Genetic variants affecting lipid and glucose metabolism in obese pediatric population

Ph.D. thesis

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1. INTRODUCTION

As a consequence of obesity the disease germs occur in the early stages of life is increasingly being observed, therefore, it means a growing challenge to pediatricians as well. The WHO (World Health Organization) based on surveys conducted in 2004 throughout the world, estimates that about 22 million children in five years are overweight or obese. Extensive epidemiological studies have shown that in Europe, America and Asia, the prevalence of childhood overweight and obesity is estimated from 10 to 20%. In Hungary, the prevalence of obesity among school children between the 1980s and 1990s, reached 15%. According to the literature, associated risk factors may be present at the 58% of obese children (high blood lipid levels, high blood pressure or insulin resistance). For the majority of cases of primary (exogenous) obesity can be said that obesity is a complex, heterogeneous condition in which both environmental and genetic factors play an important role. Twin studies show that 50-90% of the inherited factors play a role in the development of adipose tissue. Recently, approximately 200 candidate (susceptibility) gene locus or chromosome region, located on 12 different chromosomes, were found to be in relation with obesity.

1.1. The association of the pediatric obesity with cardiovascular diseases (CVD)

Data in literature show that pediatric obesity plays a critical role in early development of atherosclerosis and metabolic syndrome (MS), which significantly increases the risk of non-insulin-dependent diabetes mellitus and cardiovascular diseases. In several studies cardiovascular risk factors can be also detected in obese children such as dyslipidaemia (elevated triglycerides and cholesterol concentrations), hypertension, glucose intolerance and type 2 diabetes (T2DM). The co-occurrence of these factors is characterized at the onset of MS. In Hungarian epidemiological studies the disease was detected in 8.9% of the obese adolescents at 8-18. The onset of MS in childhood is not only a problem of adverse prognostic for cardiovascular disease risk, but it generates adverse symptoms for present, such as reduced exercise capacity. T2DM is a classic example of a complex disease as environmental and genetic factors as well as interactions among these factors all contribute to disease development. The appearance of T2DM in children and adolescents is the negative trend of obesity. The disease affects carbohydrate, lipid and even protein and nucleic acid metabolism as well. In recent years, in some child and adolescent populations cases of T2DM have epidemically increased, the proportion of new cases is close to 50%.

1.2. Molecular Genetic Background

Various studies have shown that 30-80% of the variability of body weight is genetically determined. The most important genetic factors are the gene defects coding proteins involved in lipid and glucose metabolism, which cause an abnormal metabolism and consequential resulted in cardiovascular disease.

1.2.1. The apolipoprotein A5 gene (*APOA5*)

The *APOA1-C3-A4-A5* gene cluster located on 11 chromosome contains at least three genes affecting the plasma lipoprotein metabolism. Newest member of the apolipoprotein cluster, the apolipoprotein A5 (*APOA5*) gene was reported in 2001 by comparative sequencing of mouse and human genome. Several studies analyzed the naturally occurring variants of the *APOA5* gene [*T-1131C* (rs662799), *IVS3+G476A* (rs2072560), *T1259C* (rs2266788) and *C56G* (rs3135506)], and their association with increased triglyceride concentrations in different normal and pathologic adult populations has already been established. The investigations of the *APOA5* gene revealed that the haplogroups determined by the natural variants in gene have more important and complex implications for the lipid parameters. Haplotype analysis in a healthy, adult Caucasian population showed, that the *APOA5*2* (containing the minor allele of *T-1131C, IVS3+G476A* and *T1259C* SNPs) confers risk for different cardiovascular diseases. In addition, in our previous study of adult MS the *APOA5*2* haplotype was found to exclusively confer risk for metabolic syndrome.

