THE RELATIONSHIP BETWEEN THE PRO12ALA POLYMORPHISM OF THE PPARγ2 GENE AND THE METABOLIC SYNDROME IN A POPULATION OF CENTRAL ROMANIA DIAGNOSED ACCORDING TO THE IDF CRITERIA

K. Csép[1], Gyöngyi Dudutz[1], Márta Vitay[2], Ionela Pașcanu[1], Claudia Bănescu[1], L. Korányi[2], L. Rosivall[3]

[1]University of Medicine and Pharmacy Tg. Mureș – Romania, [2]Drug Research Center Balatonfüred- Hungary, [3]University of Medicine Semmelweis Budapest – Hungary

Abstract

The nuclear receptor coding PPARγ2 (PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR-GAMMA; *601487) gene influences the lipid and carbohydrate metabolism via multiple pathways and is a candidate for the metabolic syndrome. In this paper we studied the relationship of the CCG (Pro) → GCG (Ala) polymorphism of the gene with the metabolic syndrome diagnosed according to the criteria recommended by the International Diabetes Federation (IDF) in 2005, in a population from central Romania.

We have carried out a case-control study on 144 patients and 73 control subjects. Routine biochemical assays have been carried out, fasting insulinemia was measured by ELISA, and insulin sensitivity was assessed by calculating the HOMA and QUICKI indices. Genetic analysis was done by PCR followed by digestion with the restriction enzyme BstU I. The results show that the Pro12 allele had a higher frequency in the group of patients as compared to the healthy controls (76 vs. 65.7%, $p<0.05$). The risk for developing the metabolic syndrome in the presence of the Pro12 allele in a homozygous combination was found to be low but statistically significant (PP vs. PA + AA: OR = 1.98, CI 95% 1.04 -3.78, $p = 0.046$). In conclusion, in the local population, the Pro12 allele of the PPARG2 gene seems to contribute to the hereditary predisposition of the metabolic syndrome diagnosed according to the recommendations of the IDF, most likely as part of a polygenic system. Probably the absence of the protective Ala12 allele increases the risk for developing the disease.

Key words: metabolic syndrome, PPARγ2 polymorphism.

INTRODUCTION

The metabolic syndrome has incited many discussions. It has been largely debated whether such a distinct phenotype truly exists, and it was much disputed the role of insulin resistance in the pathogenesis of the syndrome (1-6). Several diagnostic

*Correspondence to: Katalin Csép, UMF Tg. Mures - Genetics Department, 38, Gh. Marinescu Str, Tg. Mures 540042, Romania, phone: +40 265 215551, email: kcsep@rdslink.ro

approaches were recommended, however none of them gained general acceptance (7-9).

Nevertheless, the high prevalence and serious morbidity and mortality associated with the metabolic syndrome phenotype motivate the efforts of clarifying its etiology. Research data and everyday clinical practice suggest multi-factorial inheritance, at least in the majority of cases. Most probably the inherited genetic predisposition is transformed into a manifest disease in the presence of an unhealthy Western type lifestyle. As for the genetic predisposition, the association studies suggest the effect of a polygenic system with several minor genes involved.

Many candidates have been described, but among these, probably the most studied and controversial is the Pro12Ala polymorphism of the PPARG2 gene. The PPARG (PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR-GAMMA; *601487) gene is situated in 3p25, a region frequently found to be associated with type 2 diabetes (10). The members of the PPAR gene family code nuclear receptor type transcription factors that form heterodimers together with the RXR (retinoid X receptor) molecules. Activated by fatty acids, the heterodimeric structure controls the transcription of multiple genes, and thus intervenes in the carbohydrate and lipid metabolism, regulates adipocyte differentiation.

