *APOA5* haplotypes were assessed by evaluating the characteristic haplotype-tagging SNP alleles. Four of the haplotypes (APOA5*1, APOA5*2, APOA5*3 and APOA5*4) have been already described and investigated. The APOA*5 haplotype has not been published before our current observation; also, it cannot be found in the HapMap phase III project (http://www.hapmap.org/cgi-perl/gbrowse/hapmap3_B36/).

Table 1. Haplotypes of the *APOA5*.

Haplotype	*T-1131C*	*IVS3 G+476A*	*T1259C*	*C56G*
*APOA5*1*	T	G	T	C
*APOA5*2*	C	A	C	C
*APOA5*3*	T	G	T	G
*APOA5*4*	C	G	T	C
*APOA5*5*	T	G	C	C

Clinical characteristics of MS patients and control subjects are shown in Table 2. Serum triglycerides and BMI were significantly elevated in the MS group compared to the controls; while the serum total cholesterol levels of the two groups did not show a statistically significant difference.

Table 2. Major clinical parameters of the patients with MS and control subjects (means±SD).

	Controls n=284	MS patients n=343
Gender (males/females)	124/160	158/185
Age (years)	58.8±15.2	60.5±10.8
BMI (kg/m^2)	24.1±2.00	33.1±5.23*
Serum triglycerides (mmol/l)	1.40±0.37	2.32±1.38*
Serum total cholesterol (mmol/l)	5.39±1.00	5.35±1.08

* $p<0.001$

Serum triglyceride and cholesterol levels in association with the different *APOA5* haplotype-tagging variants are shown in Table 3. Except the *C56G* variant, the minor variants of all the *APOA5* alleles (*-1131C, IVS3+476A, 1259C)* were associated with increased triglyceride levels both in MS patients and in controls. The serum total cholesterol did not show allele-dependent changes. The allele frequencies of all *APOA5* variants studied were consistent with Hardy-Weinberg equilibrium expectation in both groups.

The study design enabled us to evaluate the haplotype-specific differences both in MS and in control groups (Table 4). We found significantly elevated serum triglyceride levels in association with the *APOA5*2* haplotype in both groups. In contrast, no haplotype-associated differences could be detected in the total serum cholesterol values. The studied haplotypes did not appear to have any influence on the BMI of the patient and control groups.

The prevalence of the individual haplotypes in MS patients and controls are shown in Table 5. While an approximately 2.7-fold accumulation of the *APOA5*2* haplotype could be observed in the MS group, the prevalence of the *APOA5*5* was 5.3-fold less in the MS patients compared with controls (Table 5). Crude logistic regression analysis revealed that the *APOA5*2* haplotype confers significant susceptibility for the development of MS; multiple logistic regression analysis adjusted for age, gender, total serum cholesterol, acute myocardial infarction and stroke confirmed this association. Results of the logistic regression analysis suggested that in contrast to the *APOA5*2*, the *APOA5*5* haplotype might have an independent protective effect against MS.

Table 3. Serum triglycerides, total cholesterol and HDL-C levels with body mass index (BMI) in subjects with metabolic syndrome (MS) and serum triglycerides, total cholesterol and body mass index (BMI) controls by individual genotypes (means±SD).