1.2.2. The gene of glucokinase regulator enzyme (*GCKR*)

The *GCKR* gene is located on chromosome 2p23.3-p23.2, consists of 19 exons, and encodes a protein of 625 amino acids. The glucokinase regulator protein (GCKR) regulates the function of glucokinase (GCK), the key enzyme of liver. The glucokinase enzyme has a central role in maintaining blood glucose homeostasis and catalyze the phosphorylation of glucose in hepatocytes and pancreatic cells. More data in the literature show that the *GCKR* gene has inverse effect on the glucose and triglyceride levels, according to which the indirect effect of certain polymorphisms in the gene causes elevated triglyceride levels and reduced glucose concentrations. The two most commonly studied GCKR gene polymorphisms are the intronic rs780094 (intron 16) and the rs1260326 exonic (1337) (exon 15) variant. Genome-

wide association studies (GWAS) directed to T2DM revealed that two polymorphisms in *GCKR* gene are associated with elevated triglyceride levels, lower fasting glucose rates and decreased insulin resistance. In addition, the rs780094 A and rs1260326 T alleles were shown to be associated with a decreased risk for T2DM.

2. AIMS

Analyze of genetic variants affecting lipid and glucose metabolism that have association with obesity.

1. One of the main subject of my study was the analysis of *APOA5* gene that is in association with MS and hypertriglyceridemia. My goal was to investigate the distribution of the naturally occurring variants of this gene (*T-1131C, C56G, IVS3+G476A, T1259C*) and the prevalence of haplogroups determined by these variants (*APOA5*1, APOA5*2, APOA5*3, APOA5*4, APOA5*5*) in Hungarian obese and healthy, lean children.

2. Furthermore I examined the effects of alleles (*T-1131C, C56G, IVS3+G476A, T1259C*) and haplogroups in *APOA5* gene for triglyceride- and cholesterol parameters in the patients and controls.

3. My observations were focused on the possible susceptibility risk effect of minor alleles and of haplogroups in *APOA5* gene for development of obesity in pediatric population.

4. The other main object of my study was the investigation of two natural occuring variants in the gene of glucokinase regulatory protein (*GCKR*), as an important key in glucose metabolism, directed to their allelefrequencies (rs1260326, rs780094).

5. Further observations were carried out to ascertain if the two variants in *GCKR* gene have inverse effect on triglyceride and glucose parameters in obese children similar to results of adult studies.

6. Finally, the aim of the study was to detect if the rs1260326 and rs780094 variants have any relationship with the development of obesity in children.

3. MATERIALS AND METHODS

3.1. Study population

For the *APOA5* gene, the investigations were carried out in 232 obese children (138 boys, 94 girls, age: 13.7 ± 0.16 years, BMI: 31.8 ± 0.34 kg/m^2), and in 137 healthy children with normal weight as controls (58 boys, 79 girls, age: 14.8 ± 0.19 years, BMI: 20.0 ± 0.25 kg/m^2). For the *GCKR* gene, 221 obese children (122 boys, 99 girls, age: 13.5±0.16 years, body mass index (BMI): 31.5±0.32 kg/m^2), and 115 healthy children with normal weight as controls (56 boys, 59 girls, age: 14.1±0.21 years, BMI: 20.2±0.32 kg/m^2) were examined. During the study some of DNA samples ran out, which explains the different sample sizes in the cases of the two polymorphisms studied. The subjects were included in the study after the exclusion of chronic diseases, endocrinological, nutritional, growth and renal diseases, or obesity syndromes. None of them were taking any kind of medication either in the obese or control groups. The DNA with the clinical dataset from the patients was deposited into the local biobank. The local biobank was established with the authorization of National Ethics Committee.

3.2. Anthropometric measurements and clinical parameters

Anthropometric measurements were carried out by the same investigator in the survey unit. Body height was measured to the nearest 0.1 cm by a Holtain stadiometer, while weight was obtained to the nearest 0.1 kg on a standard beam scale. BMI was calculated according to the formula: real weight (kg) divided by squared height (m^2). We considered children to be obese if their BMI exceeded the value corresponding to 25 at the age of 18 years as suggested by Cole et al. The triglycerides and total cholesterol parameters were measured by Roche Modular automatic system (Hoffmann-La Roche Ltd, Basel, Switzerland). The hypertriglyceridemia (HTG) in patients and controls was defined at TG level ≥1.1 mmol/l (de Ferranti at al).