Of the three subtypes of PPAR (α, δ and γ), PPAR γ or PPARG is expressed in large quantities in the adipocytes, and at a lower level in the bone marrow, spleen, testicles, brain, muscles and liver. The most frequent mutations of the PPARG2 gene associated with severe insulin resistance are Pro467Leu and Val290Met, while Pro115Gln is associated with obesity and Pro12Ala is involved in the development of type 2 diabetes and high blood pressure. Scanning the coding region, Yen *et al*. have identified in 1997 a missense mutation [CCG (Pro) $\rightarrow$ GCG (Ala)] resulting in a proline to alanine substitution (Pro12Ala) in the 12th codon, with an allele frequency of 0.12 in Caucasians (11). The polymorphism is situated in the ligand independent activation domain, and determines an important change in the structure of the protein that results in reduced trans-activation capacity of target genes and better insulin sensitivity. Since then, many association studies have been published, and a few meta-analyses that revealed contradictory results (10).

We found no data published regarding the allele frequency of the aforementioned single nucleotide polymorphism (SNP) in the Romanian population. Welcoming the effort of the International Diabetes Federation (IDF) to elaborate a universal system for diagnosing the metabolic syndrome, recommended including for research purposes, we proposed to study the relationship of the Pro12Ala polymorphism of the PPARG2 gene with the metabolic syndrome in the local population.

PATIENTS AND METHODS

A case-control study was designed in metabolic syndrome patients and age-matched healthy control subjects inhabiting the same region (Tg. Mures). Patients

and controls were recruited from the County Emergency Clinical Hospital and Empatia Medical Center respectively.

Anthropometric measurements were taken, and biochemical assays have been done using the Hitachi© 717 Roche analyzer from the Central Laboratory of the County Hospital. Fasting insulinemia was determined by ELISA using DakoCytomation Insulin kits. Insulin sensitivity and β-cell function was assessed by calculating the HOMA and QUICKI indices (12, 13). The metabolic syndrome diagnosis was made according to the recommendations of the IDF (14).

Genetic studies were done by PCR (polymerase chain reaction) followed by RFLP (restriction fragment length polymorphism) on DNA isolated with Epicentre MasterPure Genomic DNA Purification Kits from venous blood samples collected on EDTA. PCR reactions were carried out in a Biometra T3 system, with primers PPARGA1: 5'-GCCAATTCAAGCCCAGTC-3' and PPARG2: 5'-GATATGTTGC AGACAGTGTATCAGTGAAGAATCGCTTTCCG-3' obtained from Integrated DNA Technologies, Inc, and AmpliTaq Gold in a mix containing 1.8 μl $MgCl_2$ (15-17). After an initial denaturation at 95°C for 10 minutes, and 30 cycles of denaturation, annealing and synthesis (94°C – 45 sec, 58°C – 45 sec, 72°C - 45 sec) a final extension followed at 72°C for 5 minutes. Digestion with 5 U BstU I restriction enzyme from NewEngland Biolabs Inc. was done in 10 μl PCR product and NEB 2, at 60°C (15-17). Restriction fragments were separated by electrophoresis in 3% agarose, and captured with a Kodak EDAS 290 system.

For the statistical analysis we used Statistica version 6 (StatSoft Inc., 2001) and GraphPad InStat version 3.00 for Windows 95. We considered results statistically significant if $p < 0.05$ (all p values are two-sided).

RESULTS

The demographic and metabolic characterization of the two study groups is represented in Table 1 and Table 2.

Genotype was assessed based on the different migration of the restriction fragments caused by the polymorphism (Ala12: 223 + 47 bp and Pro12: 270 bp) as shown in a set of probes (Fig. 1). Allele frequency of the Pro12 allele and the less frequent Ala12 allele was 86.17% and 13.82% in the metabolic syndrome group and 80.82% and 19.17% in the control group, respectively. The three genotypes in the two study groups were distributed as shown in Fig. 2. Allele and genotype frequencies were in Hardy-Weinberg equilibrium. Due to the low frequency of the Ala12 allele, AA homozygotes and PA heterozygotes were considered together. Metabolic characteristics in the two groups according to the genotype are presented in Table 3.

The risk for developing the metabolic syndrome in PP homozygous combination, in the absence of the Ala12 allele (PP vs. PA + AA) was low but statistically significant: OR = 1.98 (CI 95%: 1.04-3.78, p = 0.046).

Taking into account the presence of a BMI ≥ 30, the relationship remained

Table 1. Demographic characteristics.