		T-1131C		*IVS3+G476A*		*T1259C*		*C56G*	
		TT	TC+CC	GG	GA+AA	TT	TC+CC	CC	CG+GG
		n=282	n=61	N=285	n=58	n=284	n=59	n=300	n=43
MS patients	BMI (kg/m^2)	33.2±5.16	32.6±5.38	33.2±5.15	32.7±5.41	33.2±5.16	32.6±5.41	33.2±5.20	33.0±5.22
	Serum triglycerides (mmol/l)	2.33±1.28	2.90±1.55*	2.39±1.28	2.90±1.55†	2.34±1.29	2.89±1.54‡	2.42±1.38	2.48±1.48
	Serum total cholesterol (mmol/l)	5.30±1.06	5.51±1.11	5.38±1.06	5.46±1.10	5.31±1.08	5.47±1.06	5.34±1.05	5.31±1.16
	HDL-C (mmol/l)	1.24±0.03	1.22±0.04	1.24±0.05	1.23±0.03	1.25±0.02	1.23±0.03	1.23±0.06	1.24±0.02
		TT	TC+CC	GG	GA+AA	TT	TC+CC	CC	CG+GG
		n=256	n=28	N=262	n=22	n=251	n=33	n=257	n=27
Controls	BMI (kg/m^2)	24.1±2.05	24.1±1.50	24.1±2.05	24.1±1.28	24.1±2.08	24.2±1.25	24.1±2.03	24.2±1.68
	Serum triglycerides (mmol/l)	1.38±0.34	1.66±0.54§	1.38±0.34	1.70±0.53¶	1.38±0.35	1.63±0.48#	1.41±0.38	1.32±0.33
	Serum total cholesterol (mmol/l)	5.42±1.00	5.18±1.06	5.42±0.99	5.10±1.07	5.41±0.98	5.30±1.17	5.39±1.02	5.40±0.81

* p=0.002; † p=0.033; ‡ p=0.029; § p=0.026; ¶ p=0.032; # p=0.025

Table 4. Serum triglycerides, total serum cholesterol, HDL-C levels, and body mass index (BMI) in subjects with metabolic syndrome (MS) and Serum triglycerides, total serum cholesterol, and body mass index (BMI) in controls, as a function of their *APOA5* haplotypes (means±SD). Statistical comparisons are made against haplotype APOA5*1/1.

	Haplotype variant	*APOA5*1/1*	*APOA5*1/2-2/2*	*APOA5*1/3-3/3*	*APOA5*1/4-4/4*	*APOA5*1/5-5/5*	Other haplotype variants
MS patients	Frequency	73.1%	13.1%	9.50%	1.40%	1.00%	1.90%
	BMI (kg/m^2)	33.2±5.25	33.4±5.85	33.5±4.40	31.6±5.37	30.5±2.33	27.2±2.07
	Serum triglycerides (mmol/l)	2.29±1.29	2.95±1.63*	2.57±1.43	2.84±1.51	2.68±1.50	2.43±1.87
	Serum total cholesterol (mmol/l)	5.29±1.09	5.37±1.10	5.33±1.09	6.02±0.90	5.50±1.36	5.34±0.87
	HDL-C (mmol/l)	1.24±0.03	1.23±0.04	1.24±0.02	1.26±0.11	1.29±15	1.22±0.09
Controls	Frequency	76.5%	4.90%	8.40%	3.50%	5.30%	1.40%
	BMI (kg/m^2)	24.1±2.13	23.9±1.29	24.3±1.69	24.1±2.02	24.2±1.35	24.7±0.43
	Serum triglycerides (mmol/l)	1.38±0.35	1.77±0.61†	1.32±0.33	1.46±0.49	1.39±0.25	1.45±0.21
	Serum total cholesterol (mmol/l)	5.41±1.00	5.16±1.07	5.42±0.85	5.34±1.17	5.51±1.19	4.85±0.90

* p=0.023; † p=0.042

Table 5. Prevalence of the major *APOA5* haplotypes and the odds ratios at 95% confidence intervals (CI) calculated by multiple logistic regression analysis models.

Haplotype variant	MS patients	Controls	Unadjusted model Odds ratio (95% CI)	Adjusted model Odds ratio* (95% CI)
*APOA5*1/1*	73.1%	76.5%	0.837 (0.590-1.187) p=0.318	0.878 (0.594-1.299) p=0.515
*APOA5*1/2-2/2*	13.1%	4.90%	2.880 (1.567-5.292) p=0.001	2.561 (1.295-5.066) p=0.007
*APOA5*1/3-3/3*	9.50%	8.40%	1.122 (0.659-1.911) p=0.672	1.162 (0.645-2.092) p=0.617
*APOA5*1/4-4/4*	1.40%	3.50%	0.401 (0.114-1.117) p=0.081	0.539 (0.183-1.591) p=0.263
*APOA5*1/5-5/5*	1.00%	5.30%	0.174 (0.057-0.531) p=0.002	0.182 (0.058-0.565) p=0.003
Other haplotype variants	1.90%	1.40%	0.374 (0.410-4.606) p=0.607	1.388 (0.395-4.876) p=0.609

*Adjusted for: age, gender, total serum cholesterol, acute myocardial infarction, and stroke.