3.3. Genetic analysis

Genomic DNA was extracted from peripheral blood samples anticoagulated by EDTA with a routine salting out method. The starting point for DNA analysis was the amplification performed by polymerase chain reaction (PCR) which needs sequence-specific, synthetic oligonucleotide primers, Taq polymerase, dNTP, reaction buffer, and the genomic DNA template. The amplification was in a final volume of 50 µl including 5 µl reaction buffer (500 mM KCl, 14 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0), 1 µl 50 mM $MgCl_2$, 0.2 mM of each dNTP, 1 U of Taq polymerase, 0.2 mM of the adequate specific primer pairs and 1 µg DNA sample. The amplifications were executed using an MJ Research PTC-200 thermal cycler (Bio-Rad, Hercules, CA, USA). The analysis of PCR products was performed by gel-electrophoresis, etidhium-bromid dyeing and was visualized by UV transillumination. After the amplification we used RFLP method to analyze the polymorphisms in different genes (*APOA5, GCKR*). During this method the PCR products were digested with restriction endonuclease enzymes. All methods were designed to have an obligatory cleavage site for the restriction enzyme to control the efficacy of the enzymatic digestion.

3.4.Statistical analysis

All statistical calculations were executed using the SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). All data are represented as means ± SEM. Mann-Whitney U-test was used to compare quantitative data between obese patients and the controls (*APOA5, GCKR*). The chi-square test was employed to compare the allele and haplotype frequencies. To compare metabolites data between individual polymorphisms (*APOA5, GCKR*) and haplotype cohorts (*APOA5*) we applied one-way ANOVA test. Odds ratios were calculated by multivariate logistic regression analysis to estimate the possible risk influence of polymorhisms (*APOA5, GCKR*) and haplotypes (*APOA5*) on the development of obesity in children. The p value ≤ 0.05 was considered statistically significant.

4. RESULTS

4.1. The analysis of apolipoprotein A5 gene (*APOA5*)

During the investigation of association betwween APOA5 variants and triglyceride levels I revealed that with except of the *C56G* polymorphism, all the minor alleles *(-1131C, IVS3+476A, 1259C)* were associated with increased triglyceride levels both in obese children, and in controls comparing individuals with normal and carrier genotypes. For BMI and total serum cholesterol levels, no variant-dependent changes could be detected. The prevalence of *–1131C* allelic variant was significantly elevated in obese pediatric patients as compared to healthy, normal weight children. The regression analysis revealed, that the *–1131C* minor allele alone confers an independent risk for development of pediatric obesity. Results of haplotype analysis on lipid parameters showed that, carrying *APOA5*2* and *APOA5*4* haplotype variants resulted in a significant increase in serum triglyceride levels in both cohorts. In contrast, the *APOA5*5* haplotype had a serum triglyceride level-decreasing effect. Besides, no haplotype-dependent changes were detected in BMI and total serum cholesterol levels concerning all studied haplogroups. The prevalence of the *APOA5*2* was about 2.59-fold increased in obese children, while the accumulation of the *APOA5*5* displayed 3.88-fold decrease in obese subjects compared to healthy, normal weight children. The logistic regression model showed, that the *APOA5*2* haplotype confers significant risk for the development of obesity in pediatric patients (OR: 2.87, CI: (1.29-6.37), p=0.01), in contrary, the *APOA5*5* haplotype can behave as a protective factor against obesity in pediatric patients (OR: 0.25, CI: (0.08-0.83), p=0.02).