	Metabolic syndrome group	Control subjects
Male/female (%)	54.37/45.62	50.68/49.32
Urban/rural residence (%)	60.36/39.63	57.53/42.46
Ethnicity - Romanian/Hungarian (%)	50.23/49.76	43.83/56.17
Age mean ± SD (median)	61 ± 10.63 (61)	58.53 ± 14.25 (62)

Table 2. Metabolic, anthropometric and biochemical characteristics for study and control groups.

	Metabolic syndrome group	Control subjects	p*
BMI (kg/m^2)	31.79 ± 6.75 (30.28)	25.19 ± 4.24 (24.76)	< 0.0001
Male	30.65 ± 5.22 (29.53)	26.13 ± 4.57 (25.64)	< 0.0001
Female	33.21 ± 8.13 (31.62)	24.18 ± 3.66 (23.87)	< 0.0001
W (cm)	109.7 ± 13.41 (108)	93.29 ± 12.63 (91)	< 0.0001
Male	112.08 ± 13.02 (110)	97.43 ± 13.06 (93)	< 0.0001
Female	106.63 ± 13.37 (107)	89.16 ± 10.94 (88)	< 0.0001
WHR	0.97 ±0.07 (0.97)	0.92 ± 0.08 (0.93)	< 0.0001
Male	0.97 ± 0.06 (0.98)	1.01 ± 0.04 (1.01)	< 0.0001
Female	0.92 ± 0.07 (0.92)	0.88 ± 0.07 (0.88)	< 0.0001
FG (mg/dl)	122.33 ± 42.98 (108.4)	96.43 ± 12.44 (95.75)	< 0.0001
FI (μU/ml)	13.39 ± 7.94 (11.73)	8.38 ± 7.2 (6.68)	< 0.0001
QUICKI	0.32 ± 0.03 (0.31)	0.36 ± 0.04 (0.35)	< 0.0001
IRI	3.11 ± 0.31 (3.13)	2.8 ± 0.32 (2.8)	< 0.0001
HOMA-IR	3.97 ± 3.17 (3.29)	1.59 ± 1.74 (1.08)	< 0.0001
HOMA-B	38.26 ± 25.21 (33.9)	27.6 + 23.78 (21.44)	< 0.0001
TG (mg/dl)	220.94 ± 155.16 (180)	111.45 ± 59.07 (103)	< 0.0001
HDL-C (mg/dl)	46.19 ± 17.73 (46)	53.99 ± 14.37 (52.8)	< 0.0001
TC (mg/dl)	213.42 ± 51.72 (215.5)	199.79 ± 43.1 (198)	0.025
SBP (mmHg)	155.24 ± 20.37 (155)	132.02 ± 20.84 (125)	< 0.0001
DBP (mmHg)	90.37 ± 11.47 (90)	78.94 ± 11.03 (80)	< 0.0001

*Mann-Whitney U test
FG - fasting glucose, FI - fasting insulin, SBP - systolic blood pressure, DBP - diastolic blood pressure, TG - triglyceride, C - cholesterol, BMI - body mass index, W - waist circumference, WHR - waist/hip rtio, HOMA - Homeostasis Model Assesment - IR insulin resistance and B - beta cell function index, QUICKI - quantitative insulin sensitivity check index, IRI - 1/QUICKI.

significant and independent of obesity (chi^2 = 12.81, df: 3, p = 0.0051).

DISCUSSION

Diagnosing the metabolic syndrome has been challenging. The aim of the IDF recommendations was elaborating a worldwide definition, a diagnostic tool