7. DISCUSSION

7.1. Role of theT-1131C variant found in the APOA5 gene promoter region

The most studied alteration among the frequent natural variants of APOA5 gene is the T-1131C transition respecting the promoter region. This variant was found in 6% of the healthy European population. Referring Asia, the Japanese population showed 35%, the Chinese population showed 29% while India showed 20% occurrence. Studying the variant, we found 9.89% allele frequency in the studied

Hungarian control group, which is slightly higher than the yet defined values for European population. Plasma lipid levels are important determining factors of cardio- and cerebrovascular disease susceptibility. 1 mmol/L triglyceride elevation increases the risk for CAD 14% in men, and 37% in female.

APOA5 T-1131C variant – since its discovery – was studied in different populations for several disease groups. It was referred as unambiguous risk factor for the formation of the given diseases in Chinese, Czech, Irish and Hungarian familiar hyperlipidaemia and hypertriglyceridaemia patients. According to the criteria of an international cooperating (Framingham Heart Study) genotyping and statistical analysis on individuals suffering from cardiovascular diseases defines the presence of the -1131C mutant allele as risk factor of the disease, however its role in coronary disease is controversial. In Hungarian and Chinese patient groups the mutant allele showed elevated risk in myocardial infarction.

Our study demonstrated that the presence of the mutant C allele resulted significantly elevated triglyceride levels in every observed groups. Triglyceride increasing action of –1131C was investigated by several research groups in adults and children also. Their results regarding triglyceride levels are uniform and agree our results: the mutant allele showed connection to the elevated triglyceride level, independently of the studied populations.

Vu-Dac and collaborators used bioinformatics to – among many other regulator sequences – identify a peroxisome proliferator responsive element (PPRE) in this promoter region (-272, -260), which is required for the *APOA5* gene expression. As supposed, –1131C allele can alter the affinity and binding to this or other similar regulator regions, decreasing the expression of the *APOA5* gene, thus increasing the triglyceride level.

Likely T–1131C is in strong linkage with functional variants within other genes, therefore it can exert its influence on the triglyceride level through this connection. Such associations were found between *APOA5* T-1131C and A-3G variants. This deviation is 3 bp distance from the start codon in 5' direction. This base change causes the *APOA5* mRNA translation decrease, thus lower plasma APOAV level, therefore increased triglyceride level.

T-1131C is in strong linkage not only with the variants of *APOA5* gene, but with other - *APOA5* gene near localized - variants of the apolipoprotein gene cluster. Strong linkage was found between the variant studied by us and *APOC3* gene C-482T and C–455T variants. An insulin responsive element was identified in the promoter region of the *APOC3* gene, which can be the key element of the *APOC3* regulation by insulin. Mutations in the promoter region of the gene change this important function, therefore insulin's repression on *APOC3* breaks off, thus APOCIII level increases, which imperatively increases triglyceride level.

7.2. Role of the IVS3+G476A variant of the APOA5 gene intronic region

We have slight knowledge about the variants respecting the *APOA5* gene intronic region relative to the previously discussed variants. Research made by us and studies of other European populations clearly show that this SNP elevates triglyceride level. However, in an examined Costa Rican population no triglyceride elevation was found in the minor allele presenting individuals. The mechanism, through which they influence triglyceride level, is still unknown. Assumedly strong linkage to other variants can play a role in this. In European populations full linkage was established between *APOA5* variants. The linkage was partial in the Costa Rican population, which can explain why no coherence was found between the mutant alleles and the elevated triglyceride levels.

