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NEW TRENDS IN THE BIOLOGICAL AND ECOLOGICAL RESEARCH

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PREFACE

The proccedings from the scientific conference with are part of the activity 2.3 „Dissemination of research and development centre for professional public“ within the project: „Completion of Excellence Centre of Animal and Human Ecology with emphasis on improving the quality of scientific research – the 2nd stage“ (ITMS 26220120041)

University of Prešov significantly affects the provision and conditions of education, research and development in eastern Slovakia. Today, it is a modern dynamically developing research and educational institution equipped with modern information--communication systems.

Regional development is an important condition for economic and social stability of the country. In Slovakia, great imbalance of individual regions constitutes a problem. I do not intend to present socio-economic profile of the Prešov region. My goal is rather to think about the status of science and research in relation to the strategy of the region.

Expenditure on research and development in the Prešov region in 2010 amounted to 11,588,983 €, which is almost 4,5 times less than in the Košice region, and about 18 times less than in the Bratislava region.

One way of addressing these disparities is the utilization of resources from Operational Programme Research and Development of EU Structural Funds. In May 2009, the Excellence Centre of Animal and Human Ecology was established under Priority axis: 2. Support to research and development, measure: 2.1 Support of networks of excellence in research and development as the pillars of regional development and support to international cooperation, with nonrepayable financial contribution 1,312,233.69 €.

In October 2009, the project of University of Presov „*The use of research and development for breeding new cultivars (prototypes) of medicinal plants and their varietal registration*” was approved under Priority axis: 2. Support to research and development, measure: 2.2 Transfer of knowledge and technology from research and development into practice, with nonrepayable financial contribution 428,508.22 €.

In 2010, the implementation of the project “Completion of Excellence Centre of Animal and Human Ecology with emphasis on improving the quality of scientific research – the 2nd stage” started under Priority axis: 2. Support to research and development, measure: 2.1 Support of networks of excellence in research and development as the pillars of regional development and support to international cooperation, with nonrepayable financial contribution 2,773,548.52 €, which is up to twice of the budget of stage I of building the centre. The aim of this project is the creation of new laboratories - Laboratory of Sequencing Analysis I and II, Environmental Biotechnology Laboratory I and II, Laboratory of Animal Biodiversity and Laboratory of ICT.

Official opening of the new laboratories of the Excellence Centre is planned in April 2013. A part of the project is also today’s presentation of the scientific results

obtained using the cutting-edge technology of Excellence Centre of Animal and Human Ecology - 2nd Stage.

On behalf of the editor-in-chief and editorial board I can recommend readers all articles. Most of them are first works and scientific results which can contribute to present Centre of Excellence.

Prof. Dr. Ivan Bernasovský
Director of the Excellence Centre of Animal and Human Ecology

UTILIZATION OF REAL-TIME PCR IN PREDICTION OF THE RISK OF OSTEOPOROSIS

Iveta BOROŇOVÁ – Jarmila BERNASOVSKÁ – Ivan BERNASOVSKÝ – Soňa MAČEKOVÁ – Ján KĽOC – Zlatica TOMKOVÁ – Janka PORÁČOVÁ – Marta BLAŠČÁKOVÁ – Eva PETREJČÍKOVÁ

Abstract: *Osteoporosis is a common multifactorial disease with a strong genetic component characterized by reduced bone mass and increased risk of fractures. Genetic factors play an important role in the pathogenesis of osteoporosis. Current research in genetics of osteoporosis is focused on identification of responsible genes and polymorphisms. Osteoprotegerin (OPG) gene is considered as an important candidate gene of osteoporosis playing a key role in the biological characteristics of bone. A total of 160 unrelated postmenopausal women with diagnosed osteoporosis and 160 normal controls were genotyped for 163A/G and 245T/G polymorphisms. Genomic DNA was isolated from peripheral blood leukocytes using standard methodology. Real-time PCR allelic discrimination TaqMan assay was used for genotyping analyses. Hardy-Weinberg equilibrium was tested for each SNP in groups of participants using the chi-square test. The distribution of investigated genotypes in the survey of patients with osteoporosis were as follows: AA (68.1%), AG (31.3%), GG (0.6%) for A163G polymorphism; TT (86.3%), TG (12.5%), GG (1.3%) for T245G polymorphism. In A163G polymorphism the variant G allele was more common among patients with osteoporosis: 16% versus 14% in normal controls. In T245G polymorphism the phenomenon of more frequent occurrence of G allele in the group of patients with osteoporosis was not observed. Genotype distribution in the studied populations was in Hardy-Weinberg equilibrium. Genotypes and alleles frequencies showed no significant differences. Our results represents an initial study, further studies on larger numbers of samples and associations studies will be carried out. Knowing the distribution of genotypes is important for assessing the impact of these polymorphisms on various parameters associated with osteoporosis. Screening for the identification of "at-risk" women likely to develop osteoporosis and initiating subsequent early intervention appears to be most effective strategy to substantially reduce the risks of osteoporosis.*

Key words: *osteoporosis, real-time pcr, A163G polymorphism, T245G polymorphism*

Osteoporosis is a common multifactorial disease with a strong genetic component characterized by reduced bone mass and increased risk of fractures. Osteoporosis is characterized by a combination of low bone mass and deteriorated microarchitecture

of the bone (Ling-xia et al., 2011). Bone mass is determined by interaction of genetic, metabolic, and environmental factors. Genetic factors play an important role in the pathogenesis of osteoporosis. Genetic factors have been shown to be responsible for 40-75% of the individual variation (Langdahl et al., 2002). The genetic of osteoporosis represents one of the most active areas for research in bone biology. Osteoprotegerin, vitamin D receptor, estrogen receptor alpha and LDL receptor-related protein 5 genes have been the most frequently analysed osteoporosis-related candidate genes. DNA sequence variations might contribute to bone quality in part independently of BMD (Zajíčková, Žofková, 2007). Due to its important role in bone biology, TNFRSF11B gene, coding for OPG, has been considered as a candidate gene for osteoporosis. It plays a crucial role in the control of bone resorption and its gene could be a good candidate gene for osteoporosis. Numerous TNFRSF11B gene polymorphisms have been studied in the association studies (Langdahl et al., 2002; Husted et al., 2010) (Figure 1).

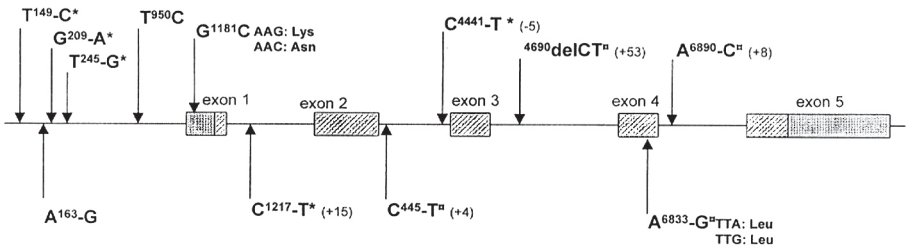

Figure 1 Gene structure and polymorphisms in the OPG gene (Langdhal et al., 2002)

As promoter polymorphisms could alter gene expression, we focused on analyses of A163G and T245G TNFRSF11B promoter gene polymorphisms in Slovak postmenopausal women.

Material and methods

One hundred sixty DNA samples from Slovak osteoporotic postmenopausal women and one hundred sixty normal controls were screened for sequence variations in the promoter region of OPG gene. Each patient was examined clinically and routine biochemical tests to exclude systematic and metabolic bone diseases other than primary osteoporosis were performed. Osteoporosis was defined according to the World Health organization criteria (WHO, 1994).

Genomic deoxyribonucleic acid (DNA) was isolated from peripheral blood leukocytes using standard methodology. Real-time PCR allelic discrimination TaqMan assay was used for genotyping analyses of A163G (rs3102735) and T245G

(rs3134069) in the promoter region of TNFRSF11B gene. Hardy-Weinberg equilibrium was tested for each SNP in groups of participants using the chi-square test. The study was approved by the ethical committee and informed consent was obtained from all patients participating in the study.

Results

The distribution of investigated genotypes in the survey of osteoporotic postmenopausal women were as follows: AA (68.1%), AG (31.3%), GG (0.6%) for A163G polymorphism; TT (86.3%), TG (12.5%), GG (1.3%) for T245G polymorphism. The distribution of genotypes in normal controls were follows: AA (73.1%), AG (25.0%), GG (1.9%) for A163G polymorphism; TT (81.3%), TG (18.1%), GG (0.6%) for T245G polymorphism (Table 1, Table 2).

Table 1 A163G polymorphism in the OPG gene: genotypes and alleles frequencies in a cohort of 160 osteoporotic postmenopausal women and normal controls

		Osteoporotic postmenopausal women		*Normal controls*		χ^2	p
Genotype distribution	AA	109	68.1%	117	73.1%	$\chi^2 = 2.326$	p = 0.313
	AG	50	31.3%	40	25.0%		
	GG	1	0.6%	3	1.9%		
Allele frequency	A	84%		86%		$\chi^2 = 0.39$	p = 0.532
	G	16%		14%			

Table 2 T245G polymorphism in the OPG gene: genotypes and alleles frequencies in a cohort of 160 osteoporotic postmenopausal women and normal controls

		Osteoporotic postmenopausal women		*Normal controls*		χ^2	p
Genotype distribution	TT	138	86.3%	130	81.3%	$\chi^2 = 3.889$	p = 0.143
	TG	20	12.5%	29	18.1%		
	GG	2	1.3%	1	0.6%		
Allele frequency	A	93%		90%		$\chi^2 = 0.97$	p = 0.323
	G	7%		10%			

In A163G polymorphism the variant G allele was more common among osteoporotic postmenopausal women than in normal controls (16% versus 14%). Genotypes and alleles frequencies in analysed surveys showed no significant differences. In T245G polymorphism the phenomenon of more frequent occurrence of G allele in the group of patients with osteoporosis was not observed. The genotypes and alleles frequencies of T245G polymorphism in the promoter among patients with osteoporosis and normal controls showed no significant differences.

Discussion

OPG gene is an important candidate gene in the pathogenesis of osteoporosis. Some polymorphisms in the OPG gene had been reported to be associated with osteoporotic fractures. The promoter region of the human OPG gene contains various binding sites that may mediate the stimulation of OPG gene expression by different calciotropic factors. Polymorphisms in this region may contribute to the genetic regulation of bone mass as suggested by several recent publications (Ling-xia et al., 2011).

The present study investigated the prevalence of informative A163G and T245G polymorphisms which located in the promoter region. Our data from Slovak postmenopausal women has shown that in A163G polymorphism the variant G allele was more common among patients with osteoporosis. In T245G polymorphism the phenomenon of more frequent occurrence of G allele in the group of patients with osteoporosis was not observed. Genotypes and alleles frequencies of A163G and T245G polymorphism showed no significant differences.

The results of the present study are compatible with results of others study (Langdahl et al., 2002, Zajíčková et al., 2008). In the study of Ling-xia et al. (2011) the G allele of A163G polymorphism was significantly more frequent among patients with osteoporosis (12.3%) than in normal individuals (6.5%) (Ling-xia et al., 2011). Also, in a study of Langdahl et al. (2002) genotypes with the rare G allele A163G and T245G have significantly prevailed in patients with vertebral fractures in comparison with controls (Zajíčková, Žofková, 2007). These findings implicate that OPG polymorphisms might be associated with bone quality parameters. Several recent publications have addressed the hypothesis that polymorphisms in the regulatory region at the 5′end of the OPG gene may contribute to the genetic regulation of various bone phenotypes (Zajíčková, Žofková, 2007, Mencej-Bedrač et al., 2007). Furthermore, several studies demonstrated that through gene-gene interactions, OPG gene affects bone mineral density (BMD) together with some others genes, like vitamin D receptor gene and TNF superfamily member 11 genes (Mencej-Bedrač et al., 2009).

The importance of TNFRSF11B gene as a candidate gene for the development of osteoporosis has been confirmed in several different studies. In our study, we did not fully cover all genetic variation in the TNFSF11B gene, since only two common

SNPs were studied. Our results represent an initial study, further investigations on different and larger populations, interaction with other genes and association's studies will be carried out. The genetic effects of the TNFRSF11B gene SNPs need further functional and clinical confirmation.

Conclusion

Knowing the distribution of genotypes is important for assessing the impact of these polymorphisms on various parameters associated with osteoporosis. Screening for the identification of "at-risk" women likely to develop osteoporosis and initiating subsequent early intervention appears to be most effective strategy to substantially reduce the risks of osteoporosis.

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DETECTION OF C677T POLYMORPHISM IN THE MTHFR GENE AND ITS ASSOCIATION WITH FETAL LOSS

Zuzana DRAGUNOVÁ – Alexandra BÔŽIKOVÁ – Katarína ŠKOVRANOVÁ – Jarmila BERNASOVSKÁ

Abstract: *Thrombophilia represents a heterogeneous group of inherited or acquired disorders of haemostasis, which are pathophysiologically associated with an increased tendency to coagulation and predispose the patient to the occurrence or recurrence of thrombosis. They are one of the causes of thromboembolic disease during gestation, significantly contributing to the pathogenesis of preeclampsia, intrauterine death and fetal growth restriction, placental abruption and recurrent pregnancy loss. Inherited forms of thrombophilia are often caused by the mutations of genes encoding factor V, prothrombin, protein C, protein S and antithrombin. Supposedly, there is an association of certain pregnancy complications and C677T polymorphism in the MTHFR gene. The risk of pregnancy complications and fetal loss may be associated with increased levels of homocysteine (hyperhomocysteinemia) which is in association with studied polymorphism. We used TaqMan SNP Genotyping analysis using real-time PCR method for detection of mutation C677T in the MTHFR gene. Studied group consisted of 115 women, including 39 women who have undergone at least one fetal loss during pregnancy, the remaining 76 women had no pregnancy complications and no fetal loss. We have not found a statistically significant increase in the incidence of a genetic predisposition to thrombophilia caused by C677T mutation in the group of women with a history of abortions when compared to women with no record of abortion.*

Key words: *trombophilia, hyperhomocysteinemi, pregnancy complications, fetal loss, mthfr c677t*

Thrombophilic states are a heterogeneous group of inherited or acquired disorders of hemostasis, which are pathophysiologically associated with an increased tendency to coagulation and predispose the patient to the occurrence or recurrence of thrombosis. They are one of the causes of thromboembolic disease during pregnancy and significantly take part in the pathogenesis of preeclampsia, intrauterine death and fetal growth retardation, placental abruption and recurrent spontaneous abortion. Inherited thrombophilias are responsible not only for complications during pregnancy, but also during delivery and puerperium. The risk of thromboembolism episodes is much higher in carriers of either congenital or acquired thrombophilic disorders compared to the healthy population of pregnant women (Procházka et al., 2004).

One of the most significant thrombophilic polymorphisms is single nucleotide substitution of cytosine to thymine in the position 677 of the gene for MTHFR (methylenetetrahydrofolate enzyme), which is located on the first chromosome. This polymorphism causes an amino acid substitution of valine for alanine (Ala222Val) and is responsible for the formation of thermolabile form of MTHFR. This leads to a reduction in activity of this enzyme approximately to 50%, causing hyperhomocysteinemia (Poljaková, 2006). The mechanism by which the amino acid homocysteine increases the risk of thrombosis is not precisely understood, laboratory tests have shown homocysteine-induced changes on the surface of endothelial cells, as well as modified activity of anticoagulant factors (Procházka et al., 2004).

Homocysteine induces expression of tissue factor on monocytes and macrophages, resulting in prothrombotic state both in venous and arterial system. It also induces an increase of fibrinolysis inhibitor PAI-1 production in the endothelium and smooth muscle cells, thereby reducing fibrinolytic activity and induces an increase in the tissue factor synthesis.

It also inhibits the expression of thrombomodulin and thus the activation of protein C on the endotelium (Kvasnička., 2003). Hyperhomocysteinemia in pregnancy causes neural tube defects in the fetus. A number of studies have confirmed the relationship between the hyperhomocysteinemia and placental abruption (26% of patients), preeclampsia (18% of patients), intrauterine fetal death (11% of patients), fetal growth retardation (38% of patients) and recurrent pregnancy loss (Kupferminc et al.,1999; Kašparová, 2009; Dekker et al., 1995). Homozygous mutation of MTHFR gene was confirmed in 22% of women with pregnancy complications, but only in 8% of women without complications (Kupferminc et al.,1999). It has been found out that hyperhomocysteinemia increases the risk of abortion up to three times and is associated with damage to the vascular supply of the placenta and the subsequent fetal death. Elevated levels of homocysteine can damage decidual or chorionic blood vessels and prevent fertilized egg implantation. It also significantly affects the incidence of spontaneous abortions by reducing the availability of methyl in the process of methylation, which plays an important role in DNA repair and genome stabilization (Vodová, 2010).

Despite the demonstrated association of MTHFR C677T variant with a mildly elevated level of homocysteine (15- 30 μmol/l), its importance for the development of venous thrombosis is still controversial. According to the results of a meta-analysis of published epidemiological prospective studies a homozygous form of the MTHFR mutation can be considered as moderate risk factor for thrombosis. The significance of the mutation without hyperhomocysteinemia for the risk of thrombosis is not clear. Substitution of folate, vitamin B6 and B12 leads to correction of the homocysteine levels (Bátorová, 2009). On the basis of ambiguous conclusions and results of individual studies the aim of our research was to try to define the effect of MTHFR C677T mutation on the risk of fetal loss in the group of pregnant Slovak women.