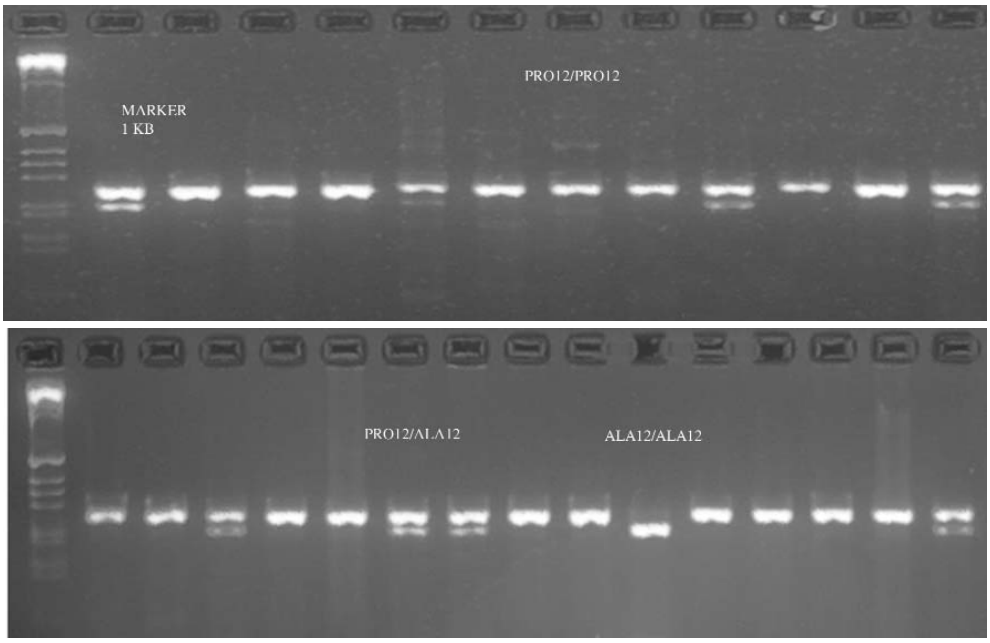

Figure 1. Electrophoretic image of the three genotypes in a set of probes (Pro12/Pro12 - PP homozygote, Pro12/Ala12 - heterozygote, Ala12/Ala12 - Ala12 homozygote)

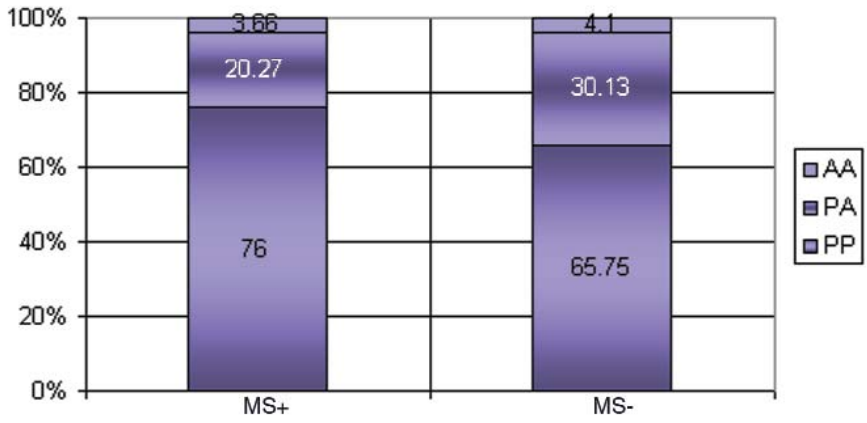

Figure 2. Genotype frequency in the two study groups
(Pro12/Pro12 - PP homozygote, Pro12/Ala12 - heterozygote, Ala12/Ala12 - Ala12 homozygote)

adequate for both the bedside and the research setting (14). In our previous studies having faced the difficulty of determining insulin resistance and interpreting the results published by various authors using different diagnostic algorithms, we initiated our genetic epidemiology studies using the new system.

Our experience is that the defining element of the syndrome is abdominal obesity, the presence of which regularly associates the clinical spectrum of the

Table 3. Metabolic characteristics in the two groups of study according to the genotype.