7.3. Role of the T1259C variant of the APOA5 gene 3' UTR region

In spite of the triglyceride level increasing effect of the T1259C variant we did not find the presence of the mutant C allele to predispose to MS. In contrary the IVS3+G476A alteration can increase the chance of MS 3-4-times. It expresses its main effect in the function change caused by the variants occurring linked to it. This is supported by the fact that in the case of APOA5*5 haplotype, when the 1259C allele is present alone, does not increase triglyceride level, and even protects against MS development.

7.4. Role of the C56G variant of the APOA5 gene first exon

The studied SNP is a C/G transition in the 56 nucleotide position of the gene's third exon. The base change regards the 19th amino acid, a serine, instead of which the mutant allele encodes a tryptophan. Allele 56G shows different occurrence in different populations. It has extremely low occurrence in Chinese and Japanese populations (<0.1%), Indian population has a 3% appearance, while Afro-american and French populations show 4.8%, Spanish populations show ~15% presence. Our research found 9.5% mutant allele presenting people in the control group in several European populations (Spanish, French, German, Austrian).

Talmud and co-workers observed 8-16% elevation of the triglyceride level in a Caucasian population in the presence of the 56G allele (Talmud et al., 2005). A Turkish population showed 18-26% increase due to the presence of the mutant allele (Hodoglugil et al., 2006). According to the results of population studies, bearing this allele means higher risk of myocardial infarction, coronary disease, metabolic syndrome. Talmud and collaborators found faster atherogenesis in the presence of the mutant allele. We found no elevated triglyceride mean values in the objects presenting the mutant allele in homo-, or heterozygote form. Also, bearing the variants did not increase the risk of MS development.

The effect of the variant on lipid parameters and the mechanism through which it can increase triglyceride level, has become central topic of many studies. According to investigations out of the *APOA5* variants the C56G is the only „functional" variant, which does not exert its effect via connections to other SNPs, but directly elevates triglycecride level. Due to the variant's action, a hydrophobe tryptophan is built into the hydrophilic domain of the APOAV signal protein instead of a hydrophilic serine. This drastically affects the protein's translocation through the endoplasmic reticulum, therefore the amount of secreted APOAV protein decreases, thus triglyceride level increase can be detected.

Of course we cannot exclude that the C56G variant may act together with other polymorphisms in the development of diseases, enhancing one another's affect. Schaefer and co-workers defined the *APOE* and *APOA5* C56G genotypes of 170 hypertriglyceridaemia patients. Almost all patients having *APOE* 2/2 genotype bleared the 56G allele. However in objects with normal lipid parameters these could not be found collectively. According to our hypothesis, C56G works as cofactor to lead to hypertriglyceridaemia. Our results suggest this mechanism as well.

7.5. Potential role of the most frequent haplotypes of the APOA5 gene

Pennacchio and colleagues, studying the natural variants of *APOA5* gene, found strong linkage between the most frequent variants, which therefore determine three main haplotypes. The two haplotypes (APOA5*2 and *3) with the wild haplotype (APOA5*1) covers ~98% of the population. Our study revealed similar data, except that we found high occurrence of APOA5*5 haplotype in the control group.

APOA5*2 haplotype is explicit risk factor for the development of hypertriglyceridaemia, different cardio-, cerebrovascular diseases. APOA5*2 haplotype in Romanian, Austrian and German adult population such as in Hungarian children clearly predisposes to MS development. APOA5*3 haplotype in many studies is a risk factor of the MS, however our investigation and more studies on child population did not prove this.

Based on the haplotype analysis one can suppose that except the C56G variant, the studied variants do not exert their action themselves, but linked to the variants of the *APOC3* gene. -1131C variant, occurring alone (APOA*4) does not increase serum triglyceride level, and has no effect on the occurrence of MS. Moreover, 1259C appearing alone protects against disease formation. Many studies dealing with the *APOA1/C3/A4* gene cluster, where *APOA5* belongs to, could find strong linkage to *APOC3* promoter region polymorphism only in the case of APOA5*2 haplotype, which polymorphisms clearly influence serum triglyceride level and glucose metabolism (Li et al., 2004; Olivieri et al., 2003b).

