PhD thesis

ASSOCIATION OF THE COMMON NATURAL VARIANTS OF APOLIPOPROTEIN A5 GENE AND SERUM TRIGLYCERIDE LEVELS IN GENERAL HUNGARIAN POPULATION, METABOLIC SYNDROME, AND STROKE PATIENTS

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Pécs, 2011

INTRODUCTION

The cardiovasculare diseases are the leader causes of death in the developed countries for several decades. Among the risk factors of these diseases – beside many others – are dyslipidemias. Dyslipidemias include the elevation of serum total cholesterol, triglyceride and the low density lipoprotein (LDL), and the lower HDL (high density lipoprotein) level.

In the last few years numerous genetic variants had been discovered in the background of the above diseases. We have already got to know genetic variants that may play a role in the pathogenesis of these diseases.

Serum lipid transportation is performed and some metabolic steps are ruled by the so called apolipoproteins (APO A-B-C-E). The ApoA-V protein encoded by *APOA5* gene plays a role in the regulation of the serum triglyceride level. The defects of *APOA5* gene can cause elevation of the triglycerides (Pennacchio 2001, 2002, Eichenbaum-Voline 2004).

In my PhD work I analyzed the possible roles of *apolipoprotein A5* gene's natural variants on serum triglyceride levels in general Hungarian population, metabolic syndrome and stroke patients.

Although the majority of the human genes among people are very similar, there is variability, a polymorphism profile that is very special in every single person. With the examination of this polymorphism in the different diseases – by our best hope – the medicine will be able to cure more and more diseases and can open a new era in the healing by the so-called personalized medicine.

MAIN AIMS OF THE STUDY

Our aims were to investigate the connections between the serum triglyceride levels and the APOA5 gene variants in general Hungarian population.

1. Our aim was to study the *APOA5* gene -1131C, 56G, IVS3+476A and 1259C alleles in general Hungarian patients, and to compare these alleles' frequencies with the literature.

2. Are there connections among the *APOA5* gene variants (T-1131C, C56G, IVS3+G476A and T1259C) and the serum triglyceride levels?

3. What is the frequency of the *APOA5* haplotypes in the general Hungarian population?

4. Are there connections between the haplotypes and the serum triglyceride levels?

In addition we wanted to investigate the APOA5 gene and serum triglyceride level connections in other diseases, namely in metabolic syndrome (MS) and stroke patients.

5. Are there or not any relations between *APOA5* gene variants of metabolic syndrome patients and serum triglyceride levels?

6. Could we find similar connections between these (*APOA5* gene variants and serum triglyceride levels) in stroke patients and is there a difference among the different subgroups of stroke?

MATERIALS AND METHODS

Patients

The DNA materials for the *APOA5* gene investigation of general Hungarian population were originated from the Markusovszky Hospital of Vas County.

The patient and control samples examined for metabolic syndrome and stroke patients were selected from the sample collection of our department's Biobank, which belongs to the Central National Biobank Network of Hungary (www.biobank.hu), and were collected in the Pandy Kalman Hospital, Gyula. During the collection and use of DNA samples the regulations of the local ethical committee and those of the Helsinki Declaration in 1975 were followed; we had an informed consent from the patients. We have put their anonymous DNA back into the Biobank for possible future use.

The allele frequencies of all *APOA5* variants studied were in Hardy-Weinberg equilibrium in each group.

Methods

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.

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 from the genotype to control the efficacy of the digestion. The fragments received were electrophoresed through an ethidium-bromide-stained 3% agarose gel and were analyzed with UVIdoc gel documentation system.

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 (Applied Biosystems, Foster City, CA, USA). The sequence alingments were made using Winstar genetic program (DNASTAR Inc., Madison, WI, USA).

Statistical evaluation

Our data were 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 discrete clinical and laboratory parameters with normal distribution χ^2 tests were used. Continuous variables with normal distribution were analyzed with Student's 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 and SAS packages (SPSS Inc, Chicago, IL; SAS Institute Inc, Cary, NC, USA).