	Metabolic syndrome group			Control subjects		
	PP	PA+AA	p*	PP	PA+AA	p*
BMI	31.16±6.14	33.82±8.27	0.08	24.34±3.65	26.92±4.82	0.02
W	112.78±14.06	112.14±3.21	>0.05	90.24±10.52	99.25±14.46	0.006
FG	122.64±42.16	121.40±46.27	>0.05	96.02±12.92	97.37±11.56	>0.05
FI	13.61±8.31	12.71±6.83	>0.05	8.52±7.37	8.74±0.007	>0.05
HOMA-IR	4.07±3.44	3.67±2.23	>0.05	1.46±1.06	1.90±2.05	>0.05
HOMA-B	38.60±25.78	37.22±23.92	>0.05	27.3±24.02	28.28±23.88	>0.05
IRI	3.12±0.33	3.11±0.25	>0.05	2.79±0.33	2.83±0.29	>0.05
TG	215.99±139.66	236.77±199.23	>0.05	108.72±50.54	116.72±73.63	>0.05
HDL-C	50.20±18.44	43.68±15.3	>0.05	54.72±14.52	52.55±14.66	>0.05
SBP	155.97±20.92	153.83±18.79	>0.05	132.88±22.69	130.50±17.4	>0.05
TBP	90.96±11.76	88.55±10.54	>0.05	78.72±1.82	79.34±9.7	>0.05

*Student's t-test

syndrome (18). Abdominal obesity, easily assessed using waist circumference is a prerequisite factor for the diagnosis of the syndrome in the new definition. On the other hand, insulin resistance, which is difficult to measure in the clinical practice, is not an essential requirement. This was in agreement with our previous results, that have shown that insulin resistance is not an obligatory element of the syndrome, it is frequently absent in patients with the complete phenotype, and it is sometimes present in persons with no signs of the metabolic syndrome (18).

As published elsewhere, the role of family history and lifestyle factors in the development of the syndrome sustain multifactorial inheritance (19). The hereditary component is probably comprised of a polygenic system, and those genes seem the best candidates that influence energy uptake and expenditure and lipid metabolism.

PPARG helps translating "*what you eat*" into "*what you are*", as their natural ligands, the alimentary fatty acids exercise their effect on gene expression through this molecule. PPARG is involved in the process of adypocyte differentiation, but receptor activation also increases the oxydated LDL uptake in the foamy cells, and so it is thought to be involved in the process of atherogenesis. Besides the metabolic effects, the gene product influences cell differentiation and monocyte biology intervening in the processes of carcinogenesis, inflammation and immunity (10). So the PPARG2 gene is an ideal candidate for the phenotype described by the metabolic syndrome.

The results concerning the frequently studied Pro12Ala polymorphism of the gene are very controversial, even in different populations of Caucasian origin. It was first reported in a Finnish population that the reduction of BMI and a better insulin sensitivity associates with the substitution (20). However, other authors have reported that obesity in the presence of the Ala12Ala genotype was more important (21). Though frequently studied, by the initiation of our studies we found no

published data about the PPARG2 gene and its Pro12Ala polymorphism in Romania. The allele frequencies found in our study are within the limits reported by other authors in various Caucasian populations.

Concerning the relationship of the different genotypes with the metabolic parameters, Masud and Ye performed a meta-analysis using data from 30 independent studies, and found that the Ala12 homozygotes had significantly higher BMI than heterozygotes and Pro12 homozygotes, which supported the hypothesis that the Pro12Ala polymorphism is a genetic modifier of obesity and the recessive model for the Ala12 allele (22). Kim *et al*. examined the effects of the Pro12Ala polymorphism on body fat in Korean females, and found that body weight, fat mass, fat percentage, BMI and WHR were significantly higher in individuals with the PA or AA genotype than those with PP. Among overweight individuals, PA/AA was associated with significantly higher abdominal subcutaneous fat, abdominal visceral fat, and subcutaneous upper and lower thigh fat. Serum lipid profiles and glucose showed no association (23). Contrary to our expectations based on the initial reports in other populations, our results confirm these observations. The Ala12 variant is associated with significant differences in the control group (and $p = 0.08$ in the metabolic syndrome group), being associated with the increase of body weight and waist circumference. However, maybe due to the different metabolic activity the syndrome does not develop.

The relationship with the complete phenotype of the metabolic syndrome was researched in the Danish population in the MONICA study (24). Allele frequency of Ala12 was 12.6% in the presence of the metabolic syndrome and 14.2% in its absence. Allele frequency was higher in the our study groups of subjects with or without the metabolic syndrome diagnosed according to the IDF recommendations; the observed values 13.76 vs. 19.17% were closer to those found by Mancini et al. in type 2 diabetes (13%) vs. healthy controls (19.6%) (25).