8. SUMMARY

We did the following observation in our study:

1. Observing the effects of *APOA5* variants on lipid parameters we can say that the presence of –1131C, IVS3+476A, 1259C mutant alleles resulted in significant elevation of triglycerides in MS patients and control objects also. C56G variant showed no relation to serum triglyceride levels in any of the groups.

2. Examining cholesterol values the variants had no effect on the whole cholesterol level for patients and for controls also.

3. We found significant accumulation of the mutant allele in case of the T–1131C, IVS3+G476A variants for metabolic syndrome patient group compared to control population.

4. We could find no difference in the occurrence of any of the alleles in case of T1259C variant neither for patients, nor for control groups, even despite that our analyses revealed significant triglycerid level increase due to the 1259C allele variant occurrence.

5. The observed variants define 5 frequent haplotypes, APOA5*5 haplotype was not yet described in other populations.

6. Amongst the haplotypes, only APOA5*2 resulted in statistically significant elevation of triglyceride concentration in metabolic syndrome patients and controls as well.

7. Regarding cholesterol values, we found no significant difference in patients and controls as a result of presence of any of the APOA5 haplotypes.

8. Studying the haplotypes' distribution we found the accumulation of the APOA5*2 haplotype in metabolic syndrome patients compared to control objects. APOA5*5 found to have protective effect against the development of MS.

9. PUBLICATIONS LIST

The thesis is based on the following publications

1. Apolipoprotein A5 T-1131C variant confers risk for metabolic syndrome. Maász A, **Kisfali P**, Horvatovich K, Mohás M, Markó L, Csöngei V, Faragó B, Járomi L, Magyari L, Sáfrány E, Sipeky C, Wittmann I, Melegh B. Pathol Oncol Res. 2007;13(3):243-7.
2. Apolipoprotein A5 IVS3+476A allelic variant associates with increased trigliceride levels and confers risk for development of metabolic syndrome in Hungarians. **Kisfali P**, Mohás M, Maasz A, Hadarits F, Markó L, Horvatovich K, Oroszlán T, Bagosi Z, Bujtor Z, Gasztonyi B, Wittmann I, Melegh B. Circ J. 2008 Jan;72(1):40-3.
3. Haplotype analysis of the apolipoprotein A5 gene in patients with the metabolic syndrome. **Kisfali P**, Mohás M, Maász A, Polgár N, Hadarits F, Markó L, Brasnyó P, Horvatovich K, Oroszlán T, Bagosi Z, Bujtor Z, Gasztonyi B, Rinfel J, Wittmann I, Melegh B. Nutr Metab Cardiovasc Dis. 2010 Sep;20(7):505-11.

Other publications

Bookchapter:

Horizons in World Cardiovascular Research. Volume 2
Chapter 3. Shared Susceptibility Genes of Metabolic Syndrome and Cardiovascular Disease.
Peter Kisfali, Eniko Safrany, Judit Bene, Bela Melegh,
pp. 57-78
Nova Science Publishers 2010

Articels:

1. Common functional variants of APOA5 and GCKR accumulate gradually in association with triglyceride increase in metabolic syndrome patients. Hadarits F, **Kisfali P**, Mohás M, Maász A, Duga B, Janicsek I, Wittmann I, Melegh B. Mol Biol Rep. 2011 Jun 4.
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4. Monitoring of group A rotaviruses in wild-living birds in Hungary. Ursu K, Papp H, **Kisfali P**, Rigó Avian Dis. 2011 Mar;55(1):123-7.
5. Cytotoxic T lymphocyte-Associated Antigen +49G Variant Confers Risk for Anti-CCP- and Rheumatoid Factor-Positive Type of Rheumatoid Arthritis Only in Combination with CT60G Allele. Farago B, **Kisfali P**, Magyari L, Polgar N, Melegh B. Autoimmune Dis. 2010;2010:285974.
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7. Haplotype analysis of the apolipoprotein A5 gene in obese pediatric patients. Horvatovich K, Bokor S, Barath A, Maasz A, **Kisfali P**, Jaromi L, Polgar N, Toth D, Repassy J, Endreffy E, Molnar D, Melegh B. Int J Pediatr Obes. 2010 Sep 30
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