4.2. The examination of glucokinase regulator gene (*GCKR*)

Previously, the rs780094 was demonstrated to be in strong linkage disequilibrium (LD) with the other non-synonymous *GCKR* variant, rs1260326, a finding we could also confirm in our examined pediatric population (LD; r^2=0.94) (Figure 1.). During the examination of individual *GCKR* variants, the minor allele carriers of both SNPs (rs1260326T and rs780094A) showed elevated triglyceride, and lowered fasting plasma glucose concentrations compared to the non-carriers in obese patients and in controls as well. We did not observe difference in allele frequencies between obese patients and controls, but the prevalence of carriers (heterozygous and homozygous subjects together) of each variants were significantly decreased in obese children compared to the healthy, lean children. The results of logistic regression models revealed that carriership for both of the *GCKR* rs780094 (GA+AA) and rs1260326 (CT+TT) minor alleles can protect against obesity in children (rs780094 A: OR: 0.49, CI: (0.28-0.84), p=0.010; rs1260326 T: OR: 0.56, CI: (0.34-1.01), p=0.035).

When the all study subjects and obese children were analyzed separately for HTG, we observed a higher frequency of the minor allele and increased prevalence of carriers of two *GCKR* variants in HTG groups comparing to NTG groups both in all subjects and in obese children. The results of logistic regression analysis showed a strong correlation of carriers (CT+TT rs1260326, GA+AA rs780094) with HTG both in obese group and in all study subjects compared to samples with NTG; but it seems that the correlation was stronger in the obese group.

1. Figure. The LD-block of two *GCKR* SNPs.

5. DISCUSSION

Obesity and insulin resistance play a central role in development of MS, one of the major public-health problems worldwide. Childhood obesity increases the risk of metabolic syndrome development in adulthood, which implies a significant role of prevention and treatment of childhood obesity in reducing risk of MS in adulthood. Elevated blood triglyceride levels also included to the criteria of MS. Therefore, the apolipoprotein-coding *APOA5* gene, which was identified as a significant determinant of plasma triglyceride levels both in human and mice, has been extensively studied in adult MS patients. The *APOA5* susceptibility variants have not been evaluated in obese children but by one Japanese study. Therefore we chose to investigate the frequencies and distributions of *APOA5* functional variants, their effect on lipid parameters and their correlation with childhood obesity in a group of pediatric patients. In agreement with results of Endo and colleagues we found an association between the *APOA5* promoter region polymorphism *-1131C* and elevated triglyceride levels in obese Hungarian Caucasian children. In addition, our results show that the triglyceride-increasing effect of *APOA5, IVS3+476A* and *1259C* minor variants are also present in obese children in agreement with results of adult studies reported. Limited data are also available about the major naturally occuring *APOA5* haplogroups in adults due to some recent studies, but we were the first who performed haplotype analysis in obese children. Our data presented here show, that both in obese children and in the control cohorts the *APOA5*2* (*-1131C, IVS3+476A, 1259C)* and *APOA5*4* (*T-1131C* alone) haplogroups are associated with elevated triglyceride levels compared to the cohort having *APOA5*1* haplotype. Additionally, multivariate logistic regression analysis revealed a susceptibility nature of the *APOA5*2* haplotype for the development of obesity in children. Consequently, our results suggest, that contrary to the current belief, the *-1131C* minor allele can contribute to disease susceptibility only in combination with *IVS3+476A* and *1259C* alleles. Moreover, our results also suggested a protective effect of the rare *APOA5*5* haplotype against obesity in pediatric groups similarly to findings reported for an adult MS population. Summarizing our results we conclude, that the risk effect of *T-1131C* allele of *APOA5* is restricted to the *APOA5*2* haplotype (*–1131C, IVS3+476A* and *1259C* minor alleles). The *APOA5*2* and *APOA5*4* haplotype variants were found to be in association with elevated triglycerides, by contrast, the *APOA5*5* haplotype had a serum triglyceride level-decreasing effect and was found to have a protective role against obesity. However further examinations in other independent population samples are needed to replicate our findings.