Materials and methods

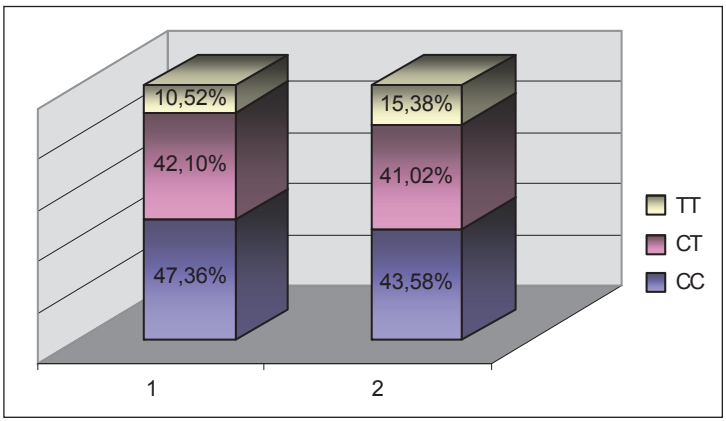
Our studied group consisted of 115 women. There were 39 women who have had at least one fetal loss (23.48% of women had one abortion, 10.43% of women had 2 abortions) in the group of female patients. Control group consisted of 76 women (66.09%) with no complications during pregnancy, in multiparous women neither previous pregnancies did not have complications possibly related to thrombophilia. Samples of DNA were collected in the form of buccal swabs to examine the presence of C677Tmutation. All patients had signed informed consent. Genomic DNA was extracted from buccal swabs using commercial set JetQuick (Genomed GmbH, Germany). We used TaqMan SNP genotyping assay for the detection of point mutation C677T in the MTHFR gene using Applied Biosystems 7500 Fast Real-time PCR System. For the statistical evaluation of the results we calculated allele frequencies from the examined genotype frequencies using Genotyping (SNPs) software (http://ihg2.helmholtz-muenchen.de/). We compared the frequencies between the two groups of women using the chi square test. The criterion for statistical significance was $p < 0.05$.

Results and discussion

We determined the frequency of the MTHFR C677T mutation in the examined group. In the group of controls we detected homozygous form CC of MTHFR C677T mutation in 36 women (47.36%), heterozygous form CT in 32 women (42.10%) and homozygous form TT in 8 (10.52%) women.

In the group of patients a homozygous genotype CC was detected in 17 women (43,58 %) and heterozygous form CT in 16 women (41,02 %). Homozygous TT genotype was detected in 6 women (15.38 %).

Graph 1: Comparison frequencies of genotypes between the group of controls (1) and group of women who had at least one fetal loss (2)



Consequently we determined the frequency of standard and mutant alleles of MTHFR C677T polymorphism in both groups of women. The comparison of standard and mutant allele frequencies is showed in table 1.

Table 1 Comparison of standard and mutant allele frequencies of MTHFR C677T polymorphism in the group of patients and controls

	Controls (women with no experience of fetal loss)	Patients (women that underwent fetal loss)
T (mutant allele)	**0,32**	**0,36**
C (standard allele)	**0,68**	**0,64**
OR (odds ratio)	**1.213**	
p (statistical significance)	**0.50979**	

The results show that although the frequency of mutant allele (T) was higher in the group of women with pregnancy complications compared to the control group, but the difference was not statistically significant ($p = 0.50979$). The level of risk (OR = odds ratio) in compared groups of women was 1.213. It means that women with C677T mutation in the MTHFR gene have 1.213 times higher risk of having fetal loss than women without the mutation. These values correspond to the results from several other authors (Pramusinto et al 2004, Jarvenpaa 2006, Prochazka et al 2007).

A meta-analysis evaluating and comparing a total of 24 studies in which 2631 women with pregnancy complications and 3422 controls were screened provide an interesting results. A significantly higher prevalence of MTHFR C677T mutation confirmed only three of these studies. We can therefore assess that our results are consistent with the results of most of the authors and confirm that the C677T polymorphism in the MTHFR gene is associated with an increased risk of pregnancy complications, but not statistically significantly.

It is, however, necessary to point out the results of the authors that have confirmed statistically significant association between homozygous form of this mutation in MTHFR gene and the risk of thrombosis occurrence and related pregnancy complications (Kašparová, Fait , 2009).

Interesting conclusions have been published in the study that was monitoring concentrations of homocysteine and folate (folic acid) in 123 women who have had at least two consecutive spontaneous abortions in the early stage of pregnancy, compared with 104 controls. Women with recurrent fetal losses had significantly lower serum folate concentrations ($p < 0.01$) when compared to controls. An elevated levels of homocysteine were also found. According to the results of this study reduced serum folate concentrations and elevated homocysteine levels represent the risk factors for recurrent pregnancy loss in the early stages of pregnancy. The importance of MTHFR mutation without hyperhomocysteinemia for the risk of fetal loss is still

not clear (Williane et al., 2000). An association of recurrent pregnancy loss in the early stage of pregnancy, hyperhomocysteinemia and MTHFR C677T mutation has been confirmed also by meta-analyses (Nelen et al., 2000; Robertson et al., 2005).

This topic is quite complex and there are many other inherited and acquired factors involved in the etiology of obstetric complications, together with a certain effect of environment. Serious pregnancy complications like recurrent pregnancy loss or preeclampsia occur also in the group of women with no thrombophilic mutations. It is therefore a probability we still do not know exactly all the possible etiological factors of obstetric complications.

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SEQUENCE ANALYSIS OF CANDIDATE GENES IN PATIENTS WITH ISOLATED (NON-SYNDROMIC) HYPODONTIA

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Abstract: *Hypodontia is agenesis of one to six teeth, and it represents the most common orofacial deformity in humans. The tooth development (odontogenesis) is very complicated and complex process. Research of the molecular basis of hypodontia is based on the detection of mutations in genes that affect the process of odontogenesis. Several candidate genes associated with isolated hypodontia have been described. The most common causes of hypodontia are mutations in transcription factors, such as the homeobox gene MSX1 and paired box gene PAX9. In this study, DNA samples of 16 patients, aged 8 to 24, with different types of tooth agenesis were analyzed. The agenesis of lateral incisors in the first and second quadrant has occurred more frequently (81.25%) than agenesis of second premolars present in the third and fourth quadrant (31.25%). Genomic DNA was isolated from buccal swabs. DNA sequence analysis was performed by the 24-capillary 3500xL Genetic Analyzer (Life Technologies). Exons, exon/intron junctions and UTRs of MSX1 and PAX9 gene were sequenced. Sequences were evaluated by SeqScape®Software and Sequencing Analysis Software (Life Technologies) and compared with reference sequences. The detected variants have been described in relation to the pathogenesis of hypodontia.*

Key words: *dental agenesis, isolated hypodontia, PAX9, MSX1, sequence analysis*

The importance of healthy teeth of individuals is functionally linked to food intake. Moreover good dental status affects the amount of sociological aspects. Tooth agenesis is the most common orofacial disorder that affects about 0.3 to 7.9% of the European population, it occurs slightly more often in females than males (Vastardis 2000; Pemberton et.al, 2005; Mattheeuws et al., 2004).

Agenesis of one to six teeth is called hypodontia (Matalova et al., 2008). Hypodontia can appear in isolated form (non-syndromic) when only teeth are affected, or in syndromic form, when it associated with symptomes (Rieger syndrome, Hypohidrotic Ectodermal Dysplasia, Witkop syndrome). Non syndromic congenital absence of teeth can be sporadic or familial. Association studies suggest an autosomal-dominant mode of inheritance, autosomal-recessive, but autosomal-recessive, X-linked type and polygenic/multifactorial inheritance has also been described (Matalova et al., 2008; Arte et al., 2004). There is large phenotype variability exists

of tooth agenesis, such as variance of tooth agenesis in different quadrants, variants in size and distributions etc. On average about 20% of individuals in a population missing third molar. Agenesis of the upper lateral incisors and second premolars is the second most common type of tooth agenesis and occurs at a frequency of 2.2% and 3.4% (respectively) (Matalova et al., 2008; Arte et al., 2001; Symons et al., 1993).

Progress in genetic research of dental agenesis in recent years has enabled the identification of candidate genes involved in isolated hypodontia. Based on these studies more than 300 genes have been found to be involved in process of odontogenesis, but only few of these genes (MSX1, PAX9, AXIN2, EDA), are related to non-syndromic hypodontia. The genetic basis of the disease, however, is not known yet.

MSX1 and PAX9 genes

MSX1 and PAX9 genes are transcription factors necessary for normal tooth development. Up to now, 27 and 11 distinct mutations in the PAX9 and MSX1 genes, respectively, have been identified in humans all associated with tooth agenesis (Mostowska et al., 2012).

MSX1 is a member of the muscle segment homeobox family, i.e. genes that can regulate the expression of other genes. Location MSX1 is on the short arm of chromosome 4 in position 4p16.3-p16.1. The gene consists of 2 exons. Mutations in this gene are associated with autosomal dominant and recessive form of hypodontia (Matalova et al., 2008). PAX9 belongs to the paired box domain gene family. It located on the long arm of the fourteenth chromosome 14q-12-q13. This transcription factor plays an important role as a regulator of cellular pluripotency and differentiation during embryonic patterning and organogenesis and in post-natal life. PAX9 gene consists of 5 exons. Mutations in the PAX9 gene are associated with autosomal-dominant hypodontia and non-syndromic oligodontia (Ogawa et al., 2005). Expression of MSX1 and PAX9 genes in the early stages of tooth development largely overlap. PAX9 gene is expressed in mesenchyme in the early stages of odontogenesis, during the so-called establishment of tooth bud and is required for the continuation of dental development from that stage on. At this stage PAX9 activates transcription of MSX1, and is also essential for the expression of mesenchymal Bmp4 (Kapadia et al., 2007).

Other candidate genes are studies that may be associated with hypodontic phenotype. Candidate genes for isolated hypodontia are also genes associated with syndromic forms of hypodontia (EDA, EDAR, EDARADD, WNT10A) (Vastardis et al. 2000; Stockton et al., 2000; Lammi et al., 2004). For example, mutation in EDA gene, originally associated with HED (Hypohidrotic Ectodermal Dysplasia) has been detected recently in connection with isolated hypodontia (Tao et al., 2006).

Materials and methods

In this study, DNA samples were analyzed in 16 unrelated patients, aged 8-24 years, with different types of dental agenesis. The sampling was carried out in cooperation with a comprehensive dental clinics and orthodontics. Patients were included to a scientific study based on informed consent. Tooth agenesis was confirmed by

panoramic radiographs and personal and family medical history of the patient. The type and frequency of missing teeth was subsequently identified.

DNA isolation: Genomic DNA was isolated from buccal swabs. DNA was extracted using a commercial isolation kit JetQuick (Genomed) according to the manufacturer's instructions.

Sequence analysis of candidate genes: Candidate genes MSX1 and PAX9 were selected to detect the mutations. Exons, exon/intron junctions and UTRs of MSX1 and PAX9 gene were sequenced in genomic DNA. Primers were designed by software Primer 3 and were supplied by a commercial supplier (Table 1). Sequencing reaction was preceded by standard PCR. PCR products were visualized by electrophoresis (1.2% agarose gel in 1x TBE buffer 16A and 120V), and purified by

Table 1 Sequences of primers for amplification and sequencing reaction of MSX1 and PAX9 gene

Gene	Exon	Primer	Sequence	Primer´s lenght (bp)	Lenght of PCR product cca (bp)
MSX1	exon1	F	5´ctggcctcgccttattagc 3´	19	930
		R	5´aggtctggaaccttcttcctg 3´	21	
	exon2a	F	5´acttggcggcactcaatatc 3´	20	850
		R	5´caattctgctggggacctta 3´	20	
	exon2b	F	5´tcacctctttgctccctgag 3´	20	837
		R	5´tctgtcgtgggtgttcaaaa 3´	20	
PAX9	exon1	F	5´cgctaatatggggaaactgaa 3´	21	566
		R	5´gcggctaaaaggagcagtc 3´	19	
	exon2	F	5´accagcctgattttgctgtc 3´	20	584
		R	5´agaatgtgagcgcctagtgg 3´	20	
	exon3	F	5´ggggacagccccagtagtta 3´	20	627
		R	5´tgtccctgaggctgcagata 3´	20	
	exon4	F	5´ggtctaagccctccagctct 3´	20	140
		R	5´ gaaggatctggctcgtagca 3´	20	
	exon5a	F	5´gagcattgctggcttactca 3´	20	976
		R	5´ gtcaaaacaccagggagagc 3´	20	
	exon5b	F	5´tgctacaccctctaatcaaatatgg 3´	25	991
		R	5´actcacatgctcacacacaca 3´	21	

Notes: (F) Forward, (R) Reverse

Exonucelase and FastSap (Thermo Scientific, Rocford, USA). BigDye®Terminator v3.1. Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) was used for sequencing reaction. Purification was carried out by Sure-Clean (Promega Corporation, Madison, WI, USA) and 70% ethanol. Sequence analysis was carried by 24-capillary genetic analyzer 3500xL (Life Technologies, Foster City, California, USA). SeqScape®Software and Sequencing Analysis Software (Life Technologies, Foster City, California, USA) were used for evaluation of the data. Mutation detection were performed using reference sequence for PAX9 (GenBank NG_013357.1) and MSX1 (GenBank NG_008121.1), both available in NCBI database. Table 1 shows the sequence of primers for amplification and sequencing reaction of MSX1 and PAX9 gene.

Results

In the group of 16 patients with isolated hypodontia an increased incidence of agenesis of lateral incisors (81.25%) in quadrant 1 and 2 was observed, less often agenesis of the second premolars (31.25%) and in quadrant 3 and 4. On average, patients were missing two teeth. In one patient microdontia of lateral incisors in the first quadrant was present (Table 2). In 50% of patients familial tooth missing (dental agenesis occurred in at least one of the two parents) was confirmed.

Sequence analysis of candidate genes MSX1 and PAX9 showed the presence of several polymorphisms. All identified nucleotide variants of these genes are known and available in databases (Table 3).

Five polymorphisms have been identified in PAX9 gene. Cytosine insertion 99insC was found in exon1. Due to the presence of three cytosine consecutive bases at position 99-101, it could not be determined in what position the base is inserted. An interesting finding is that in patients with cytosine insertion, cytosine to guanine substitution at position 272 (rs4904155) was always found. In the group of patients 99insC mutated allele frequency was 50%. In exon 4, which encodes the protein, variant G/C at position 1444 (rs4904210) was detected in 12 patients. Substitution of bases ***G***CG→***C***CG causes a change of amino acid alanine to proline (Ala240Pro) (Figure 1). It should be noted that the variant occurred concurrently with insC99--insC101 and 272 G/C, always in the same patients. Described variants are probably part of jointly inheriting haplotype. In exon 4 substitution of cytosine for thymine (C/T) at position 1443 (rs12881240) was observed. It represents a silent mutation, which does not cause a change in the amino acid, histidine remains coded even after substituted bases CA***C***→CA***T*** (His239His). It is interesting that all wild-types for Ala240Pro were heterozygotes for His239His. Variant at position 2570 (C/T) in 3'UTR region of PAX9 was found in three patients.

Table 2 Summary of missing teeth in 16 patients with isolated hypodontia

Patient	1 quadrant (right)							2 quandrant (left)						
	17	16	15	14	13	12	11	21	22	23	24	25	26	27
	37	36	35	34	33	32	31	41	42	43	44	45	46	47
	3 quadrant (right)							4 quadrant (left)						
1						*					*			
			*											
2			*											
3						*			*					
						*								
4				*										
5														
				*								*		
6						*			*					
7						*			*					
8						*								
9						*			*					
10						*								
11						*			*					
12						*			*					
			*									*		
13														
14									*					
15						m			*					
												*		
16						*			*					

Notes: (*) dental agenesis, (m) microdontia

Table 3 Overview of the identified polymorphisms in candidate genes MSX1 and PAX9

		Polymorphisms	SNP	Number of patients
PAX9	exon1	insC99-insC101	rs138135767	12
		272 C/G (S)	rs4904155	12
	exon4	1443 C/T (His239His)Y	rs12881240	4
		1444 G/C (Ala240Pro)S	rs4904210	12
	exon5	2570 C/T (Y)	rs11847165	3
MSX1	exon2	1152 C/T (Y)	CR067843	8
		1421 A/G (R)	rs12532	8

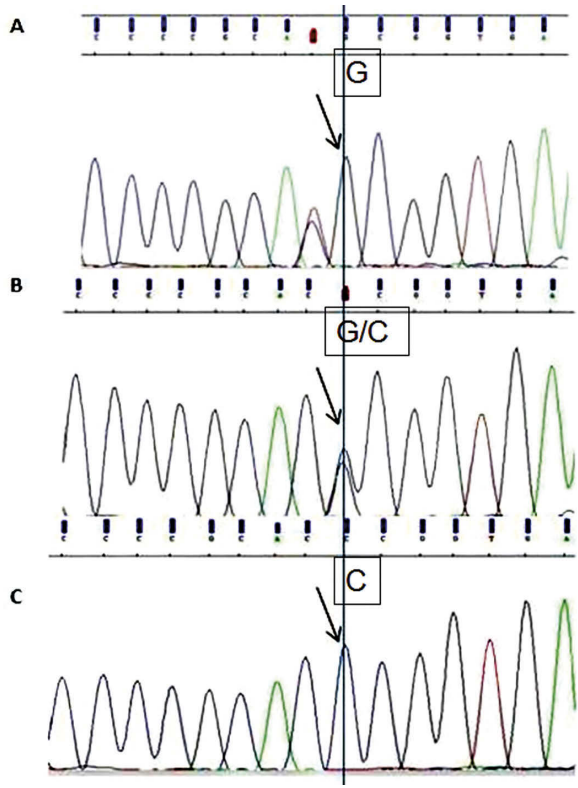

Figure 1 Sequence analysis of PAX9 in patients.
The position of polymorphism is indicated with an arrow.
The patient was A) wild-type B) heterozygote, C) homozygote for the amino acid (Ala240Pro) caused by substitution G/C (rs4904210)

In MSX1 gene two variants (CR067843, rs12532) were identified in exon 2, in untranslated region (3'UTR). Substitution of C/T (CR067843) was present in half of the patients, rs12532 polymorphism was present in the remaining half of the patients, both variants are therefore not present together in any of the patients. Exon 1 sequences of MSX1 gene could not be analyzed due to nonspecific PCR products.

Discussion

Agenesis of the second premolars and upper lateral incisors is the second most common type of dental agenesis (Matalova et al., 2008, Arte et al., 2001). Symons et al. (1993) describes the second premolars and upper lateral incisors agenesis as the dominant type of tooth agenesis.

In the group of 16 patients with isolated hypodontia higher incidence of agenesis of the lateral incisors was observed, less frequently agenesis of the second premolars. Microdoncia of lateral incisors together with agenesis of the opposite lateral incisors was present in one patient. By Pinho et al. (2010) the concurrent lack of opposing teeth is relatively common in primary genetically conditional hypodontia and is often associated with structural changes in the contralateral tooth (microdontia, sharp shape).