RESULTS

The connections between the *APOA5* natural allelic variants of metabolic syndrome patients and serum triglyceride levels

This section of our study had three parts:

A. The IVS3+G476A and T1259C allelic variants and their relations with serum triglyceride levels.

B. The *APOA5* gene haplotypes in metabolic syndrome patients.

C. The frequencies of *APOA5* gene allelic variants in metabolic syndrome patients by serum triglyceride quartiles.

A. IVS3+G476A and T1259C allelic variants by different serum triglyceride levels in metabolic syndrome patients

213 MS patients – 99 male and 114 female – were aged of 61,09 $\pm$ 1,01 years (25 – 82 year).

We found significantly higher serum triglyceride level in IVS3+G476A and T1259C natural allelic variants of MS patients. However by multiple logistic regression analysis there was significant connection found only between IVS3+G476A and serum triglyceride level in MS patients but not in T1259C allelic variant.

B. Different haplotypes of MS patients

Of the 343 MS patients were 158 male and 185 female (aged: 60,5 $\pm$ 10,08 years (23 – 74 year).

The *APOA5* gene haplotypes are shown in the following chart.

Allelic variants Haplotypes	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

In our results we found significant correlation only with the *2 haplotype and the serum triglyceride levels in MS patients.

C. APOA5 gene allelic variants in quartiles of serum triglyceride levels

In this part of our investigation DNA material used was obtained from 141 male and 184 female MS patients, aged 60,5 $\pm$ 10,8 years (23 – 74 year).

The quartiles were as follows: q1: TG < 1,38 mmol/L, q2: 1,38 – 1,93 mmol/L, q3: 1,94 – 2,83 mmol/L and q4: > 2,83 mmol/L.

We found a step wise connection with TG levels by all allelic variants except for C56G. These significant connections were in the third and fourth quartiles.

Investigation of the connections between *APOA5* natural allelic variants and serum triglyceride levels in stroke patients

1. Investigation of T-1131C APOA5 allele

The results show a significantly higher serum triglyceride level at the -1131C allelic variant-carrier patients than at the non-carrier ones.

2. The C56G APOA5 variant

We examined the connection between *APOA5* gene C56G variant and serum triglyceride level in different stroke subgroups (the grouping was by Adams 1993).

Our results show a significantly higher serum triglyceride level in all stroke subgroup patients with *APOA5* 56G variant than in the non-carrier patients.

3. Investigation of the T1259C and IVS3+G476A allelic variants

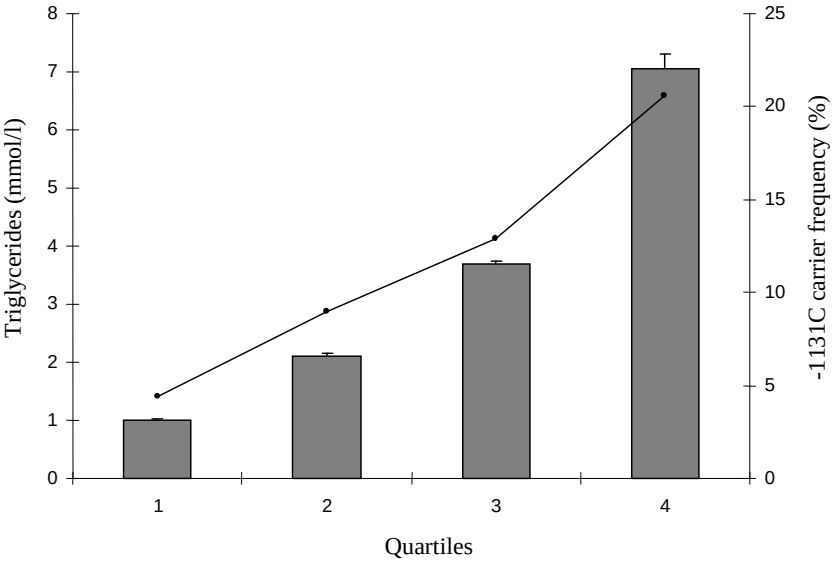
Finally we have investigated the connection between the other two frequent variants (T1259C and IVS3+G476A) of *APOA5* gene and the serum triglyceride level in stroke patients.