The presence of the PP genotype vs. the PA and AA genotypes determines a modest but significant risk to the development of the metabolic syndrome. We have considered together the Ala homo- and heterozygotes as done in the other studies due to the low frequency of the AA genotype. This approach however, may be arguable if the Ala12 allele is recessive as considered by some authors and if complete dominance characterizes the Pro12 allele. From this point of view the results are inconclusive, especially in the absence of published data regarding the allele frequency in our population. Studies carried out on larger samples, eventually stratified by sex, ethnicity and body weight would be necessary to elucidate this topic.

To explain the controversial results obtained in the different studies, a couple of meta-analyses have been done, usually following the relationship of the polymorphism with type 2 diabetes, and not the metabolic syndrome. After evaluating 16 studies, Altshuler *et al*. suggested that the Pro12 allele occurs at such high frequency that the modest effect may translate into a large population-attributable risk which may influence as much as 25% of the cases in the general population (26). Ardlie *et al.* examined 4 case-control samples and their results provided insight into the factors affecting the replication of association studies. They

suggested that carefully matched, moderate-sized case-control samples in cosmopolitan U.S. and European populations are unlikely to contain levels of structure that would result in significantly inflated numbers of false-positive associations (27). Deficiencies related to the stratification of the studied populations, sample size, allele frequency differences, the minor effect of the polymorphism and its interactions with other genes and extrinsic factors, the recessive nature of the Ala12 gene, the multiple effect on the various components of the syndrome, may explain the inconsistencies and, sometimes, contradictory results of the published data. Some of these factors may, as well, influence our results.

In conclusion, it seems that the PP genotype contributes with a modest effect to the development of the metabolic syndrome phenotype defined according to the IDF criteria in the studied Romanian population, and although the presence of the Ala12 allele associates with higher bodyweight, it exercises a protective effect against the metabolic abnormalities.

Acknowledgments: Herewith, we wish to thank for the grants obtained from the Illyés Foundation, the Pro Diabetologia Foundation and the Hungarian Hypertension Society that made the molecular investigations possible.

References

1. Cheal KL, Abbasi F, Lamendola C. Relationship to insulin resistance of the Adult Treatment Panel III diagnostic criteria for identification of the metabolic syndrome. Diabetes 2004; 53:1195-1200.
2. Jarrett RJ. Is the metabolic syndrome a relevant entity? International Diabetes Monitor 1998; 10:3.
3. Laaksonen DE, Lakka HM, Niskanen LK et al. Metabolic syndrome and development of diabetes mellitus: application and validation of recently suggested definitions of the metabolic syndrome in a prospective cohort study. Am J Epidemiol 2002; 156: 1070 -1077.
4. Maison P, Byrne CD, Hales CN, Day NE, Wareham NJ. Do different dimensions of the metabolic syndrome change together over time? Evidence supporting obesity as the central feature. Diabetes Care 2001; 24:1758-1763.
5. Meigs J. The metabolic syndrome. BMJ 2003; 327:61-62.
6. Reaven GM. Insulin resistance, cardiovascular disease, and the metabolic syndrome - How well do the emperor's clothes fit? Diabetes Care 2004; 27:1011-1012.
7. Alberti KGMM, Zimmet PZ. The WHO Consultation: Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus: provisional report of a WHO consultation. Diabet Med 1988; 15:539-553.
8. American College of Endocrinology Task Force on the Insulin Resistance Syndrome: American College of Endocrinology position statement on the insulin resistance syndrome. Endocr Pract 2003; 9:236-252.
9. Third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation and treatment of high blood cholesterol in adults (Adult Treatment Panel III). Final report. Circulation 2002; 24:3144-3421.
10. OMIM-Mendelian Inheritance in Man. (Accessed July 04, 2008, at site www3.ncbi.nlm. nih.gov/OMIM/).