Exons sequence of PAX9 gene and MSX1 gene was obtained by sequence analysis in this study. The several common polymorphisms were identified in group of 16 patients with isolated hypodontia.

Cytosine insertion, cytosine to guanine substitution at position 272 (rs4904155) was always found in exon 1 of PAX9 gene. Exon1 represents untranslated region (5'UTR). It is so difficult to describe accurately what effect has the presence of polymorphisms producing protein product. By Mignone et al. (2002) UTR regions have a regulatory function. Mutations in this region may affect transport and mRNA stability and translation efficiency and the amount of protein product. The frequency of mutant allele (insC) was 50% in study group. We observed a higher frequency of the mutant allele in the group of patients with isolated hypodontia in comparison with allele frequency (insC) (MAF-Minor alelle frequency) in European and global population, where does not exceed 38% (www.ensembl.org). This could indicate a possible association with the disease in terms of changes in the regulatory function.

One of the identified polymorphisms of PAX9 gene (rs4904210) causes substitution of alanine to proline at position 240 (Ala240Pro). The polymorphism was occurred in parallel with insC99-insC101 and 272 G/C in the same patient at the same genotypes represented. Our results suggest that the described variants are probably part of jointly inheriting haplotype, which may be responsible for causing tooth agenesis.

Pawlowska et al. (2010) suggest that the polymorphism rs4904210 may be considered the cause of tooth agenesis. Earlier family studies have shown that Ala240Pro mutation has a recessive model of inheritance (Kula et al., 2008; Trimmell et al., 2004). Other studies suggest that the 240Pro homozygotes can form a protein with a slightly reduced DNA-binding capacity, which could be specifically associated

with the absence of third molars and lateral incisors (Rodrigues Paixao-Cortes et al., 2011; Pereira et al., 2006). There was found the association of polymorphism rs4904155 (272 C/G) and rs4904210 (Ala240Pro) with a crown size of incisor in the current study of the Korean population (Lee et al., 2012). The results of our study do not exclude the possible role of polymorphisms in the development of incisors. There was present the absence of lateral incisor in all 240Pro homozygous carriers and observed the microdontia of lateral incisors with agenesis of contralateral incisors in one case. However, there was occurred the agenesis of incisors even in 240Pro heterozygotes. Identified was also the polymorphism rs12881240 (His239His) in 4 wild-type patients for Ala240Pro and rs11847165 polymorphism in the 3'UTR in PAX9 gene without significant association.

Polymorphism CR067843 occurred in parallel with rs8670 and rs12532 polymorphisms in the 3'UTR of MSX1 gene. The polymorphisms in the non-coding region may participate on the sporadic and familiar tooth agenesis according to current study (Pawlowska et al., 2009). New findings could support the hypothesis that the non-coding regions may influence the process of odontogenesis.

The precise mechanism of non-syndromic hypodontia is not known yet. The disease is also characterized by a variable phenotype and is conditioned by a heterogeneous complex of factors. To describe the importance of polymorphisms with certainty is difficult because mutations detected by different authors vary. Our results suggest that some of the identified polymorphisms of candidate genes MSX1 and PAX9 could participate in the development of incisors. In order to associate specific mutations with particular types of missing teeth more comprehensive analysis of larger group of patients is needed.

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THE USAGE OF HRM METHOD FOR MOLECULAR GENETIC ANALYSIS OF "SPORTS GENE" ACTN3

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Abstract: *The aim of the research was to test suitability of real-time PCR High--Resolution Melting for molecular genetic analysis of the "sports gene" ACTN3. ACTN3 gene is the gene encoding the protein alpha-actinin-3. Polymorphism R577X found in this gene leads to two versions of the ACTN3 protein in humans. Allele 577R is functional allele of the gene, where the presence of alpha-actinin-3 predisposes individuals to speed-power sports, while 577X is dysfunctional allele, protein is absent, which is predisposing to endurance sports. The real-time PCR High-Resolution Melting method was used for polymorphism detection of ACTN3 gene. It's a simple post-PCR method for mutation screening and genotyping. The principle of the HRM method is the thermal dissociation of PCR amplicon in the presence of fluorescent dye to allow effective monitoring of dissociation behaviour. Genotypes were determined by melting curves which software automatically normalized. Software allows creating reports of any analysis, where information of the specific analysis, normalized and differential graphs and information about the samples are shown. Our analysis confirmed suitability of this method. It is quick, easy, affordable and in the future could be used to determine the predispositions of each athlete.*

Key words: *ACTN3 gene, polymorphism R577X, alpha-actinin-3, High-Resolution Melting*

One of the basic characteristics of living organisms is the ability to move. Muscles are elegant and efficient organs that allow body movement; the more complex is their structure, the less complicated is their activity. The basic unit of skeletal muscle is muscle fiber, consisting of elongated cylindrical cells (Grasgruber, Cacek, 2008). Muscle fibers are interconnected with thin fibrous tissue and form the bundles (fascia) (Merkunová, Orel, 2008), which cluster together to form a muscle, attached with tendons to the bone. Muscle fibers are divided into three basic types: I, IIa and IIb (IIx). Slow (oxidative) type I fibers are necessary for endurance aerobic muscle activity (ie, long-term, less intensive activity in the presence of oxygen). Fast fibers (type II) are divided into slower type IIa (oxidative-glycolytic), which have also some potential for aerobic activity, and faster type IIx and IIb (glycolytic), which are important for anaerobic activities, where explosive energy is necessary, such as short sprints and jumps. Fibers of type IIa present some kind of transition between fibers

I and IIb. Type IIb fibers have the greatest dynamic power of all of the three types (Grasgruber, Cacek, 2008).

Physical fitness is a very complex phenotype influenced by many genetic and environmental factors that contribute to interindividual variability not only in athletes, but also in the general population (Myburgh, 2003). Athletic performance is determined by many factors, with genetic compound accounting for 20 - 80% (MacArthur, North, 2007). The human genetic map of performance and health consists of more than 150 genes and gene regions associated with athletic performance and fitness. R577X polymorphism of the ACTN3 gene for alpha-actinin-3 is a potential precondition contributing to differences in the structure and function of muscle performance (Mills et al., 2001). It is a substitution of cytosine for thymine at the position 1747 in exon 16 (Livingstone, 2006), which results in the change of codon for arginine (R) at position 577 to premature stop codon (X) (North et al., 1999). It causes premature transcription termination, subsequently shorter mRNA molecule and dysfunctional protein (Livingstone, 2006). This variation results in two versions of human ACTN3 protein (North et al., 1999). Allele 577R is functional, while 577X is dysfunctional allele. This polymorphism generates three possible genotypes: XX, RX and RR.

• **Genotype XX**: R577X variant is present in both copies of the ACTN3 gene. This genotype is associated with a natural predisposition for endurance disciplines (e.g. triathlon, swimming ≥ 400m) (GenEffect, 2012a; GenEffect, 2012b).

• **Genotype RR**: R577X variant is not present in any copy of the gene ACTN3. This absence is associated with a natural predisposition for sprint/power discipline (e.g. running ≤ 800 m, swimming ≤ 200m, track cycling for short distances, rugby, body building and weight lifting) (GenEffect, 2012a; GenEffect, 2012b).

• **Genotype RX**: R577X variant is located in one of the two copies of the ACTN3 gene. This combination is suitable for sports, where the power and speed is needed and as well as endurance (e.g. football, handball, tennis or basketball) (GenEffect, 2012a; GenEffect, 2012b).

The prevalence of allele X associated with endurance performance was demonstrated in all studied populations in the world (North et al., 1999). Frequency of genotype XX varies from 25% in the Asian population for <1% in the population of the African tribe Bantu. In European population it is approximately 18% (Cuticchia, 2009). The most comprehensive study of the association of ACTN3 gene with a sport performance in Caucasoid population by Yang et al. (2003) followed a set of professional athletes (from which 50 athletes participated in the Olympic Games) and compared them with a common population. Significant variations in the frequency of ACTN3 genotypes between different groups of athletes were observed. RR genotype was present in higher frequency in the group of speed athletes, while presence of genotype XX was higher in the group of endurance athletes (Yang et al., 2003).

In search for simple, fast and cheap method of molecular genetic analysis of R577X variant in the “sports gene” ACTN3 we have optimized a protocol for real-time PCR

High-Resolution Melting. High Resolution Melting (HRM) is a homogeneous, highly powerful method for SNP genotyping, mutation scanning and sequence scanning in DNA samples. Enabled by the recent availability of improved double-stranded DNA (dsDNA)–binding dyes and next-generation real-time PCR instrumentation, High Resolution Melting Analysis is based on PCR melting (dissociation) curve techniques. The HRM technology characterizes nucleic acid samples based on their dissociation behaviour and detects small sequence differences in PCR amplified sequences, just by direct melting. Samples are also discriminated according to their sequence length, GC content and strand complementarity. With the use of specific DNA dyes, high-end instrumentation and sophisticated analysis software, these differences are detected. High Resolution Melting Analysis (HRM) is a post PCR method.

The region of interest within the DNA sequence is first amplified using the polymerase chain reaction. During this process, special saturation dyes are added to the reaction, that fluoresce only in the presence of double stranded DNA. Such dyes are known as intercalating dyes. During PCR, the region of interest amplified is known as amplicon. As the amplicon concentration in the reaction tube increases the fluorescence exhibited by the double stranded amplified product also increases. After the PCR process the HRM analysis begins. In this process DNA amplicons are heated gradually from around 50°C up to around 95°C. As the melting point temperature of the amplicon is reached, DNA sample denatures and the double stranded DNA melts apart. The fluorescence decreases due to the absence of double stranded DNA - the intercalating dyes have nothing to bind to and they only fluoresce at a low level. This observation is plotted showing the level of fluorescence vs. the temperature,

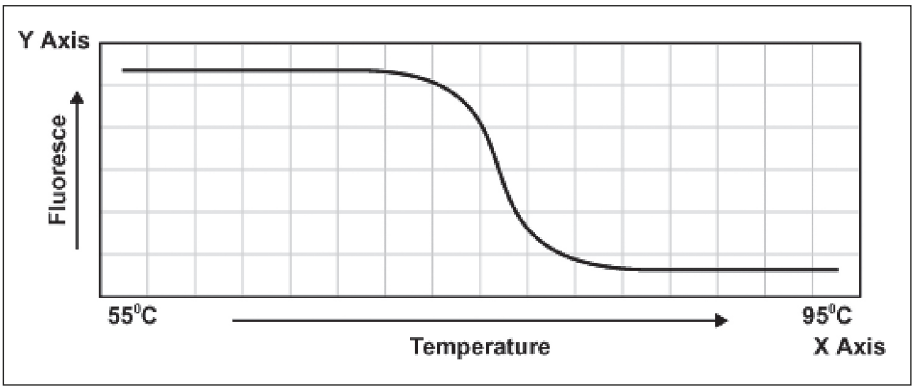

Figure 1 DNA Melt Curve for High Resolution Melting Analysis

generating a melting curve. Even a single base change in the DNA sample sequence causes differences in the HRM curve. Since different DNA sequences melt at slightly different rates, they can be viewed, compared, and detected using these curves (Premier Biosoft, 2012).

Melt curves generated after High Resolution Melting analysis are normally plotted with fluorescence on the Y axis and temperature on the X axis. These are similar to real-time PCR amplification plots but with the substitution of temperature for cycle number (12).

Material and methods

We used 279 samples for our analysis. DNA was obtained from buccal swabs and extracted by available commercial kits according to the standard protocol, then the concentration of DNA samples was measured by a spectrophotometer Nanodrop ND 2000. Polymorphism of ACTN3 gene was detected by a real-time PCR High--Resolution method using LightCycler ® 480 Real-Time PCR System (Roche). 2xPlatinum SuperMix qPCR/UDG (Life Technologies, Foster City, USA) was used for performing PCR and LC Green (Idaho Technology Inc, Salt Lake City, Utah, USA) was used as an intercalating dye to visualize the reaction in real-time (Table 1). Primers were produced by Sigma Aldrich (Bratislava, Slovakia), primer sequences are available upon request. Reaction temperature profile is summarized in Table 2. As a quality control, every sample was analyzed twice.

Table 1 HRM components for LightCycler ® 480 RT-PCR System

COMPONENT	CONCENTRATION	VOLUME (15 µl)
water		2,4
2xPlatinum SuperMix qPCR/UDG	1x	7,5
5µM forward primer	500 nM	1,5
5µM reverse primer	500 nM	1,5
10x LC Green	1x	1,5
DNA	variable	0,6

Table 2 Temperature profile

The initial denaturation	95°C 3 min
Cycle 20x	95°C 15 s
	60°C 5 s
The data were obtained for FAM channel	
HRM	80-90°C
The data we obtained for HRM channel.	

Results and discussion

The genotypes were determined by melting curve analysis. The LightCycler ® 480 Real-Time PCR System software automatically normalized melting curves, created reports from each analysis that contained information about the specific analysis, normalized (Fig.n.1) and differential graphs (Fig.n.2) and information about the samples - each sample was identified by color and number . Based on the use of positive controls the software automatically assigned a particular genotype to every sample (Fig.n.2). The genotype was successfully detected in all 279 samples. Repeated genotyping confirmed the results with 100% concordance. We confirmed the suitability of HRM method for this kind of analysis. It is a simple method for detection of known polymorphisms as well as for screening for new mutations, DNA methylation analysis and rapid genotyping of individuals. HRM method is cost effective compared to the other genotyping technologies such as sequencing and Taqman SNP genotyping. This method is fast and powerful thus able to accurately genotype many samples rapidly.

The specific analysis of ACTN3 gene may be helpful in choosing the appropriate sports activities. The results of sports performance genetic research are useful for precise profiling of athletic training and the creation of individual training plans for elite athletes. The genetic test can provide athletes with the relevant information that can help them to decide which kind of sport to pursue, in order to achieve the best utilization of their abilities.

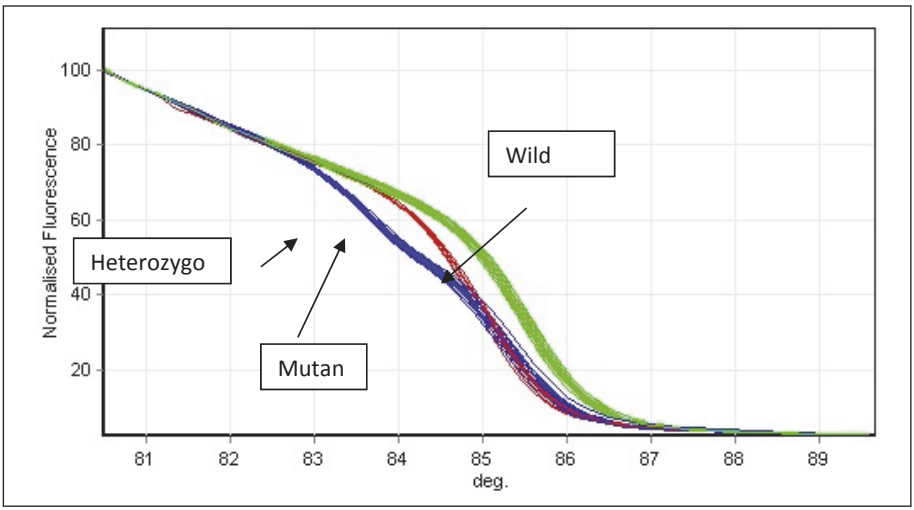

Figure 2 Normalized graph for HRM (wild type = RR, mutant = XX, heterozygot = RX)

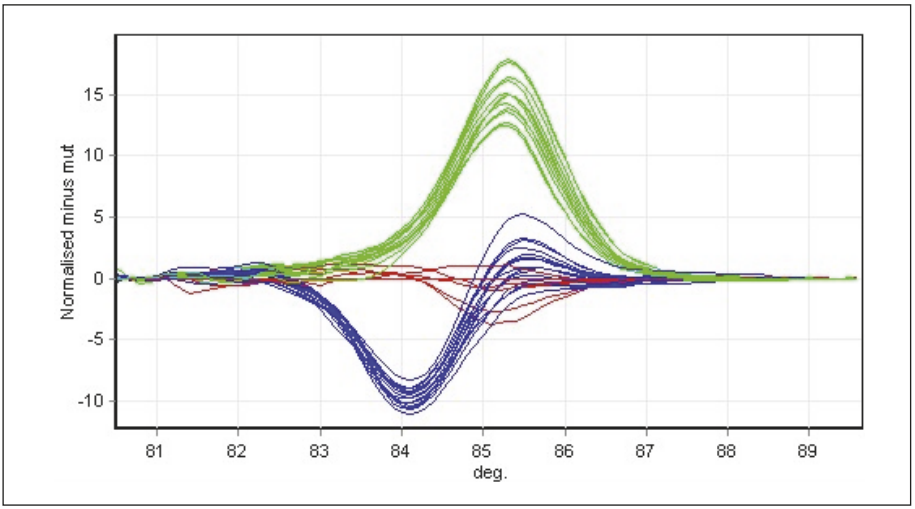

Figure 3 Differentiation graph

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FACTORS INFLUENCING PANDEMIC POTENTIAL OF INFLUENZA A VIRUSES

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Abstract: *Influenza A viruses (IAV) cause an acute respiratory disease, which represents one of the most important public health problems at presence. A high variability of IAVs spreading in human population and their broad host specificity impede to predict influenza virus subtype which will occur and will cause epidemic or pandemic. New viruses to which human population has no immunity arise by cumulative substitutions in virus genome or by reassortment among segments of human and avian viral genomes. The lacking of immunity in humans specific to new emerging IAV, as well as their interaction with human host determine the danger which new emerging viruses represent. Factors, which influence virus-host interactions (like receptor specificity, RNA polymerase activity, cleavability of haemagglutinin (HA) by host proteases, glycosylation of viral proteins, properties of PB1-F2 protein, etc.) are studied on theoretical and on experimental levels. Our group is focused on HA, particularly its conserved HA2 glycopolypeptide (gp). HA is initially expressed as a precursor molecule, HA0, which is proteolytically cleaved into HA1 and HA2 subunits, linked by a single disulphide bond. The HA1 subunit is responsible for virus attachment to susceptible cells, while HA2 gp mediates the fusion of viral and endosomal membranes. The fusion potential is activated by structural change of HA due to reduced pH in endosome, leading to insertion of fusion peptide into target membrane resulting in fusion of both membranes. The structural change of HA, which is a result of release of intermolecular interactions in the HA trimer, influences its fusion activity. It can consequently influence the virulence of virus. The aim of our study was to examine how the differences in the fusion activity of HA influence in vitro and in vivo replication ability of two antigenically identical viruses of H3 subtype.*

Keywords: *conserved region of haemagglutinin, fusion activity, influenza A virus and pandemic potential.*

Haemagglutinin, the surface glycoprotein of influenza A virus, mediates the initial steps of influenza virus infection: binding of virus particle to sialic acid, its endocytosis and virus and endosome membranes fusion. HA is a trimer created by 3 identical monomers, each of them composed of two disulfide-linked glycopolypeptides, HA1 and HA2, generated by proteolytic cleavage of precursor HA0 (Shekel, Wiley, 2000). Fusion of viral and endosomal membranes requires restructuralization of HA resulting in the exposition of conserved hydrophobic N-terminus of HA2 out

from the interface of HA trimer, which is triggered by low pH in endosomes. Then N-terminus of HA2 is inserted into the target (endosomal) membrane and mediates the connection of both viral and endosomal membranes, thus starting the process of their fusion. Low pH in endosomes simultaneously opens the proton channel, M2 protein, which enables the uptake of protons into the virion. Consequently, the viral ribonucleoprotein (RNP) dissociates from M1 protein (Plotch et al., 1999) and RNP is released into the cytosol and transported to the nucleus, the site of vRNA replication. The conformation change of HA trimer, which is essential for the process of membrane fusion, is triggered by low pH between 5 and 6. The pH optimum of fusion is strain-dependent. In this study we examined how differences in the fusion activity of virus influence its virulence and pathogenicity. We compared two viruses with different pH optimum of fusion. As a model we used mutant virus Ab4, derived from influenza virus X-31 (H3N2) with the substitution of histidin (H) to arginine (R) in position 17 of HA1 gp and virus A/Dunedin 4/73 (H3N2), antigenically identical with parental virus. It was shown that Ab4 mutant has higher pH optimum of fusion than parental virus (Daniels et al., 1985). Therefore, *in vitro* and *in vivo* replication activity of these two viruses was compared.