The connection between the *APOA5* allelic variant stroke (each subgroup) patients and their serum triglyceride level is significant. This significance is more powerful when the comparison is performed for the overall patient group (all subgroups) compared to separate analysis of the stokre subgroups.

Correlations between general Hungarian population's *APOA5* allelic polymorphism and serum triglyceride levels

436 patients' (235 men and 201 women) – 60,5 ± 101 years, the youngest was 23, the oldest was 74 – EDTA- anticoagulated samples were analyzed. Four quartiles (q1-4) were created according to the serum triglyceride level: q1: TG < 1,31 mmol/L, q2: TG = 1,31 – 2,90 mmol/L, q3: TG = 2,91 – 4, 85 mmol/L and q4: TG > 4,85 mmol/L.

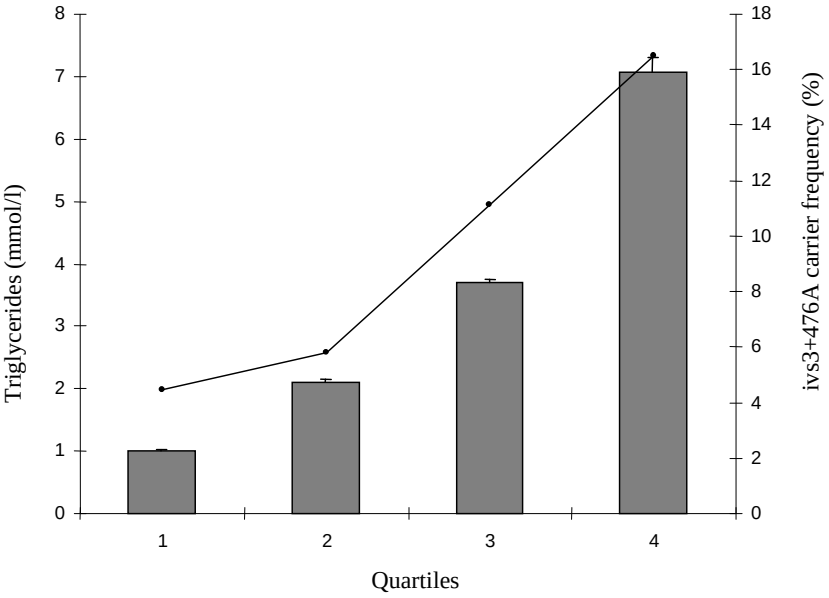
Investigation of the APOA5 gene promoter region's T-1131C allelic variant



The continuous line links the data points of frequency of T-1131C minor variant in the quartiles, in the different serum triglyceride level patients.

We can conclude that the -1131C *APOA5* variant's frequency is significantly higher in the third and fourth quartiles' patient groups, compared to the first quartile's. The significance is more powerful in the fourth quartile than in the third quartile ($p < 0.001$ and $p = 0.001$, respectively). We didn't find a significant difference in the second quartile's patients with regard to this allelic variant.