Genome-wide association studies executed in recent years (2007, 2008) revealed that rs780094 and rs1260326 polymorphisms at the *GCKR* locus are associated with decreased fasting glucose and insulin concentrations and with elevated triglyceride levels. In addition, results of several studies in literature showed that rs780094 A and rs1260326 T alleles were shown to be associated with a decreased risk for T2DM. The aim of my work was to investigate the possible associations of *GCKR* gene variants rs780094 and rs1260326 with triglyceride and fasting glucose levels, and to study their allele distributions in Hungarian obese pediatric patients and controls. My results of present study carried out in pediatric population confirmed the inverse effect of both rs780094 and rs1260326 functional *GCKR* gene variants on serum triglyceride and plasma glucose levels similar to data in the literature. The prevalence of carriers for both SNPs were significantly decreased in obese pediatric patients compared to the healthy, lean children, although, the minor allele frequencies did not show significant differences between the patient and control groups. In my study, using logistic regression model, I detected that the carrier form of both *GCKR* variants confer lower risk for development of obesity in this population, partly in agreement with results of a Chinese study evaluating the rs780094 polymorphism in adults. Based on the association of pediatric obesity and risk for development of T2D, our findings suggest that the functional minor alleles of the *GCKR* gene protect against the development of pediatric obesity, and as a consequence show a protective effect against development of T2D and metabolic syndrome in adults. Further examinations in other large pediatric population samples are needed to confirm our findings of GCKR gene study.

6. CONCLUSION

1. We conclude that with except of the *C56G* polymorphism, all the minor alleles *(-1131C, IVS3+476A, 1259C)* of *APOA5* variants were associated with increased triglyceride levels both in obese children and in controls as well.

2. During the analysis of the same population, we revealed that the *–1131C* minor allele alone confers an independent risk for development of pediatric obesity.

3. Based on results of haplotype-analysis, we can conclude that carrying of *APOA5*2* and *APOA5*4* haplogroup variants results in elevated triglycerid levels in both patients and controls.

4. After association-analysis between haplogroups and obesity we revealed a susceptibility nature of the *APOA5*2* haplotype for the development of obesity in children, by contrast, the *APOA5*5* haplotype with low prevalence was found to have a protective role against obesity similar to results in our previous adult study with MS.

5. During the investigation of *GCKR* functional variants we detected that both of rs780094 and rs1260326 SNPs have inverse effect on serum triglyceride and plasma glucose levels. Children carrying rs780094A, or rs1260326T variants showed elevated triglycerid and decreased glucose-concentrations.

6. Results detected from analysis of relationship between *GCKR* functional variants and obesity revealed that carrying of each variants reduce risk for obesity despite the elevated triglyceride levels.

7. LIST OF PUBLICATIONS

7.1. The thesis is based on the following publications

1. **Horvatovich K**, Bokor Sz, Barath A, Maasz A, Kisfali P, Jaromi L, Polgar N, Toth D, Repasy J Endreffy E, Molnar D, Melegh D, Haplotype analysis of the apolipoprotein A5 gene in obese pediatric patients. Int J Pediatr Obes. 2010 septembre 30.IF: 3.53

2. **Horvatovich K,** Bokor Sz, Polgar N, Kisfali P, Hadarits F, Jaromi L, Csongei V, Repasy J, Molnar D, Melegh B, Functional GCKR gene variants have inverse effects on triglyceride and glucose levels and decreases the risk of obesity in children. Diab Met. 2011 IF: 2.43 (accepted for publishing).

7.2. Other publications

1. Maász A, **Horvatovich K**, Magyari L, Talián C G, Bokor S, Laczy B, Tamaskó M,Molnár D, Wittmann I, Melegh B. Search for mitochondrial DNA T4291Cmutation in Hungarian patients with metabolic syndrome. Orv Hetil.2006;147(15):693-696.

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 suggests susceptibility for small-vessel-associated ischemic stroke. Neuromolecular Med. 2006;8(3):353-60. IF: 2.631

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7. Kisfali P, Mohás M, Maasz A, Hadarits F, Markó L, **Horvatovich K,** Oroszlán T, Bagosi Z, Bujtor Z, Gasztonyi B, Wittmann I, Melegh B., Apolipoprotein A5 IVS3+476A allelic variant associates with increased trigliceride levels and confers risk for development of metabolic syndrome in Hungarians.Circ J. 2008 Jan;72(1):40-3. 2.387.

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7.3. Abstracts

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