Materials and methods

Cells: MDCK (Madine Darby Canine Kidney) cells were cultured in Dulbecco´s modified Eagle medium supplemented with 5% fetal bovine serum, 0,1% gentamicin and 1% L-Glutamine at 37°C in 5% CO_2.

Viruses: A/Dunedin/4/73 (H3N2) (abbreviated "Dun") originates from collection of Institute of Virology, Bratislava, SR and amantadine resistant mutant Ab4 virus with H to R mutation at position 17 in HA1 derived from influenza A virus X-31 originates from NIMR, London, UK (abbreviated "Ab4") (Daniels et al., 1985). Viruses were propagated in fertilized chicken eggs and purified from allantoic fluid by differential sucrose density gradient centrifugation (**Russ, et al., 1974**).

Monoclonal antibodies MAb: MAbs 107L specific to IAV nucleoprotein (**V**arečková, 1995) and HA monomer-specific MAb (Varečková et al., 1993), which recognizes 125-175 aa of HA2 gp (**Varečková et al., 2003**) were prepared at the Institute of Virology, Bratislava, SR.

Erythrocytes (RBC): Human (0 group. Rh+) or guinea pig erythrocytes were used as 1% (for haemagglutination test) or 5% (for haemolysis test) suspension in sodium chloride.

Infection of mice: Six-week-old BALB/c mice were infected under light anaesthesia with 40µl of infectious allantoic fluids at the appropriate dilution. vRNA and infectious virus in mouse lungs were evaluated two days after the infection. Lungs were homogenized with 1ml of PBS and after removing cell debris by centrifugation, the supernatant was used for vRNA detection by RT-PCR and infectious virus estimation by rapid culture assay (RCA) as described (Tkáčová et al., 1997). In all animal experiments mice were treated according to the European Union standard and the fundamental ethical principles including animal welfare requirements were respected.

Haemagglutination test (HA test): was performed in a micro-method modification according the standard procedure (Varečková et al., 2006) using 1% RBC. One HA unit (HAU) in the HA titration represents the reciprocal value of the highest dilution of virus which still agglutinates RBC.

Haemolysis: the pH at which different viruses lysed erythrocytes was determined as described (Daniels et al., 1985) and optical density of the supernatant was measured at 540 nm as described (Formanowski et al., 1990).

Plaque titration: the method for determination of plaque forming unit was done as described (Fislová et al., 2009).

Rapid culture assay (RCA): RCA was used for evaluation of virus titer in infectious allantoic fluid or for titer of infectious virus in mouse lungs. NP-specific MAb 107L was used for detection of virus on MDCK cell monolayer after adding the Goat-anti-mouse IgG-peroxidase conjugate and substrate solution containing 3-amino-9-ethylcarbazole with 0,03% H_2O_2. The differentiated red coloured cells, identified by light microscopy, were evaluated as positive for infection. The titer of infectious virus represents a reciprocal value of the highest sample dilution at which infected red coloured cells could be observed (Tkáčová et al. 1997).

Enzyme immunoassay (ELISA): 300ng of purified virus adsorbed onto wells of a microtitration plate overnight at 4 °C was incubated for 30 min/25 °C with McIlvaine buffer of appropriate pH decreasing from 6.5 to pH5. In the case of antibody titer estimation, treatment of virus with McIlvaine buffer was eliminated. Plates were then saturated for 90 min/25 °C with phosphate buffer saline pH 7.2 (PBS) containing 0,5% ovalbumine and specific antibodies (MAb IIF4) 100ng/100µl/well or 2-fold dilutions of sera from infected mice 100µl/well in PBS with 0,5% ovalbumine were added for 90 min incubation at 25 °C. Specific antibodies bound to antigen were detected by a HRP-conjugated goat anti-mouse IgG after 90 minutes incubation and addition of the substrate solution containing ortho-phenylendiamine with 0,03% H_2O_2. The reaction was stopped with 2M H_2SO_4 and OD492 was measured.

RT-PCR: was used for detection of vRNA in lungs of infected mice on the 2nd day after infection. RNA was isolated according to Total RNA Isolation Kit protocol provided by E. coli and transcribed into cDNA by Moloney murine leukemia virus reverse transcriptase. Virus-specific cDNA was detected after PCR amplification using NP specific primers as before (Fislová et al., 2009) and product of 509 bp was identified in 1.0 % agarose gel.

Results

The activation of fusion potential of IAV is triggered by low pH and is accompanied by restructuralization of influenza HA. Therefore, we followed the pH dependence of fusion activity of HA in relation to its structural changes.

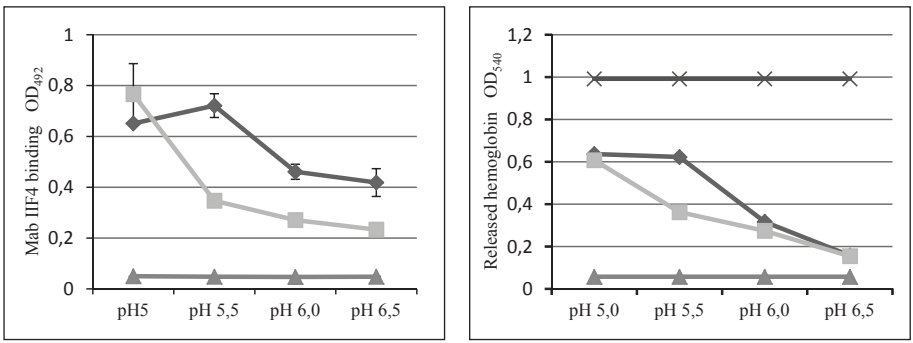
The pH dependence of haemolysis mediated by virus Ab4 and Dun

For monitoring the fusion we used haemolytic test. It is based on the ability of IAV to cause RBC lysis after its binding to RBC and subsequent exposition to low

pH. The amount of released haemoglobin, which is measured in supernatant of the reaction mixture at A540, represents the measure of fusion. We monitored the fusion at descending pH of reaction mixture from pH 6.5 to 5.0 (ΔpH 0,5) and showed that RBC lysis mediated by Ab4 virus and Dun differed. For virus Ab4, maximum of RBC lysis was achieved already at pH 5.5, while pH maximum of RBC lysis mediated by Dun virus required further lowering of pH to pH 5.0 (Table 1, Figure 1B).

Table 1 Comparison of fusion activity of Dun and Ab4 viruses with conformational change of HA

Fusion activity [%]	Mab IIF4 ELISA binding [%]				
pH	Ab4	Dun	pH	Ab4	Dun
5,0	100	100	5,0	90,3	100
5,5	97,8	62,5	5,5	100	45,3
6,0	50	42	6,0	64	35,4
6,5	25	25,5	6,5	58	30,5



1A 1B

Figure 1 pH dependence of conformational change (1A) and fusion (1B) activity of mutant virus Ab4 (♦) and virus Dun (■)

1A: MAb IIF4 binding in ELISA was used for monitoring of conformational change of HA trimer. As a negative control (▲) irrelevant MAb specific to Herpes virus (Bystrická et al., 1991) was used.

1B: Fusion was monitored by RBC haemolysis at different pH range. As a negative control (▲) we used 5% erythrocytes in PBS and as a positive control (×), 5% erythrocytes in 0,2% Nonidet were used.. The released haemoglobin was measured at A540.

The conformation change of HA of viruses Ab4 and Dun triggered by decreased pH

To monitor conformation change of HA we used MAb IIF4 as a probe for detection of conformationally changed HA. This MAb recognizes HA monomer, but does not effectively bind to HA trimer. Thus MAb IIF4 binding to virus reflects the pH – dependent conformation change of HA trimer and can be used for monitoring of structural changes on HA trimer under the appropriate pH. We observed maximum of MAb IIF4 binding to Ab4 virus at pH 5.5, while for Dun maximal reaction with HA probe was achieved at pH 5.0 (Table 1, Figure 1A).

Comparison of replication activity of Ab4 and Dun viruses in vitro

Further we compared the replication ability of these two viruses Ab4 and Dun, which differed in the fusion activity. MDCK cells were infected with viruses of comparable dose and titers of viruses were determined by RCA in three intervals post infection: 6hrs, 12hrs and 18hrs. We observed significant higher titer of Ab4 virus than that of Dun. The difference in titer increased with time p.i. (Table 2).

Table 2 *In vitro* replication activity of Ab4 and Dun viruses determined by RCA

Time p.i [hrs]	Titer	
	Ab4	Dun
6	204 800	12 800
12	819 200	102 400
18	1 638 400	102 400

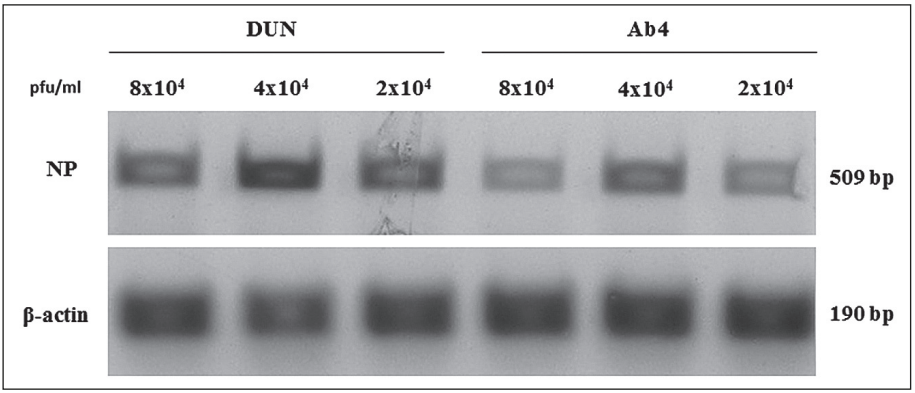


Figure 2 Detection of v RNA in mouse lungs infected with Ab4 and Dun viruses
Infectious dose: 8x104, 4x104, 2x104 pfu/ml

Comparison of replication activity of Ab4 and Dun virus in vivo

BALB/c mice were infected intranasally with various doses of viruses Ab4 or Dun. Two days post infection, two mice of each group were sacrificed and infectious virus as well as the presence of vRNA in cell lung homogenates were estimated (Figure 2). No significant dose dependence of virus titer in mouse lungs for Ab4 virus was found (Table 3). Mice infected with Dun virus revealed the highest titer in the group of mice infected with 4x104 pfu/ml. This was in correlation also with the highest signal corresponding to vRNA detected in these groups of mice. In all other groups no significant difference of virus titer was observed even between groups of mice infected with any used dose of Ab4 and Dun viruses.

Table 3 Infectious titer of Ab4 and Dun viruses in mouse lungs determined by RCA

Infectious dose [pfu/ml]	Titer	
	Ab4	Dun
8x104	800	800-1600
4x104	800	6400
2x104	1600	800

Comparison of antibody response in mice infected with Ab4 and Dun viruses

We tested sera obtained from infected mice 20 days after the infection and examined them by ELISA binding test with purified viruses to determine titer of specific antibodies. We did not find significant difference in titer of virus specific antibodies in dependence on the infective virus dose of Ab4 or Dun viruses, as well as no marked difference was observed in titers of antibodies specific to virus Ab4 in comparison with that of specific to Dun.

Discussion

The activation of fusion potential of IAV is the essential step for successful replication of IAV. Consequently, it can influence its virulence and pathogenicity. Fusion process is accompanied with restructuralization of HA trimer at which epitopes of HA2 gp hidden in the native HA are exposed out from HA trimer and become accessible for antibodies. For monitoring the fusion process we used haemolytic test. To follow the conformational changes of HA during pH alteration, we used MAb IIF4 which binds to fusion active form of HA, but not to the native HA trimer. In this work we compared two viruses antigenically identical, with different pH dependence of fusion activity accompanied with conformational changed HA trimer at corresponding pH decrease. At pH 6.5 already 58% of Ab4 HA was conformationally altered, whereas of Dun virus only 30,5%. Maximal difference was noticed at pH 5.5 when

100% of HA of Ab4 virus had a low pH conformation, while only 45,3% of HA of virus Dun has been changed. In our further studies we showed, that the difference in fusion activity had an impact on replication activity of viruses in MDCK cells. Orderly 15-16 times higher titer of virus was achieved after infection of MDCK cells with Ab4 virus, which revealed a maximum of fusion at higher pH (5.5), than virus Dun (pH 5.0). However, we did not recognize a difference in *in vivo* replication of viruses Ab4 and Dun in mouse lungs on day 2 p.i. Similarly no marked difference in antibody response was recorded. Here, it must be stressed that viruses Dun and Ab4 represent viruses of low virulence (Fislová et al., 2009) and did not cause any clinical symptoms of disease or death. In our further studies, therefore we shall examine how changes in the fusion activity, introduced by site-directed mutations to HA of viruses of medium (A/Mississippi/1/85(H3N2)) or of high virulence (A/PR8/34(H1N1)) can influence their pathogenicity.

Acknowledgements

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ASSOCIATION ANALYSIS OF THE COMMON FTO VARIANT RS9939609 WITH OBESITY IN THE SLOVAK POPULATION

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Abstract: *One of the most recently discovered candidate genes for obesity is FTO gene. Up to now, many replication studies confirmed an association between common genetic variants of FTO gene and obesity in many different European populations. The aim of our study was to examine the association of the rs9939609 polymorphism in FTO gene with obesity phenotype in Slovak population. Our study involved 335 unrelated adults (mean age 32.47±11.49 years). Genomic DNA was extracted from buccal swabs. TaqMan assay SNP genotyping was performed using Applied Biosystems StepOne™ Real-Time PCR system (Applied Biosystems Foster City, CA, USA). Frequency of the FTO rs9939609 polymorphism risk allele A was 0.44 and the genotype distribution was in agreement with Hardy-Weinberg equilibrium. Our results showed significant differences in mean values of obesity related anthropometric parameters such as BMI, waist circumference and WHR between different genotypes (p=0.004, p=0.001 and p=0.014, respectively). In conclusion, the results of this study revealed a significant association between FTO polymorphism rs9939609 and obesity in Slovak population.*

Key words: *FTO, polymorphism, association, obesity, population*

In the last two decades, the prevalence of obesity has increased significantly and currently represents a serious global health problem. Obesity is a complex multifactorial disease that results from an imbalance between energy intake and expenditure, and this delicate balance is affected by the interaction of a number of environmental risk factors and genetic background. The development of molecular genetics has resulted in the progress of obesity research, which recently focuses on the identification of candidate genes responsible for its development. One of the last identified candidate genes responsible for polygenic form of obesity is FTO gene. FTO gene represents the first example of the common gene predisposing to obesity at the population level. FTO gene is located in the position 12.2 on chromosome 16 (16q 12.2), it consists of 9 exons and a total length of over 400 kb. Although the biological function of the FTO gene and its product is not known yet, expression gene profile reveals that the human FTO gene is expressed in many tissues, including the pancreatic islets, adipose tissue and muscles (Frayling et al., 2007; Dina et al., 2007; Wåhlen et al.,

2008; Andreasen et al., 2008, Gerken et al., 2007). Cross-sectional studies focusing on the research of gene variants association with specific phenotypic expression help to elucidate the mechanism by which the risk of obesity occurrence increases. The aim of this study was to determine the allele and genotype frequencies of rs9939609 T>A polymorphism located in the first intron of FTO gene in the Slovak population and to analyze the association of this polymorphism with selected obesity indices.

Material and methods

Our presented study involved 335 randomly selected, unrelated adult subjects (mean age 32.47 ± 11.49 years) living in Presov region in the northeastern Slovakia. The study sample consisted of 112 men and 223 women. The study was conducted after obtaining written informed consent from each volunteer of study. All anthropometric measures including body weight, height, waist circumference (WC), hip circumference, body mass index (BMI) and waist-to-hip ratio (WHR) were taken using standard methods by qualified anthropologists. For the need of genetic testing the genomic DNA was extracted from buccal swabs using JetQuick kit (Genomed). The rs9939609 SNP in the first intron of the FTO gene was selected for analysis in our study population (Frayling et al., 2007). SNP genotyping was performed by the TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA, USA) using the Applied Biosystems StepOne™ Real-Time PCR system (Applied Biosystems Foster City, CA, USA). The allele frequencies were determined by the direct gene counting method. Descriptive statistics are given as sample size and percentages or arithmetic mean ± SD (standard deviation). All statistical analyses were performed with Predictive Analytics Software (version 17.0, SPSS Inc., Chicago, IL). P values < 0.05 were considered statistically significant.