Examination of APOA5 gene IVS3+G476A intronic variant

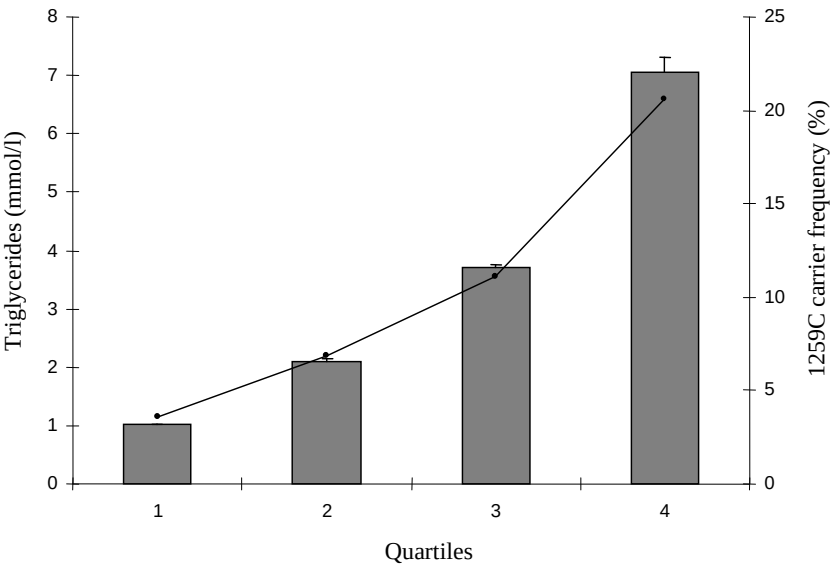


Our results were similar to the T-1131C allelic variant in the examination of the IVS3+G476A minor variant as well.

The frequency of this two allelic variant was higher in the third and fourth quartiles compared to the first one. The significance was stronger in the fourth than in the third quartiles ($p < 0.001$ and $p = 0.006$, respectively).

Similarly to the T-1131C allele, the IVS3+G465A minor allele carrier patients of the second quartile also did not show significant difference compared to those belonging to the first one.

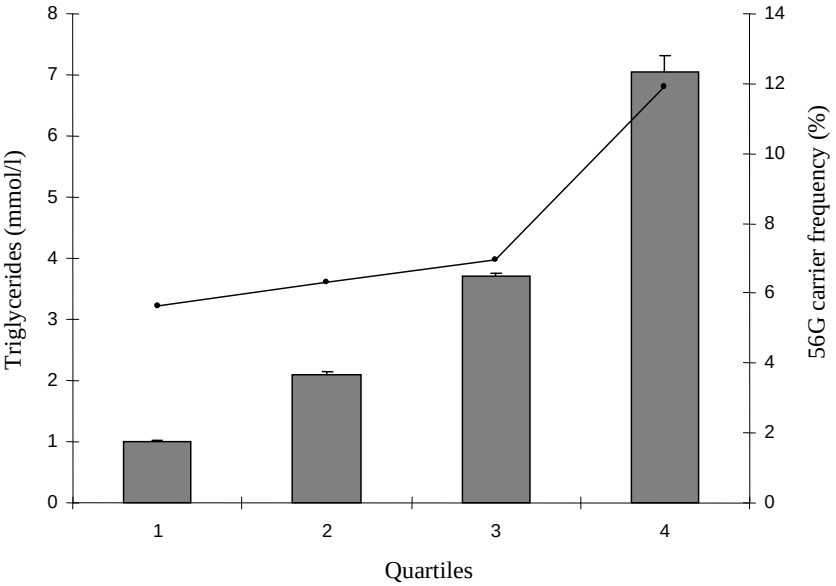
Investigation of the APOA5 gene T1259C allelic variant



The results were almost the same as they were in the case of the two previous *APOA5* minor variants. Namely, the frequency of T1259C allelic variant was significantly higher only in the third and in the fourth quartiles compared to the first one.

The allelic frequency in the third quartile was 11,1% and in the fourth quartile 20,6%.

Examination of the APOA5 gene C56G variant located in the third exon



In this quartile, in the frequency of C56G *APOA5* minor variant was significantly different ($p < 0.001$) only in the fourth quartile compared to the first one. The frequency of this variant was 5,64% in the first, 6,31% in the second, 6,94% in the third and 11,9% in the fourth quartile.