11. Yen CJ, Beamer BA, Negri C, Silver K, Brown KA, Yarnall DP, Burns DK, Roth J, Shuldiner RA. Molecular scanning of the human peroxisome proliferator activated receptor gamma (hPPAR-gamma) gene in diabetic Caucasians: identification of a pro12ala PPAR-gamma-2 missense mutation. Biochem. Biophys. Res. Commun. 1997; 241:270-274.
12. Fukushima M, Taniguchi A, Sakai M et al. Assessment of insulin sensitivity from a single sample. Diabetes Care 2000; 23:1434-1435
13. Katz A, Nambi SS, Mather K et al. Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. J Clin Endocrinol Metab 2000; 85: 2402-2410
14. International Diabetes Federation. The IDF consensus worldwide definition of the metabolic syndrome. IDF, Brussels. 2005. (Accessed May 15, 2005, at site <http://www.idf.org/webdata/docs/IDF_Metasyndrome_definition.pdf>).
15. Hasstedt SJ, Ren QF, Teng K, Elbein SC. Effect of the PPARG2 Pro12Ala variant on obesity, glucose homeostasis and blood pressure in members of familial type 2 diabetic kindreds. J Clin Endocrinol Metab 2001; 86:536-541.
16. Kara K, Okada T, Tobe K. The Pro12Ala polymorphism in PPARG2 may confer resistance to type 2 diabetes. Biochemical and Biophysical Research Communications 2000; 271:212-216.
17. Oh EY, Min KM, Chung JH. Significance of Pro12Ala mutation in PPARG2 in Korean diabetic and obese subjects. J Clin Endocrinol Metab 2000; 85:1801-1804.
18. Csép K. Characterization of pathological modifications of metabolic syndrome patients and their relatives.(in Hungarian) Orvostudományi Értesítõ 2003; 3:543-548.
19. Csép K, Darkó Zs, Budán I, Pașcanu I, Mureșan C, Brassai Z. The effect of inherited and acquired factors on insulin sensitivity(in Hungarian). Orvostudományi Értesítõ 2005; 2(78):255-261.
20. Deeb SS, Fajas L, Nemoto M, Pihlajamaki J, Mykkanen L, Kuusisto J, Laakso M, Fujimoto W, Auwerx J. A pro12ala substitution in PPAR-gamma-2 associated with decreased receptor activity, lower body mass index and improved insulin sensitivity. Nature Genet. 1998; 20:284-287.
21. Valve R, Sivenius K, Miettinen R, Pihlajamaki J, Rissanen A, Deeb SS, Auwerx J, Uusitupa M, Laakso M. Two polymorphisms in the peroxisome proliferator-activated receptor-gamma gene are associated with severe overweight among obese women. J. Clin. Endocr. Metab. 1999; 84: 3708-3712.
22. Masud S, Ye S. Effect of the peroxisome proliferator activated receptor-gamma gene pro12ala variant on body mass index: a meta-analysis. J Med Genet 2003; 40:773-780.
23. Kim KS, Choi S M, Shin S U, Yang HS, Yoon Y. Effects of peroxisome proliferator-activated receptor-gamma-2 pro12ala polymorphism on body fat distribution in female Korean subjects. Metabolism 2004; 53:1538-1543.
24. Frederiksen L, Brodbaek K, Fenger M. Studies of the pro12-to-ala polymorphism of the PPAR-gamma gene in the Danish MONICA cohort: homozygosity of the ala allele confers a decreased risk of the insulin resistance syndrome. J Clin Endocr Metab 2002; 87:3989-3992.
25. Mancini FP, Vaccaro O, Sabatino L. Pro12Ala substitution in PPARG2 is not associated with type 2 diabetes. Diabetes 1999; 48:1466-1468.
26. Altshuler D, Hirschhorn JN, Klannemark M. The common PPAR-gamma pro12ala polymorphism is associated with decreased risk of type 2 diabetes. Nature Genet 2000; 26:76-80.
27. Ardlie K G, Lunetta KL, Seielstad M. Testing for population subdivision and association in four case-control studies. Am. J. Hum. Genet. 2002; 71: 304-311.