Results and discussion

The distribution of allele and genotype frequencies for rs9939609 polymorphism of FTO gene in 335 analyzed samples is shown in table 1. In the Slovak population the minor allele frequency (MAF) of rs9939609 was 0.44. Our findings are in agreement with the MAF reported in Caucasian population which corresponds to the geographic localization of Slovak Republic in Europe (http://www.ncbi.nlm.nih.gov/SNP/). The frequency of genotypes for the FTO rs9939609 was AA 17.3%; AT 53.7%; TT 29.0% in Slovak study subjects. We found no evidence for deviation of genotype distribution from the Hardy-Weinberg equilibrium.

Obesity is a disease resulting from the complex interaction between genes and the environment. Obesity has a heterogeneous origin and pathways to its development and progression are different, therefore it is necessary to perform a number of studies in different ethnic populations, which could help clarify the mechanism that forms the basis of the association between the genetic component and obesity traits. Andreasen et al. (2008) confirmed an association of rs9939609 polymorphism with increased body weight, BMI and waist circumference in Danish population. Gonzáles-Sán-

chez et al. (2009) presented a strong association of rs9939609 polymorphism with obesity in Spanish population. Very similar results of association of FTO genetic variants with obesity confirmed also Dina et al. (2007) in the French population, Hubacek et al. (2008) in the Czech population, Villalobos-Comparan et al. (2008) in the Mexican population and Peeters et al. (2008) in the Belgian population. There are also studies that indicate significant differences in the association between the FTO gene variants and obesity. Li et al. (2008) in the Chinese population, Ohashi et al. (2007) in the Oceanic populations and Horikoshi et al. (2007) in the Japanese population have not been successful in confirming the association of FTO variant with obesity traits, possibly due to the relatively low frequency of risk allele in these populations. However, other studies (Chang et al., 2008, Cha et al., 2008, Hotta et al., 2008) have confirmed a strong association of FTO genetic variants with obesity by which they disproved the findings that FTO gene is not associated with obesity in population of Asian descent. Although the frequency of the risk allele vary in different ethnic populations, the power of its association with obesity is relatively stable and the extent of the influence of mutant allele on the risk of obesity and higher BMI is comparable to findings in European populations.

Table 1 Frequency of FTO rs9939609 in the Slovak population

FTO rs9939609	Number of subject	Frequency of genotypes	Frequency of allele A
Homozygote AA	58/335	17.3%	
Heterozygote AT	180/335	53.7%	0.44
Homozygote TT	97/335	29.0%	

Table 2 Comparison of anthropometric parameters among different genotypes of FTO rs9939609 in the Slovak population

Parameters	Genotype (mean ± SD)			P value
	TT	AT	AA	
Age (years)	33.72 ± 12.40	31.88 ± 10.87	32.22 ± 11.80	0.440
Height (cm)	169.99 ± 8.77	170.61 ± 9.09	171.52 ± 9.22	0.594
Weight (kg)	65.75 ± 12.77	68.14 ± 15.38	73.53 ± 14.65	0.006
Waist circumference (cm)	79.75 ± 11.50	83.21 ± 12.91	87.66 ± 12.25	0.001
Hip circumference (cm)	98.01 ± 8.59	100.69 ± 8.75	102.21 ± 9.14	0.009
BMI (kg/m²)	22.67 ± 3.58	23.36 ± 4.11	24.86 ± 3.87	0.004
WHR	0.817 ± 0.08	0.823 ± 0.09	0.856 ± 0.09	0.014

Data are presented as mean ± standard deviation. BMI-body mass index in kg/m², WHR-waist to hip ratio. P values were analyzed using ANOVA test.

In our study we tested the possible association between obesity and the FTO rs9939609 SNP in the Slovak population. The average value of BMI index in individuals with risk genotype AA was 24.86 ± 3.87 kg/m², while the individuals with protective TT genotype had a BMI index of 2.19 ± 0.29 kg/m² lower. The average value of waist circumference in individuals with AA risk genotype was about 7.91 ± 0.75 cm higher than in individuals with protective TT genotype. Genotype-phenotype association analysis showed statistically significant differences between genotypes in body weight, BMI, waist circumference and hip circumference ($p = 0.006$, $p = 0.004$, $p = 0.001$ and $p = 0.009$). The differences between the genotypes in the WHR index ($p = 0.014$) were also significant which points to an increased risk of obesity for individuals with mutant AA genotype.

Conclusion

In this study we reported the allele and genotype frequencies of rs9939609 FTO variant in the Slovak population and we also demonstrated its association with obesity indices such as BMI, waist circumference and WHR implying that the presence of mutant allele is inflicting higher risk of obesity. Obesity is a serious health problem and a significant number of obese people die of diseases caused by complications from increased weight. Therefore we suppose that knowing the risk genotype may help to identify individuals susceptible to obesity what could improve the overall approach to preventing and treating obesity in clinical practice.

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ARCHAEOGENETIC RESEARCH OF THE CONTACT ZONE FROM THE 10TH CENTURY IN SLOVAKIA

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Abstract: *Ancient DNA studies significantly increase our knowledge about medieval populations obtained by archaeological, historical and anthropological methods. It is particularly appropriate to use mitochondrial DNA isolated from bones or teeth, which provides significant information about the maternal lineage. Mitochondrial DNA can provide information about the origin of population, ancestors from the maternal side, the geographical distribution of haplogroups and the familial relationships between individuals. From that perspective, it is interesting to analyze the multi-ethnic populations living in the so-called contact zone in the territory of the Great Moravian and later Hungarian state formations. These populations probably lived genetically isolated for some time. The aim of this study is to identify the origin of individuals from early-medieval cemeteries localized in the contact zone based on the analysis of ancient mtDNA markers. Obtained data can supplement former archaeological investigations.*

Key words: *archaeogenetics, ancient DNA, mitochondrial DNA, haplotype, mutation*

New findings in molecular genetics affect many other sciences such as botany, microbiology, zoology, medicine, veterinary medicine, forensics, evolutionary biology, etc.. Molecular analysis of aDNA (ancient DNA) increasingly penetrate into archaeology and allows refining the initial findings, identifying individuals, determining the sex, detecting diseases, assessing the relative genetic distance of populations, thereby determining familial relationships between the findings. The archaeogeneticists gain knowledge that provide new perspectives in recognition of our history. DNA amplification by polymerase chain reaction (PCR) also significantly affected the research of historical DNA. This method is applicable to the analysis of nuclear and extra-nuclear DNA in cells. Analysis of extra-nuclear DNA, such as mitochondrial DNA (mtDNA) is especially recommended in the study of historical findings. Nuclear DNA is much more vulnerable, less resistant to physical impact acting on it over the centuries. On the contrary, the mitochondrial DNA genome is very compact, containing little repetitive DNA, and codes for 37 genes (Figure 1). The genetic code of the mtDNA differs slightly from that of nuclear DNA. The mitochon-

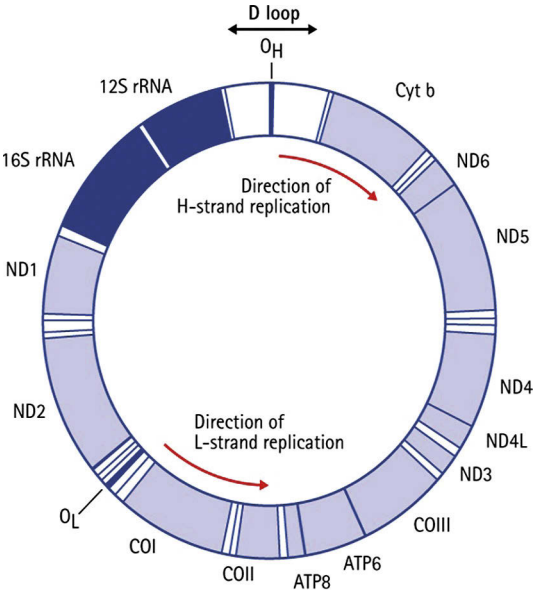

Figure 1: The sampling of bone tissue of archaeological stock

dria of the fertilized zygote are almost exclusively inherited from the oocyte, leading to the maternal pattern of inheritance (Turnpenny**,** Ellard, 2007).

Mitochondrial genomes with a less effective reparation mechanism are subjected to a more intense mutation process than the nuclear genome containing histones, which protect the DNA against mutations. Based on the analysis of frequency of mtDNA mutations in samples of the current population and comparison with aDNA the Mitochondrial Eve Theory (Out of Africa) was created, under which the Mitochondrial Eve (mt-MRCA) is the name of the woman, who is defined as the matrilineal most recent common ancestor (MRCA) for all currently living humans. Passed down from a mother to her offspring, all mtDNA is derived from her in every living person.

Based on analysis of a set of specific mutations in DNA, the so-called haplotypes can be determined. These specific mutations are point mutations - SNP (single nucleotide polymorphisms), which are a significant source of variability in DNA. The similar haploptypes derived from a common ancestor are called haplogroups. Understanding the evolutionary path of the female lineage has helped population geneticists trace the matrilineal inheritance of modern humans back to human origins in Africa. The origin of haplogroups formation and their extension path is already known. Accordingly, there are sub-Saharan (African) haplogroups: L0, L1, L2, L3, L4, L5, L6, L7, Western Eurasian haplogroups: H, T, U, V, X, K, I, J, W, East-Eurasian haplogroups: A, B, C, D, E, F, G, haplogroups of Native Americans: A, B, C, D, X, and the haplogroup of the South Pacific: P, Q, S. This phylogenetic tree is based on the van Oven (Van Oven, Kayser, 2009) tree and subsequently published papers.

The aim of our study is the origin and the examination of family ties in the territory of the Great Moravian and Hungarian state clusters in the early Middle Ages using anthropogenetical methods. The goal is to expand the opportunities for archaeological research examining the selected samples from the cemeteries in the contact zone with modern methods such as analysis of mitochondrial DNA markers. This study will be realised in cooperation with Constantine the Philosopher Univer-

sity in Nitra, J. Selye University in Komarno, Hungarian Academy of Science in Budapest and the Archeological Institute SAS in Nitra.

Materials and methods

Samples shall be taken from small family burial sites from the 10th century, which are well defined, and the number of individuals within the repository does not exceed 20. Samples will be collected from long bones or teeth. Then in the laboratory the cleaning the bone samples is followed by their milling with a grinding ball. We have to acquire the compact part of the bone from the area between the epiphysis and diaphysis with an electric sawing machine (Figure 2), and also withdraw a sample from a tooth. During the sampling we must follow strict procedures to minimize the contamination of samples with recent DNA.

Figure 2: The sampling of bone tissue

Data-records will continuously be made, and a photo-documentation will be prepared about bones. The prepared bone powder will be used for the isolation of mtDNA, then the 360 bp hypervariable region of mtDNA (HVR-I) will be amplified by PCR (Tomory, Csanyi, Bogacsisyabo, et al.,2007). PCR fragments will be electrophoretically separated and subjected to fragment analysis and/or sequenced. It is important to check the efficiency of DNA amplification and the quality of PCR products. The DNA sequences of mtDNA will be compared with the revised Cambridge Reference Sequence (rCRS) (Andrews, Kubacka, Chinnery, et al. 1999) and classify the corresponding haplogroup. In addition, determined genetic markers in the mtDNA might be useful in diagnostics of specific diseases.

Expected results

The genetic diversity can be used to provide information concerning female--specific patterns and migrations that occurred in the past and possible family relationships between individual samples. Results supplemented by archaeological data from a burying ground permit a more accurate characterization of the population, than would be possible without genetic research.

In human genetics, a human mitochondrial DNA haplogroup is a haplogroup defined by differences in human mitochondrial DNA. Haplogroups are used to represent the major branch points on the mitochondrial phylogenetic tree. These mitochondrial haplogroups (clusters) arose thousands of years ago and show a characteristic geographical distribution. In progress of time and accumulation of mutations these groups are more differentiated, more and more subgroups and additional variants have appeared. If two samples belong to the same haplogroup, but their mutation positions do not show complete similarity, they belong to different haplotypes within the haplogroup. The less significant difference between two mitochondrial patterns of findings assign more closely related to each other on maternal line. Understanding the evolutionary path of the female lineage has helped population geneticists trace the matrilineal inheritance of modern humans back to the human lineage of its origins in Africa and the subsequent expansion across the globe (Figure 4).

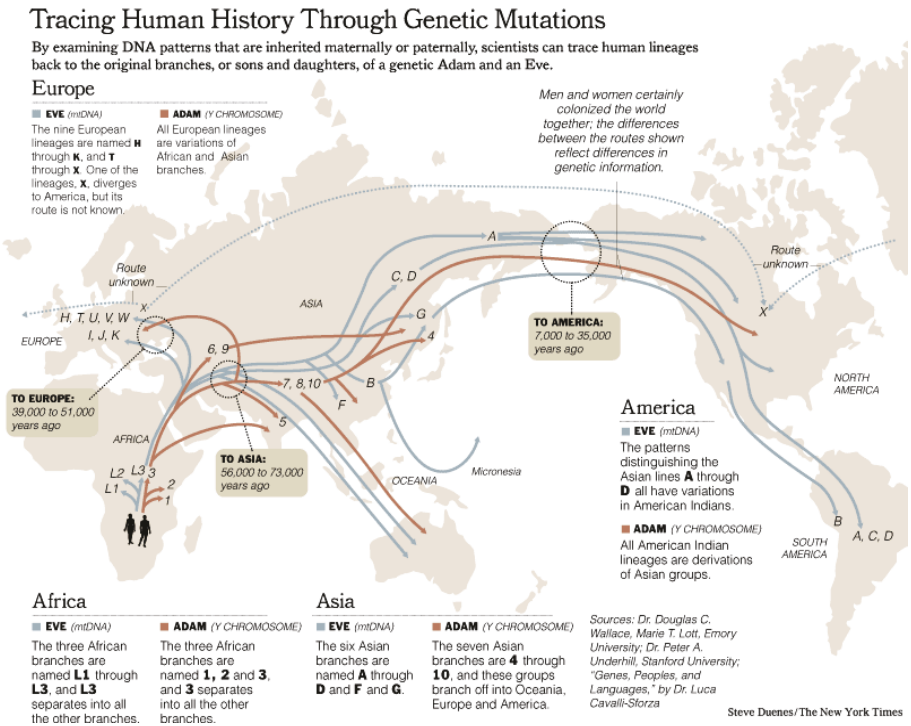

Figure 4: Track of the human migration (Wade, 2000)

We expected interesting information about haplogroups, which is characterised by specific mutations. Even if the patterns are completely similar it is not certain that two dead people have a direct family relationship. If the mtDNA profile of two people

is completely identical, they share a common maternal ancestor but this ancestor might have lived a generation before or thousands of years ago.

The origins and spread of haplogroups can be associated with certain geographic regions. Therefore, the research of haplogroups allows the monitoring of the wanderings of some populations, and their mixing with each other.

We will try to identify which haplogroups belong based on our samples. Some of mtDNA mutations cause genetic diseases with physiological expression and through the mutations we can characterize the individuals of investigated ancient population. We will find if our samples carriers of mtDNA mutations. But a clear correlation between the mutation and disease can be regarded as a very rare exception because of the properties of mitochondrial genetics. That the disease is always behind the same mutation, or that the mutation is always associated with the appearance of the same disease, is very rare. Because heteroplasmic cells cause most diseases, the various tissues may show different results based on the tests. These cases can be quite difficult to interpret. (Venetianer) Therefore, the detection of recent mtDNA mutations has limited importance in laboratory practice compared to the nuclear DNA-testing. In most cases we usually have so little information about the archaeological samples that we are trying examine all the possibilities.

Through this method archaeologists are now gaining new insight and information to open new areas concerning examining our past in such dimensions that previously could not be taken into account.

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COMPARISON OF METHODS OF Y-HAPLOGROUP DETERMINATION IN THE SLOVAK ROMANY POPULATION

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Abstract: *Y-chromosomal polymorphisms are valuable markers to study the origin and history of human populations. Approaches to predicting the Y-chromosome haplogroup from a set of Y-STR markers is presented and compared to results of Y-haplogroup determination by SNP analysis. In this study the efficiency of two programs (Athey`s Haplogroup Predictor, Cullen`s Haplogroup Predictor) for Y-haplogroup prediction was tested with 130 samples of know haplotypes and haplogroups from the Slovak Romany population. One subclade of Haplogroup H, H1a- M82, was the most frequent in the studied population with the frequency 43.07%. Our results showed that Y-chromosome haplogroup prediction based on Y-STRs is less accurate. 3.84% of error has revealed in the studied samples by using haplogroup predictor. Typing a set of SNPs precisely define a phylogenetic branch and is reliable method to establish, to which haplogroup a given sample belongs.*

Key words: *haplogroup, Y-polymorphisms, prediction, Romany population*

Romanies (also Gypsies) constitute the largest minority in the Slovak population. On the basis of historical and social data, they represent a genetic isolate whose gene pool was influenced by strong genetic drift and relatively small population size. These predictions can be tested using a genetic approach (Pamjav et al., 2011).

Determining human Y-haplogroups is the most useful tool in tracing human histories that have arisen a single time in human evolution. In study we determinated the Y-chromosome haplogroups in the Slovak Romany population using Y-SNPs. These binary markers are characterized by a low mutation rate and therefore their occurrence represents a unique event in geographic or ethnic origin of paternal human lineage (Brion et al., 2005). The selected SNPs were to permit the classification of the analyzed samples according to the main haplogroups, which are described in the 2008 haplogroup tree of the International Society of Genetic Genealogy (Y-DNA Haplogroup Tree, 2012).