APOA5 haplotypes

We didn't find significant difference among the quartiles with regard to the frequency of *APOA5**1 haplotype. But our results showed significant difference in the frequency of *APOA5**2 haplotype among patients of the third and fourth quartiles, while in the fourth quartile the *APOA5**3 haplotype showed an increased prevalence as well compared to the first quartile. The level of significance in all three groups was $p \leq 0.05$.

SUMMARY

1. The serum triglyceride level was significantly higher in the case of carriers of the *APOA5* gene –1131C, IVS3+476A, 1259C allelic variants but not in the case of C56G variant in metabolic syndrome patients.
2. Our analysis revealed a significant correlation between the carriership of *APOA5**1/2-2/2 haplotype and the serum triglyceride level in metabolic syndrome patients but no such correlation was found in case of the other haplotypes.
3. In stroke patients – in each subgroup – we got a significant association between the serum triglyceride level and each *APOA5* allelic variant.
4. In general Hungarian population the connection of the serum triglyceride level and the frequency of all four allelic variants (–1131C, IVS3+476A, 1259C and 56G) of *APOA5* gene is significant. This connection shows a stepwise elevation from quartile to quartile.
5. But this significance at the –1131C, IVS3+476A, 1259C allelic variants was noticed only at q3 and q4 group (serum triglyceride > 2,91 mmol/L) compared to the q1 (serum triglyceride level < 1,31 mmol/L). The association of the 56G variant with serum triglyceride was significant only in q4.
6. The haplotype analysis of *APOA5* gene in average Hungarian patients with different serum triglyceride level revealed that:
    a. There was no significant difference in the prevalence of *APOA5**1/1 haplotype among the quartiles.
    b. The *APOA5**1/2-2/2 haplotype frequency was significantly higher in patients with serum triglyceride level higher than 2,91 mmol/l (q3; q4); while the *APOA5**1/3-3/3 haplotype frequency was significantly elevated only among q4 patients (where serum triglyceride level is higher than 4,85 mmol/L).

LIST OF PUBLICATIONS

The thesis is based on the following publications:

1. Kisfali P, Mohas M, Maasz A, ***Hadarits F,*** Marko L, Horvatovich K, Oroszlan T, Bagosi Z, Bujtor Z, Gasztonyi B, Wittmann I, Melegh B. *Apolipoprotein A5* IVS3+476A allelic variant associates with increased triglyceridee levels and confers risk for development of metabolic syndrome in Hungarians. Circ J. 2008; 72(1):40-3

   Impact Factor: 2,387 (2008)

2. Maasz A, Kisfali P, Szolnoki Z, ***Hadarits F***, Melegh B. *Apolipoprotein A5* gene C56G variant confers risk for the development of large-vessel associated ischemic stroke. J Neurol. 2008; 255(5):649-54.

   Impact Factor: 2,536 (2008)

3. 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.

   Impact Factor: 2,387 (2008)

4. Kisfali P, Mohas M, Maasz A, Polgar N, ***Hadarits F***, Marko L, Brasnyo P, Horvatovich K, Oroszlan T, Bagosi Z, Bujtor Z, Gasztonyi B, Rinfel J, Wittmann I, Melegh B. Haplotype analysis of the *apolipoprotein A5* gene in patients with the metabolic syndrome. Nutr Metab Cardiovasc Dis. 2010;20(7):505-11.

   Impact Factor: 3.517 (2009)

5. ***Hadarits F***, Kisfali P, Mohas M, Maasz A, Sumegi K, Szabo M, Hetyesy K, Valasek A, Janicsek I, Wittmann I, Melegh B. Stepwise Positive Association Between *APOA5* Minor Allele Frequencies and Increasing Plasma Triglyceride Quartiles in Random Patients with Hypertriglyceridemia of Unclarified Origin. Pathol Oncol Res. 2011;17(1):39-44

   Impact Factor: 1.152 (2009)

6. ***Hadarits F,*** Kisfali P, Mohas M, Maasz A, Duga B, Janicsek I, Wittmann I, Melegh B. Common functional variants of *APOA5* and GCKR accumulate gradually in association with triglyceride increase in metabolic syndrome patients. Molecular Biology Reports. 2011. [Epub ahead of print]

   Impact Factor: 2.040 (2010)

Other publications:

1. Brittig F., Garzuly F., Mazlo M., ***Hadarits F***. Fabry-kór arteria basilaris thrombosissal. Morph. és Ig. Orv. Szemle. 26 :15-24; 1986.