Concurrently we confirmed the Y-chromosome haplogroups through Y-STRs by using haplogroup predictor. The efficiency of two programs for haplogroup prediction was tested in the Romany individuals of known haplotypes. One of them is Whit Atheys' Haplogroup Predictor (Athey, 2006.) which has been employed in

previous studies (Salas et al., 2008; Mertens , 2007; Goff et al., 2006) to estimate the ethnic composition of different populations and diagnostic STR values of a given haplogroup. Whit Athey's Haplogroup Predictor version 5, which is based on the Bayesian allele-frequency approach. The second is Jim Cullen`s Haplogroup Predictor. The predictor utilizes 86 haplotypes (Y-STR-37 markers) representing the world's haplogroups / subhaplogroups, and 56 haplotypes (Y-STR-67 markers) of Haplo-I subclades, for a total of 142 modal haplotypes used for comparison to selected Y-STR signature.

The purpose of this study was to establish the accuracy of the both software systems and compare them with obtained results from Y-SNP typing.

Materials and methods

The samples for this study were Slovak Romanies (men) (N=130) from several part of Eastern Slovakia. Genomic DNA was extracted from buccal swabs using a Jet Quick DNA Tissue Kit (Genomed). Informed consent was obtained from all individuals participating in the study. Table 1 shows the Y-SNP markers tested using TaqMan probes in 7500 Fast Real Time PCR system (Applied Biosystems, Foster City, CA). PCR was performed according to the manufacturer's instructions. The haplogroup frequencies were estimated by the counting method. Haplogroup diversity was calculated according to Nei (Nei, 1973). Median joining (MJ) networks based on the Y-STR profiles were constructed with the NETWORK 4.6.1.0 software package available at www.fluxus-engineering.com. Networks were generated using the MJ algorithm, with the microsatellite loci weighted proportionally to the inverse of the repeat variance observed in each Y-haplogroup lineage (Martinez et al., 2007).

Y-haplotypes of studied samples were defined by analyze of 12 Y-STR markers DYS391, DYS389I, DYS439, DYS389II, DYS438, DYS437, DYS19, DYS392, DYS393, DYS390, DYS385a/b) (PowerPlex® Y System, Promega). These data were obtained from our previous study (Petrejcikova et al., 2009). Haplotypes were submitted to the Athey`s and Cullen`s Haplogroup Predictor, with equal priors, obtaining probabilities for inferred haplogroups.

Results and discussion

The Y-SNP data from 130 Romanies is presented in Table 1. The assignment of haplogroups presented in this study follows the phylogenetic tree of the Y Chromosome Consortium (Y-DNA Haplogroup Tree, 2012). Table 1 shows Y-chromosomal haplogroup frequencies and diversity value for the examined Romany population. The haplogroup with the highest frequency was haplogroup H (43.07% - 56 males), particularly H1a-M82, followed by haplogroup E, sublineage E1b1b1a-M78 (19.23% - 23 males). Distributions of other haplogroups in the order of their relative frequency were: J2a2-M67 (10.76% -14 males), R1a1-M198 (9.23% -12 males), I1-M253 (6.92-9 males), R1b1-P25 (5.38% -7 males), I2a1-P37.2 (4.61-6 males), G2a-P15 (0.76%

- 1 males), I2b-M223 (0.76% - 1males) and J2-M172 (0.76% - 1males). Haplogroup diversity for the studied population was 0.75902.

Table 1 Y-chromosomal haplogroup distribution and haplogroup diversity of the Slovak Romany population

Y-Haplogroups	Mutation	Slovak Romanies (N=130)	
		N	%
E1b1b1a	M78	23	17.69
G2a	P15	1	0.76
H1a	M82	56	43.07
I1	M253	9	6.92
I2a1	P37.2	6	4.61
I2b	M223	1	0.76
J2	M172	1	0.76
J2a2	M67	14	10.76
R1a1	M198	12	9.23
R1b1	P25	7	5.38
Haplogroup diversity		0,75902	

A phylogenetic network (Median Joining) comprising all 130 Romany haplotypes showed separation of detected haplogroups into two Y-chromosomal lineages (see Fig. 1) These lineages include ancestral Indian Y-lineage (H1a-M78) and present day Eurasian Y-lineages (E1b1b1a, R1a1, R1b, J2, J2a2, I1, I2a1, I2b) (Zalán et al., 2011). The presence of Eurasian Y-lineages in Romany gene pool can be explained by their migration across the Near East and Balkan Peninsula (Pamjav et al., 2011; Zalán et al., 2011; Gresham et al., 2001; Pericic et al., 2005). The network program computed the estimate the time to the most common resent ancestor (TMRCA) for haplogroup H (H1a). TMRCA was 926 (138-2783) years ago in the Romany population. This coalescent date corresponds to the date when Romanies arrived in the Byzantine Empire 900-1000 years ago (Gresham et al., 2001). Our result has also been confirmed by the previous publications (Pamjav et al., 2011; Gresham et al., 2001; Sengupta et al., 2006).

Figure 2 showed MJ network of H1a-M82 sublineages and compared 3 Roma population groups (Hungary, Bulgaria and Croatia) with Slovakian Romanies and two Indian populations (Southern India and Malaysia). Results demonstrated the Gypsy modal haplotype 15-15/17-22-10-11-12-14-9-11 (DYS19, DYS385a/b, DYS390, DYS391, DYS392, DYS393, DYS437, DYS438, DYS439) was shared by all Romani group and by both the Indian populations. Studies supported that Indian may have contributed as an ancestral source population for the proto-Romany group (Gresham

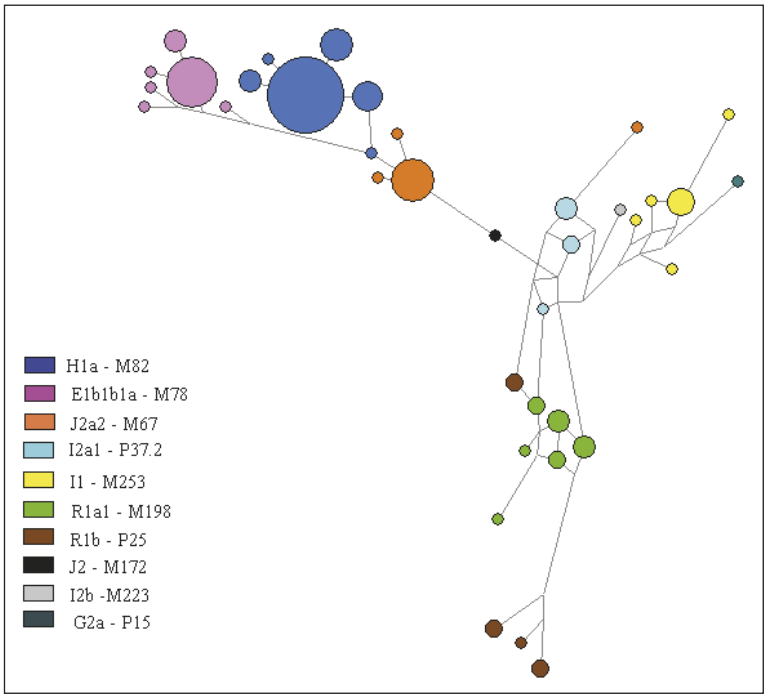

Figure 1 Median joining network for haplotypes from the Slovak Republic with the assigned generic haplogroup. Circles represent microsatelite haplotypes with an area proportion to frequency and colors indicate the different haplogroups

et al., 2001). The Romany populations in Europe are the main source of haplogroup H. The ratio of haplogroup H in the European populations is less than 5% (Wells et al., 2001).

SNP determinated haplogroups were compared with those provided by the software (Athey`s Haplogroup Predictor, Cullen`s Haplogroup Predictor). When comparing our results from Y-SNP typing to the haplogroup prediction, all haplogroups were correctly assigned except 5 samples representing 3.84% of the total. Results have revealed a 4 samples with error in haplogroup prediction based on Y-STR haplotypes using Athey`s Haplogroup Predictor. 2 errors in haplogroup prediction were determined by using Cullen`s Haplogroup Predictor. Table 2 shows haplogroups incorrectly assigned with Haplogroup Predictors.

According Y-SNP typing the male lineage 10-14-11-30-10-16-15-11-13-21-13/14 (DYS391, DYS389I, DYS439, DYS389II, DYS438, DYS437, DYS19, DYS392, DYS393, DYS390, DYS385a/b) was characterized by the C->T base exchange at M253 locus defining haplogroup I1. Athey`s Haplogroup Predictor incorrect assig-

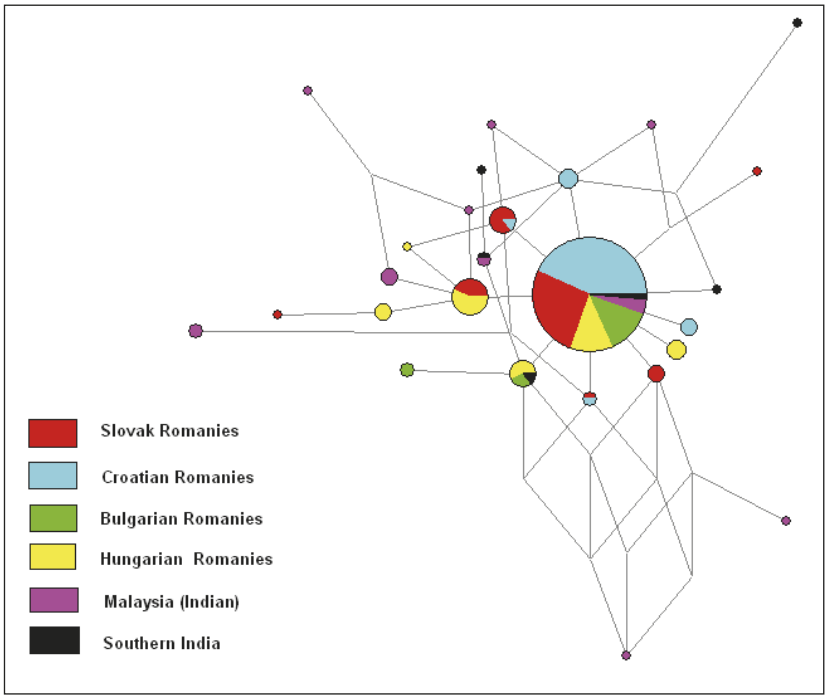

Figure 2 Median joining network based upon haplotypes of individuals within haplogroup H1a using 10 Y-STR loci.
Romany population data were compared with that previously published for Croatian Romanies (Klaric´ et al., 2009), Bulgarian Romanies (Gresham et al., 2001), Hungarian Romanies (Nagy et al., 2007), Indian populations from Malaysia (Chang et al., 2007) and Southern India (Frank et al., 2008). Circles represent microsatelite haplotypes with an area proportion to frequency and colors indicate the population of origin

ned this haplotype to haplogroup G2a. It also failed when predicting haplogroup J, sublineage J2a2, (for haplotype 10-13-12-29-9-15-14-11-12-24-13/15) a haplogroup Q (for haplotype 11-13-11-30-11-14-13-11-13-24-17/18) for samples, while the Y-SNP typing excluded these possibilities and determined haplogroups J2-M172 (T->G base exchange) and E1b1b1a-M78 (C->T base exchange). Cullen`s Haplogroup predictor failed to classify one sample to haplogroup I1-M253 for the male lineage 10-12-12-28-10-16-15-11-14-22-12/16. The data of Y-SNP analysis showed, that the sample belongs to sublineage G2a (haplogroup G). In our dataset, the R1b-P25 subclade was detected in the paternal gene of 7 Romany individuals. In both haplogroup predictors, one sample of them was assigned to haplogroup R1a in the male lineage 10-13-12-30-11-14-16-11-13-25-11/14.

Table 2 Haplogroups incorrectly assigned with Haplogroup Predictors and the Y-SNP haplogroup for each case

Prediction haplogroup		Y-SNP typing	
Athey`s predictor	**Cullen`s predictor**	**Y-Haplogroup**	**N**
G2a	I1-M253	I1-M253	1
G2a	I1-M253	G2a-P15	1
J2a2	J2-M172	J2-M172	1
Q	E1b1b1a-M78	E1b1b1a-M78	1
R1a	R1a	R1b1 – P25	1

Both of predictors proved to be a good estimators for haplogroup prediction, but Cullen`s method gives deeper prediction than Whit's, because his results from Y-haplotypes contained information about corresponding mutation of haplogroup subclade. Haplogroup prediction could be quick and easy method, when Y-SNP information is not available (Núñez et al., 2012). The proportion of error observed in the allele-frequency haplogroup prediction method may not be so critical for anthropological studies (Núñez et al., 2012) and may help to estimate the population of origin. But an inaccurate prediction cannot be ignored in forensic applications, where Y-SNP typing is necessary. Muzzio et al. (Muzzio et al., 2011) confirmed a high probability error in R haplogroup based on haplogroup prediction. It is assumed that an increase in the number of Y-STR/Y-SNP studies could reduce haplogroup prediction inaccuracies (Muzzio et al., 2011). At present, haplogroup determination by SNP analysis remains the best approach, considering the lower reliability of prediction of software available (Muzzio et al., 2011).

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A SHORT REGION OF PB1-F2 INFLUENZA A VIRUS PROTEIN SEEMS TO BE IMPORTANT FOR INTERACTION WITH VIRUS POLYMERASE SUBUNIT PB1

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Abstract: *Influenza A virus is an important pathogen, which causes widespread infections in birds, mammals. In humans, influenza causes frequently epidemics and pandemics because of antigenic changes in surface glycoproteins. A major proapoptic virulent and pathogenic factor of influenza A virus is PB1-F2 nonstructural protein that can induce cell death and potent bacterial secondary infections. PB1-F2 is localized in infected cells in mitochondria, cytoplasm and nucleus. It was shown that PB1-F2 interacts with PB1 subunit by N-terminal region and enhances expression of catalytic polymerase subunit. This interaction influences the intracellular localization of PB1. The deletions specify was prepared by mutagenic PCR to identify short 10aa long PB1-F2 region responsible for interaction with influenza A virus catalytic subunit. Lost of interaction between particular deletion mutants of PB1-F2 protein and catalytic polymerase subunit (PB1) was tested by proximity ligation assay. This information will be used to design and test therapeutic peptides which could hinder PB1-F2 interaction with-PB1 and thus decrease virus replication.*

Key words: *deletion library, Influenza A virus, PB1-F2 protein, proximity ligation assay*

Influenza A virus (IAV) is an important human pathogen that causes yearly epidemics and sporadic pandemics, due to its antigenic changes. Genome of IAV is ssRNA of negative polarity and divided into 8 segments. The replication and transcription of IAV genome in nucleus of infected cells "runs" through ribonucleoprotein complex. Every segment is associated with nucleoprotein and with the RNA-dependent RNA polymerase (Resa-Infant et al., 2010). The RNA polymerase consists of three subunits encoded by virus: polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase acidic protein (PA) (Muratore et al., 2012). PB1 protein is coded by segment 2 of IAV genome and is involved in catalytic activity including nucleotide polymerization and chain elongation. By comparative sequence analysis was found out that PB1 possesses four conserved motifs (Siddhartha et al., 1944). In a year 2001 was discovered eleventh protein of IAV during the systematic search for peptides recognized by CD8+T lymphocytes (Chen et al., 2001). It is PB1-F2 non--structural protein (10,5 kDa). PB1-F2 is encoded by the +1 open reading frames of

the PB1 genomic segment. In infected cells PB1-F2 shows a predominant localization to mitochondria but also in the cytoplasm and the nucleus (Krumbholz et al., 2011). This protein localizes in mitochondria through its C-terminal region and induces cell apoptosis and loss of mitochondrial membrane potential (Wise et al., 2009). Due to its properties is considered as virulence factor. PB1-F2 enhances inflammation during the primary viral infection of mice and increases both the frequency and severity of secondary bacterial pneumonia (Mcauley et al., 2007). The absence of PB1-F2 results in an altered localization of catalytic polymerase subunit (Mazur et al., 2008). It was shown that N-terminal region of PB1-F2 protein was responsible for the increase in PB1 protein expression (Košík et al., 2011). Following this findings in our laboratory we prepared deletion mutants of PB1-F2 protein for identify of region responsible for interaction with IAV catalytic polymerase subunit. This information will be used to design and test therapeutic peptides which could compete PB1-F2 to PB1 interaction and thus decrease virus replication.

Materials and methods

Cells and viruses: Madin-Darby Canine Kidney cells (MDCK) were grown in DMEM (Dulbecco´s Modified Eagle´s Medium, BioWitthaker) suplemented with 5% fetal bovine serum (HyClone), gentamicin 40 μ/ml at 37°C in 5% CO_2. Allantoic fluid containing IAV A/Puerto Rico/8/34 (PR8) (H_1N_1), A/Puerto Rico/8/34 conferring site mutation in the position T120C, C153G on the 2 segment (PR8ΔPB1-F2) was kindly provided by Bennick JR and Yewdell JW (NIH, Bethesda, USA) and was prepared by Palese P.

Deletion library preparation: It was used multifunctional expression vector pTriEx-4 with PB1-F2 sequence (87 aa) from PR8. Deletion library was prepared by PCR mutagenesis (Finnzymes, Phusion® Site-Directed Mutagenesis Kit) by the recommendations of manufacturer. Primers were designed that the next deletion partially overlaps with previous deletion. It was designed 17 pairs of primers (5´-phosporylated) which introduced 10 aa deletion into PB1-F2 protein.

Cell transfection: MDCK cells were propagated on 24-well plates to 70-80% confluence. Turbofect ™ *in vitro* Transfection Reagent (Fermentas) was used as recommended by the manufacturer and after 24 h post transfection the samples were used in further experiments

Polyclonal and monoclonal antibodies: Polyclonal rabbit serum specific for the N- terminal part of PB1-F2, was prepared by rabbit subcutaneous immunization with KLH conjugated peptide corresponding to aa position 3 to 13 of PB1-F2, polyclonal rabbit serum raised against full-length PB1-F2 (PR8) was kindly provided by (Dr. Yewdell J, Bethesda, NIH), polyclonal serum against PB1 protein was prepared by the DNA vaccination, complete immune mouse serum (IMS) was prepared by intraperitoneal immunization of BALB/c mouse with whole purified virus PR8, mouse anti-β-actin monoclonal antibody (Sigma-Aldrich), was used to detection of the β-actin (loading control), monoclonal antibodies (MAb) AG55 anti PB1-F2 (Krenusová et al., 2009) and 107L2 anti-NP (Varečková et al., 1995), MAb M21 anti-

M1 and MAb NS1 anti-NS1 (Yewdell J, Bethesda, NIH) were used for detection of IAV proteins, polyclonal rabbit anti-mouse IgG FITC conjugate (DAKO), polyclonal rabbit anti-mouse HRP conjugate (DAKO) were used as secondary antibodies in indirect immunofluorescence and western blot.

Western blot: Cells for Western blot analysis were lysed directly on the Petri dishes with 5x sample buffer, loaded onto a 12% SDS PAGE gel. Separated proteins were transferred on the nitrocellulose membrane. The blot was then washed in PBS, blocked overnight in 5% non-fat dried milk at 4°C, incubated with primary antibody for 90 mins at RT, washed in PBS three times for 15 mins, incubated for 90 mins with secondary antibody at RT, washed three times in 0,2% NP-40 in PBS, developed with luminol (Sigma-Aldrich) solution and exposed to the photography film.

Indirect immunofluorescence: MDCK cells were grown on glass cover slips to 60-70% confluence. The cells were transfected with plasmid DNA using Turbofect (Fermentas) or infected with PR8 MOI (multiplicity of infection) 10 PFU or transfected and co-infected. 3-7 hrs post infection (p.i.) the cells were fixed by methanol for 5 mins at -20°C. Samples were washed three times with PBS and incubated with the primary antibody 90 mins at RT. Then samples were washed and were incubated for 90 mins at RT with secondary antibody. After final wash step, the samples were mounted with DAPI (4´6´-diamino-2-phenylindole) containing mounting medium (Santa Cruz Biotechnologies). Fluorescence was visualized with confocal microscope LSM Zeiss 510 Meta or with fluorescence microscop Leica.

Proximity ligation assay: MDCK cells were grown on glass coves slips to 60-70% confluence and were transfected with deletion mutants of PB1-F2. After 24 hrs were infected with PR8 virus and 6 hrs p.i. were fixed with 2% paraphormaldehyde 10 mins than permeabilized with 1% Triton X100 (Sigma Aldrich) in PBS 1 mins and blocked for 30 mins. The samples were incubated with primary antibody in a humidity chamber (1 h at 37°C). To the samples were added secondary antibody PLA+ and PLA- probes and incubated in a humidity chamber (1 h at 37°C). To the samples was added ligation-ligase solution and incubated 30 min at 37°C. Next to the samples was added amplification-polymerase solution and incubated 100 min at 37°C. Samples were washed between each step two times for 5 mins. The slides were mounted with mounting medium.

Results

We have shown that the presence of PB1-F2 dramatically increases the expression of catalytic polymerase subunit (Fig.1). PB1-F2 also enhances the expression of other IAV proteins (NP, NS1 and M1) (Fig.2). In the left row of the figure are cells only infected with PR8ΔPB1-F2 (MOI 10 PFU) and on the right are cells transfected with pTriEx-4 PB1-F2 and infected with PR8ΔPB1-F2 (MOI 10 PFU). The expression of NP, NS1, M1 and PB1 is represented by green colour and the presence of PB1-F2 is represented by red colour because of Fitc or Texas red conjugated secondary antibodies were used respectively. It was prepared 17 deletion mutants of PB1-F2 protein (Fig. 3) by PCR mutagenesis and DNA sequencing was used for the cloning

correctness confirmation of the resulted expressing plasmids. Immunofluorescence was used for the confirmation of expression ability (data not shown). The interaction between catalytic polymerase subunit (PB1) and particular deletion mutants of PB1-F2 was defined by proximity ligation assay (Fig. 4, 5). MDCK cells were transfected with deletion mutants, after 24hrs transfection was infected with PR8ΔPB1-F2 (MOI 10 PFU). Over all expression of PB1-F2 is visible as green fluorescence (left row of the figure), the red dots represent the interaction between PB1 and deletion mutants of PB1-F2 and in the right row are detected nucleus of cells (blue). As it shown on the figure the deletion mutant B seems to be responsible for this interaction. The positive control was used plasmid expressing PB1-F2 protein and as negative control served plasmid with STOP mutation after 3 amino acids.

Discussion

In this paper it was confirmed that the presence of PB1-F2 increases the expression of catalytic polymerase subunit and the others IAV proteins. These findings supported the role of PB1-F2 in pathogenicity of IAV, because the increase of expression the IAV proteins during replication cycle could influence the course of infection. Our experimental results are in agreement with other groups observations. Virus expressing PB1-F2 has prolonged clearens from infected animals in comparison to PB1-F2 expression deficient virus (Zamarin et al., 2006). It is reasonable to suggest that increased IAV protein expression could lead to increased virion production and prolonged presence of virus in the infected animals as well. Deletion library was used for identification of PB1-F2 domain interacting with PB1 protein. Meaning of deletion library grounds in possibility to determinate which deletion is responsible for lost of interaction. Thus aminoacids corresponding to deletion are involved in PB1-F2 to PB1 interaction and biological effects resulting from this interaction. We had identified such deletion, is concerned delB mutant. The deletion of 10 amino acids caused the lost of ability of PB1-F2 to interacts with catalytic polymerase subunit. The acquired cognitions will be used to design and test potentially therapeutic peptides blocking interaction of PB1-F2 and PB1, thus silencing expression of IAV proteins.

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SUPPLEMENT

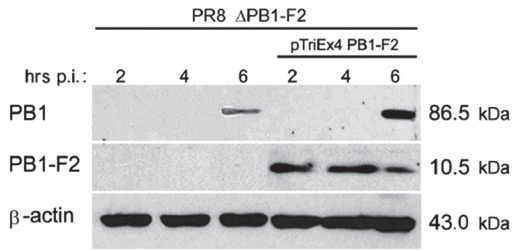

Figure 1: Effect of the PB1-F2 presence or absence on expression level of the PB1 protein respectively

MDCK cells were infected with PR8ΔPB1-F2 or transfected with pTriEx-4 PB1-F2 and 24 hrs post transfection cells were infected with PR8ΔPB1-F2. Over expression of the PB1-F2 protein in cells infected with PR8ΔPB1-F2 virus dramatically increases PB1 protein level. As a loading control detection of the β-actin was used.

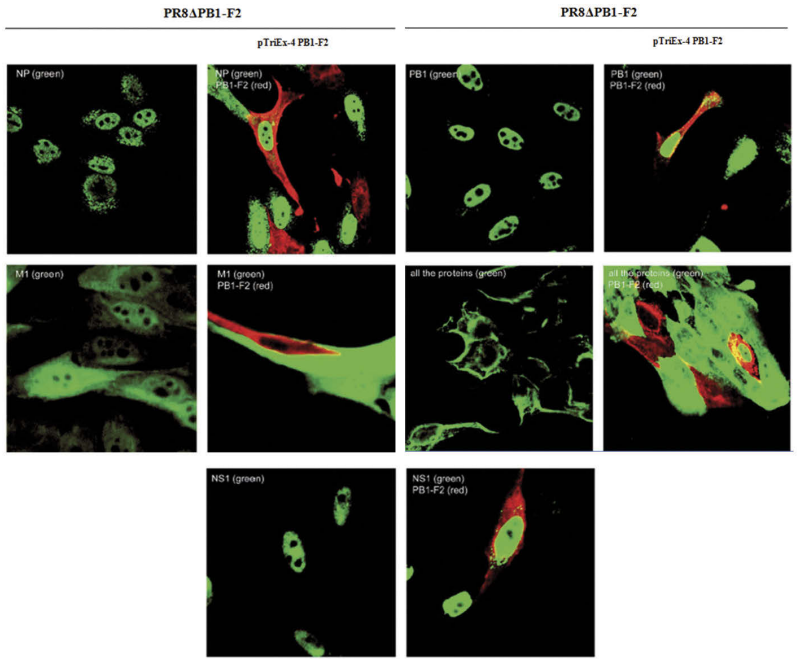

Figure 2: Effect of PB1-F2 on expression of the other viral proteins

Cells were infected with PR8ΔPB1-F2 or transfected with pTriEx-4 PB1-F2 and later infected with PR8ΔPB1-F2. 7hrs p.i. expression of NP, M1, NS1, PB1 (green) were detected in absence or presence PB1-F2 (red) respectively. Additionally the cells were also analyzed with antibodies induced by whole purified virus.

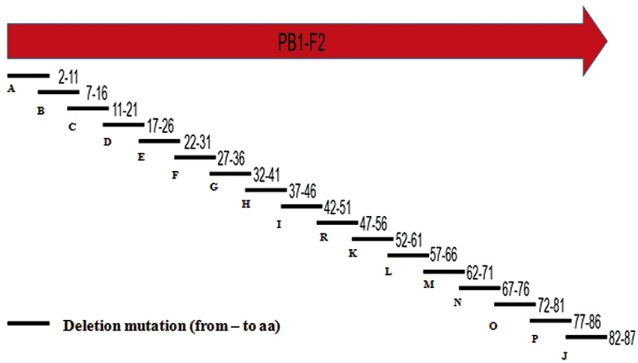

Figure 3: The scheme of deletion mutants

Schematic illustration the 17-th deletion considering full length of PB1-F2 protein.

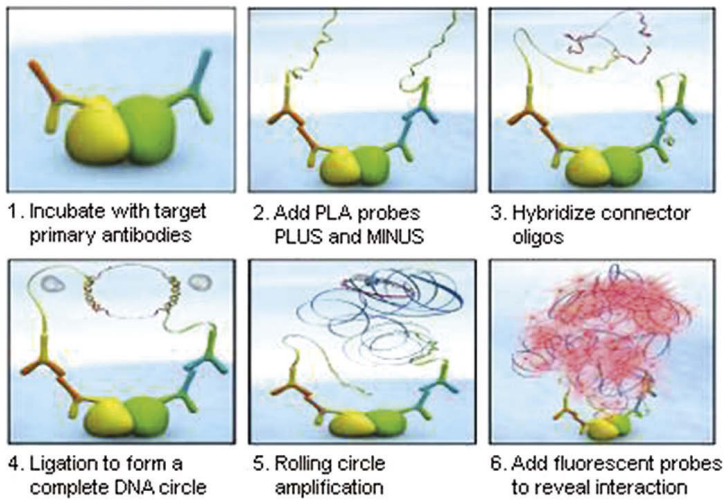

Figure 4: Proximity ligation assay

Typical starting materials are adherent cells, on a glass slide. Primary antibodies bind to the protein(s) to be detected. Secondary antibodies are conjugated with oligonucleotides (PLA probe MINUS and PLA probe PLUS). During the ligation oligonucleotides will hybridize to the two PLA probes and join to a closed circle if they are in close proximity. The amplification solution consists of nucleotides, fluorescently labeled oligonucleotides and the polymerase is added. The oligonucleotide arm of one of the PLA probes acts as a primer for a rolling-circle amplification (RCA) reaction using the ligated circle as a template, generating a concatemeric (repeated sequence) product. The fluorescently labeled oligonucleotides will hybridize to the RCA product. The signal is easily visible as a distinct fluorescent spot and analyzed by fluorescence microscopy.

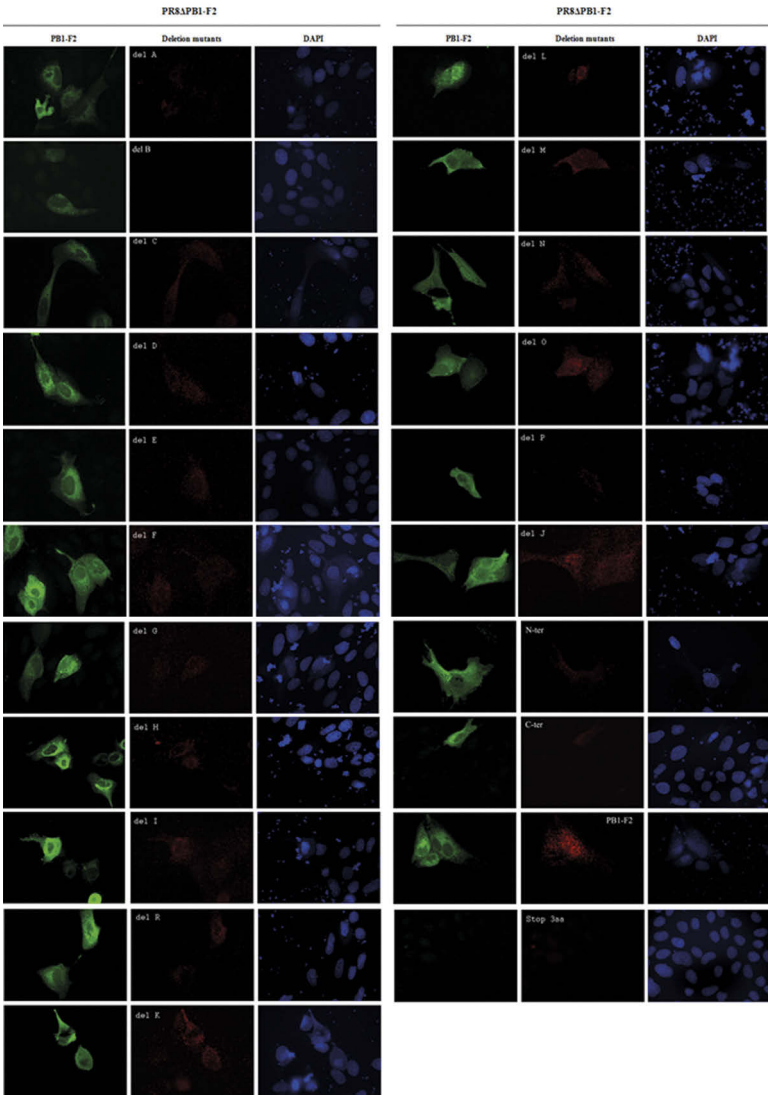

Figure 5: Fine mapping of interaction between PB1-F2 deletants and PB1 by proximity ligation assay.

Cells were transfected with PB1-F2 deletion mutants, full length PB1-F2 (positive control) and pTriEx-4 Stop 3aa (negative control). After 24 hrs were cells infected with PR8ΔPB1-F2 (MOI 10 PFU) and 6 hrs p.i. were cells incubated with primary antibodies (IMS anti-PB1, IRS anti PB1-F2) and then were incubated with secondary antibodies (PLA+ and PLA- probes, goat anti rabbit Fitc). It was identified PB1-F2 protein (green), the interaction between PB1 and deletion mutants of PB1-F2 (red) and the nucleus of cells (blue).

MOLECULAR STUDIES DONE WITH *LEUCOJUM VERNUM* L. (*AMARYLLIDACEAE*)

Mária TULENKOVÁ – Irena ŠUTIAKOVÁ

Abstract: *The work presents molecular studies of taxa Leucojum vernum L. of Amaryllidaceae. The purpose of these studies was: (1) to study the phylogenetic relationships of Leucojum and Galanthus in greater detail and elucidate relationships among the rest of Mediterranean Amaryllidaceae using a combination of the plastid gene matK and the internal transcribed spacers (ITS) of nuclear ribosomal DNA sequences and a morphological dataset; (2) to produce a well supported phylogenetic hypothesis for Amaryllidaceae based on combined analyses of matK, ITS and additional plastid trnL-F sequences; and (3) to use the phylogenetic framework to evaluate the distribution of alkaloids and acetylcholinesterase inhibitory activity.*

Key words: *molecular studies, Leucojum vernum L., Amaryllidaceae, hylogenetic relationships, alkaloids*

Family *Amaryllidaceae* has 60 genera and 800 species according to recent records (Watson, Dallwits, 2005). Genus *Leucojum* has only two species: *L. vernum and L. aestivum* (Crellin, 2005). *L. vernum* L. *and L. aestivum* L. are widespread in central and northen Europe and also found in Turkey and the Caucasus. They are also widely cultivated in the temperate zone world – wide (Ledó et al., 2004). *L. vernum* is spread naturally in the France, Belgium, Italy, Germany, Czech Republic, Poland, Slovakia, Hungary, Ukraine and Romania (Tulenková, 2000). *L. vernum* L. had two sub–species: *Leucojum vernum subsp. vernum* L. and *Leucojum vernum* L. subsp. *carpaticum* (Spring) O. Schwarz (Dostál, Červenka, 1992). *L. vernum* L. is represented by „*L. vernum subsp. carpaticum*" in Slovakia. This species distributed in few very specific habitats in eastern Slovakia. Population of *Leucojum vernum* L. subsp. *carpaticum* (Spring) O. Schwarz are located on south parts of Vihorlat mountain, close to inundation area of Latorica river (Tulenková, 2000).

In recent years molecular studies done by Lledó et al. (2004) and Larsen et al. (2010) also included *L. vernum* L. The purpose of study Lledó et al. (2004) was to study the phylogenetic relationships of *Leucojum* and *Galanthus* in greater detail and elucidate relationships among the rest of Mediterranean *Amaryllidaceae* using a combination of the plastid gene *mat*K and the internal transcribed spacers (ITS) of nuclear ribosomal DNA sequences and a morphological dataset. The purpose of study Larsen et al. (2010) was (1) to produce a well supported phylogenetic hypothesis for *Amaryllidaceae* based on combined analyses of *matK*, ITS and additional

plastid *trnL-F* sequences; and (2) to use the phylogenetic framework to evaluate the distribution of alkaloids and acetylcholinesterase inhibitory activity.

Molecular studies done with *Leucojum vernum* l. (*Amaryllidaceae*) and molecular markers - plastid gene *mat*K, the internal transcribed spacers (ITS) of nuclear ribosomal DNA and plastid *trnL-F*

In studies Lledó et al. (2004) and Larsen et al. (2010) a chloroplast gene (*matK* and *trnL-F*) and nuclear DNA region (ITS) were used in phylogenetic reconstruction. All three give DNA sequences that are useful for comparing species and closely related genera (Soltis et al., 1998).