2. ***Hadarits F.*** Balogh M. Oroszlan G. Kovacs L. G. Hyperimmunglobulinaemia E (Jób) syndroma. Klinikai és Kísérletes Laboratóriumi Medicina, 1998:25,4:187-189.

3. ***Hadarits Ferenc:*** Human Papillomavírusok (HPV) és cervixrák szűrés. Új Bábakalauz, 1998; II., 4, 27-32.

4. Horvath B. ***Hadarits F.*** Szabo L. Hüvelyi fertőzések kezelése, 144 beteg Gynoflor-kezelésének prospektív vizsgálata. Magyar Nőorvosok Lapja, 2004, 67, 85-91.

5. Z Szolnoki, A Maasz, L Magyari, K Horvatovich, B Farago, F Somogyvari, A Kondacs, M Szabo, L Fodor, A Bodor, ***F Hadarits***, B Melegh. Coexistence of angiotensin II type 1 receptor A1166C and angiotensin-converting enzyme D/D polymorphism represets susceptibility for small-vessel-associated ischaemic stroke. NeuroMolecular Medicine, 2006, 8 (3), 353-60.

Impact Factor: 3,396 (2006)

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.

Impact Factor: 1,735 (2007)

7. Szolnoki Z, Maasz A, Magyari L, Horvatovich K, Farago B, Kondacs A, Bodor A, ***Hadarits F***, Orosz P, Ille A, Melegh B. Galectin-2 3279TT variant protects against the lymphotoxin-alpha 252GG genotype associated ischaemic stroke. Clin Neurol Neurosurg. 2009;111(3):227-30.

Impact Factor: 1,323 (2008)

8. ***Hadarits F***, Ivan, A., Markus, Cs. and Nagy, L. Experiences with cardiac troponin assessments. Orvosi Hetilap 2009;150(43):1988-1993

Citable abstracts:

Maasz A, Kisfali P, Jaromi L, Szolnoki Z, ***Hadarits F***, Melegh B. *Apolipoprotein A5* gene IVS3+G476A allelic variant confers

susceptibility for development of ischemic stroke. Eur J Hum Genet, 2008;16(S2):293.

Impact Factor: 3,925 (2008)

Kisfali P, Mohas M, Maasz A, ***Hadarits F***, Marko L, Kesoi I, Oroszlan T, Bagosi Z, Bujtor Z, Rinfel J, Gasztonyi B, Wittmann I, Melegh B. *Apolipoprotein A5* gene *APOA5**2 haplotype variant confers risk for the development of metabolic syndrome. Eur J Hum Genet, 2008;16(S2):328.

Impact Factor: 3,925 (2008)

Hadarits F., Horvath M., Nyuli L. and Kovacs L.G. Abnormalities of cellular and humoral immune parameters in chronic alcoholic patients with delirium tremens. 3rd Alpe-Adria Congress on Clinical Chemistry and Laboratory Medicine, Hungary, Pécs, 1994.

Hadarits F, Csanaky G, Donhoffer A. Kovacs L. G. HPV-DNA detection and cervical cancer screening. 20th World Congress of Pathology and Laboratory Medicine. Brazil, Sao Paulo, 1999.

Hadarits, F., Hajnal, A., Norgren, R. Conditioned taste aversion affects gustatory taste neuron responses in awake rats. Society for Neuroscience, 31st Annual Meeting, USA, San Diego, California, 2001.

Cumulative impact factor (without citable abstract): 20,473

Cumulative impact factor (with citable abstract): 28,323