The *matK* gene is located in the large single -copy region of the chloroplast genome (Soltis et al., 1998). The gene is approximately 1 550 bp in length and encodes a maturase involved in splicing type II introns from RNA transcripts. The evolution rate of *matK* makes it appropriate for resolving intergeneric or interspecific relationships in plants. The information content of this gene is similar to or greater than that of ITS. However, given that *matK* is 3,1 times longer, *matK* sequences may be informative at the generic and species levels (Chat et al., 2004; Järvinen et al., 2004; Lledó et al., 2004; Barfuss et al., 2005; Samuel et al., 2005; Shaw et al., 2005), and even familial level (Ito et al., 1999; Freudenstein et al., 2004; Wojciechowski et al., 2004). The upper limits of the phylogenetic utility of *matK* are still being explored as seen from mentioned reports. The retrieving of phylogeny within families and genera of land plants has great potential when comparing sequences of *matK* (Soltis et al., 1998). Well resolved phylogenies have been obtained in most studies by using approximately two-thirds of the 1 550 bp gene. Some studies have used considerably less. In several plant families, *matK* data has been combined with data of other genes or DNA regions, providing enhanced resolution, shortened run times and increased internal support for clades when compared to the separate data sets.

Noncoding sequences that include the *trnL* (UAA) intron and the intergenic spacer between the *trnL* (UAA) 3' exon and the *trnF* (GAA) gene also has phylogenetic potential (Soltis et al. 1998). These DNA regions are easily amplified and sequenced. They are relatively small, with the *trnL* intron ranging from 350-600 bp and the *trnL-F* spacer ranging from approximately 120-350 bp. Sequences of the *trnL-F* region may be informative at the generic and species levels (Gielly, Taberlet, 1996; Meerow et al., 2003; Chat et al., 2004; Graham, Barrett, 2004; Lihová et al., 2004; Mansion, Zeltner, 2004; Mayuzumi, Ohba, 2004; Alejandro et al., 2005; Barfuss et al. ,2005; Shaw et al., 2005), and even familial level (Meerow et al., 1999; Pfosser, Speta, 1999; Wojciechowski et al., 2004). Data sets of this DNA region have been readily combined with other chloroplast or nuclear genes as seen from mentioned reports. The combination of the data sets can be useful in the analysis of very large data sets.

The small size of the ITS region, approximately 600-700 bp, and the presence of highly conserved sequences flanking each of the two spacers make this region easy to amplify (Baldwin et al., 1995). ITS regions have become a major focus of comparative sequencing at the generic and species levels (Baldwin, 1993; Suh et al.,

1993; Baldwin et al., 1995; Bogler, Simpson, 1996; Wen, Zimmer, 1996, Douzery et al., 1999; Meerow et al., 2000; Meerow, Snijman, 2001; Ran et al., 2001; Meerow et al., 2003; Dobeš et al., 2004; Lihová et al., 2004; Mansion, Zeltner, 2004; Mayuzumi, Ohba, 2004; Meerow, Van der Werff, 2004; Alejandro et al., 2005; Muellner et al., 2005; Oh, Potter, 2005). Sequencing of these regions can be difficult because it is G + C rich and prone to secondary structure (Soltis et al., 1998).

Results

According Lledó et al. (2004) phylogenetic analyses of the monocotyledonous genera *Leucojum* and *Galanthus* (*Amaryllidaceae, Asparagales*), using plastid (trnL-F and matK) and largely non-coding nuclear ribosomal (ITS) DNA sequences

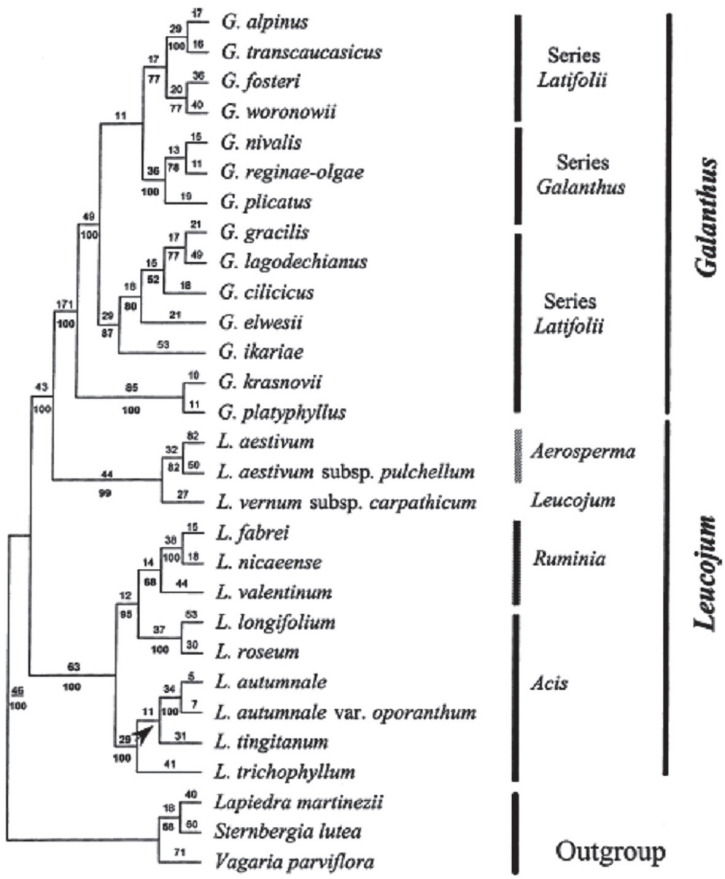

Figure 1 One of the two most parsimonious trees found with the combined matK, ITS and morphological data
(tree lengthĽ1709 steps; CIĽ0.69; RIĽ0.82) (Lledó et al., 2004)

show the two to be closely related to *Lapiedra, Narcissus, Vagaria, Pancratium* and *Sternbergia* (Figure 1). Lledó et al. (2004) compare the results obtained with a combined parsimony analysis of these nucleotide sequences with that of a matrix of morphological characters. The sampling included all species of *Leucojum* and most species of *Galanthus* (representing all series and subseries of the genus) and used as outgroup the above mentioned genera of *Amaryllidaceae* shown to be close relatives. The plastid, nuclear and morphological data were analyzed independently and in combination, showing that the boundaries between the two genera are not appropriate. *Galanthus* is monophyletic but embedded in *Leucojum*. On the basis of chromosome numbers and floral characters *Leucojum* has been previously divided into four subgenera, which have been accepted as genera by some authors. In phy-

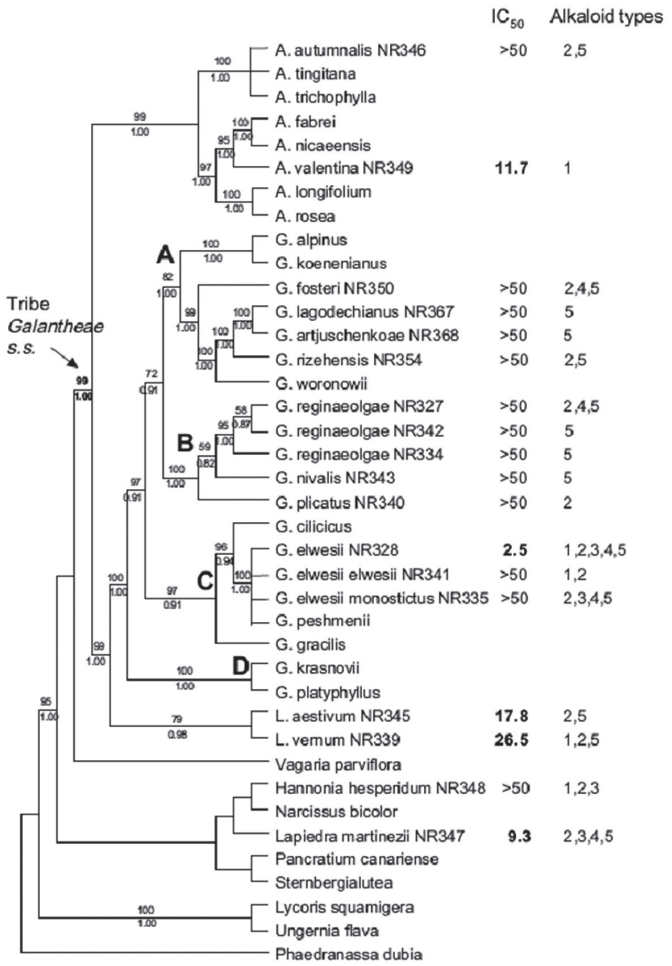

Figure 2 Strict consensus MP tree (Larsen et al., 2010)

logenetic analyses (separate as well as combined) of Lledó et al. (2004), *Leucojum* species are separated in two primary clades corresponding to L. subgenera *Ruminia* + *Acis* and L. *Leucojum* + *Aerosperma*. The taxonomic implications of this pattern are discussed, and an alternative classification is proposed.

Fitch lengths (ACCTRAN optimization) are shown above the branches, and bootstrap percentages greater than 50% and consistent with the strict consensus tree are given below. Clades not present in the strict consensus are marked with an arrowhead.

Study of Larsen et al. (2010) present phylogenetic analyses of 32 taxa of *Amaryllidaceae* tribe *Galantheae*, 6 taxa of other Eurasian genera of *Amaryllidaceae* and *Phaedranassa dubia* as outgroup in order to provide a phylogenetic framework for selection of candidate plants for lead discovery in relation to Alzheimer's disease (Figure 2). Larsen et al. (2010) used DNA sequences from the nuclear ribosomal internal transcribed spacer (ITS) and the plastid *matK* and *trnL-F* regions. Phylogenetic analyses using maximum parsimony and Bayesian inference strongly support a monophyletic tribe *Galantheae* in a narrow sense, including only *Acis*, *Galanthus* and *Leucojum*. Infrageneric relationships of *Galanthus* only partly support previous classifications Alkaloid profiles and inhibition of acetylcholinesterase (AChE) were investigated for 18 taxa using gas chromatography–mass spectrometry (GC–MS) and an assay measuring inhibition of AChE activity. AChE inhibitory activity was found in all investigated clades and was correlated with alkaloid profiles of the plants. Lowest IC_{50} values were expressed by extracts containing either galanthamine or lycorine type compounds. Evaluation of available chemistry and activity data in a phylogenetic framework could be used to select target species for further investigation.

Bootstrap support and Bayesian posterior probabilities indicated above and below branches respectively. Distribution of AChE inhibitory activity expressed as IC50 (mg dry weight of plant material/ml) and content of alkaloids types according to Jin (2007, 2009) of different tribe Galantheae bulb extracts. AChE inhibitory activities shown as >50 mg/ml were not dose-dependent. Alkaloid types are: (1) galanthamine, (2) lycorine, (3) homolycorine, (4) tazettine, (5) other alkaloid types.

Discussion

Phylogenetic analyses on the genera *Leucojum* and *Galanthus* done by Lledó et al. (2004) using *matK* and ITS sequences and done by Larsen et al. (2010) using *matK, trnL-F* and ITS sequences showed that plastid and nuclear data were analysed independently and in combination and they showing that the boundaries between these two genera are not appropriate. The combined *matK* and ITS sequences or *matK, trnL-F* and ITS sequences gave better results than the separate *matK,* ITS, *trnL-F* analysis. The results of between the two combined matrices were very similar but the different species were better resolved in the combined matrix of all three DNA regions. Simultaneous evaluation of bioactivity and alkaloid profiles in a phylogenetic framework can potentially be used as selection tool in drug discovery.

Phylogenetic analyses strongly support a monophyletic tribe Galantheae in a narrow sense, including only *Acis*, *Galanthus* and *Leucojum*. *Galanthus* is mono-

phyletic, but *Leucojum* is paraphyletic to *Galanthus*. An alternative classification for *Leucojum* was proposed. A single genus would accommodate *Leucojum* subgenera *Acis* (Salisb.) Baker and *Ruminia* (Parl.) Baker. *Galanthus* would remain as it is. The name *Leucojum* would be applied to only *L. vernum* L. and *L. aestivum* L. *Galanthus*, *Leucojum* and *Acis* exhibit different biogeographical patterns. The whole group has a Mediterranean distribution. The genera have overlapping distributions, which could be explained by re-colonization after the clades were established in isolation.

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THE INCIDENCE OF FACTOR V LEIDEN IN THE GROUP OF SLOVAK WOMEN WITH SPONTANEOUS ABORTIONS

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Abstract: *Hereditary thrombophilia represents an innate predisposition for thromboembolic disease. It plays an important role in the pathogenesis of many obstetric complications and is associated with an increased risk of spontaneous abortion. One of the most common causes of hereditary thrombophilia is G1691A mutation in the gene for factor V (Leiden mutation). The aim of our study was to examine the association of this important thrombophilic mutation with spontaneous fetal loss in the group of Slovak women. Our studied group consisted of 64 patients with a history of at least one pregnancy loss and 105 controls (women with no complications in pregnancy). Mutation was detected using real-time PCR method using TaqMan probes. The frequency of the risk allele A was 8.6% in the group of women with pregnancy loss and 1.4% in the control group. Factor V Leiden frequency was significantly higher in patients compared to controls (p = 0.001), and the risk allele A is associated with 6.5-fold higher risk of fetal loss.*

Key words: *thrombophilia, factor V Leiden, obstetrics, spontaneous abortion*

Thrombophilia can be defined as pathologically increased tendency for blood clotting. It is a result of inherited or acquired malfunction of hemostatic balance when procoagulant factors outweigh anticoagulant (Penka, 2004). The most serious clinical manifestations include myocardial infarction, stroke, peripheral artery disease, pulmonary embolism, but also pregnancy complications. The coagulation system of a pregnant woman is a subject to adaptive changes that lead to „physiological“ hypercoagulable state. This state acts as a protective factor for potential blood loss during pregnancy and childbirth. On the other hand, it increases the risk of venous thromboembolism and other related complications during pregnancy and puerperium (recurrent pregnancy loss, preterm delivery, preeclampsia, placental abruption, placental insuficiencia and fetal growth retardation including intrauterine fetal death) (Kvasnička, 2003). One of the forms of hereditary thrombophilia is Factor V Leiden, a common mutation G→A in the nucleotide position 1691 in the Factor V gene, which is associated with a significant increased risk for systemic venous thrombosis (Dahlback, 1995). Factor V Leiden has also been reported in association with placental thrombosis (Dizon et al., 1997). Recurrent pregnancy loss, defined as two or more spontaneous abortions, affects 5% of all women of reproductive age.

Epidemiological studies suggest that inherited and acquired thrombophilia of the mother, such as that caused by the Leiden polymorphism in blood coagulation factor V, contributes to the pathogenesis of fetal loss, as well as other adverse pregnancy outcomes (Kovalevsky et al., 2004; Dudding and Attia, 2004).

Material and methods

The studied group consisted of 169 women hospitalized at the Department of Gynecology and Obstetrics, J.A. Reiman Faculty Hospital in Prešov in the years 2007 - 2011, including 64 patients who had a fetal loss in the second or third trimester [of that, 43 patients (67,19%) had history of one fetal loss, 21 patients (32,81%) had two or more fetal losses]. Patients with known cause of abortion, for example birth defects, were excluded from research. The control group consisted of 105 women with no complications in pregnancy. All probands had signed an informed consent. DNA was extracted from buccal swabs, the concentration of DNA in the sample was measured spectrophotometrically. We used real-time PCR method for detection of selected polymorphism. It is based on the principle of the target area amplification of examined gene by PCR, and the fluorescent labeling of amplified segment with TaqMan probes. For statistical evaluation of the results, we calculated allele frequencies from examined genotypes. We compared the allele frequencies between the two groups of women by $\chi 2$ test. The criterion for statistical significance was $p < 0.05$.

Results and discussion

In the group of women with a history of at least one fetal loss we detected Factor V Leiden mutant allele A with a frequency of 8.6%, in the control group the frequency was 1.4%. The prevalence of factor V Leiden was significantly higher in patients compared to controls ($p = 0.001$, OR = 6.5), which means that the A allele is associated with a nearly 6.5-fold higher risk of abortion (Table 1).

Kasparová et al. (2012) evaluated the importance of screening for thrombophilic mutations in the group of women after the first early abortion in the Czech population. They investigated four most common thrombophilic polymorphisms in a group of 100 women after one abortion and compared the results with frequency in the healthy population. FV Leiden in heterozygous form was found in 9% of patients, what was 1.8 times more than in the controls. On the other hand, Šubrt et al. (2008) did not confirm the association of FV Leiden with recurrent abortions in the group of 206 Czech patients. Several studies concerning with pregnancy loss and thrombophilia have been summarized in meta-analyzes (Dudding and Attia, 2004; Rey et al., 2003), which described the increased odds ratio (OR) for recurrent pregnancy loss for factor V Leiden carriers: OR = 2.0 for the group of women with early recurrent fetal loss, OR = 1.7 for non-recurring fetal loss and OR = 3.3 for fetal loss after 19th week of pregnancy. Many other studies confirm a higher frequency of factor V Leiden in women with a history of recurrent abortions (Grandone et al., 1997; Meinardi et al., 1999; Younis et al., 2000). Confirmation of the association of thrombophilic poly-

Table 1 Allele and genotype frequencies of factor V Leiden in the group of patients and controls and their comparison

Factor V Leiden genotype	**patients** (n=64)	**controls** (n=105)
Homozygote 1691 **GG**	**53** (82.81%)	**102** (97.14%)
Heterozygote 1691 **GA**	**11** (17.19%)	**3** (2.86%)
Homozygote 1691 **AA**	**0**	**0**
f (G)	91.41%	98.59%
f (A)	**8.59%**	**1.41%**
p (Pearson) for HWE	0.452	0.882
χ^2	10.28	
OR (odds ratio)	**6.487**	
Statistical significance	p=0.00134	

morphisms with pregnancy complications can help select out risk group of women for whom early diagnosis of these thrombophilic factors, together with subsequent prevention and thromboprophylaxis can lower or completely eliminate the risk of fetal loss during pregnancy.

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BIRTH WEIGHT OF ROMANY AND NON – ROMANY NEWBORNS DEPENDING ON THE AGE OF MOTHER FROM KEŽMAROK DISTRICT

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Abstract: *Birth weight is usually the most affordable and widely used indicator, reflecting the whole range of effects of intrauterine environment. Interpretation of factors that affect growth and development of newborn requires also to bear in mind the ethnic aspect. The main aim of this study was to compare the birth weight of Romany and non-Romany newborns according to the mother's age. We investigated a group of 578 Romany fullterm newborns (280 girls and 298 boys) and control group consisted of 574 non-Romany fullterm newborns (297 girls and 277 boys). All newborns were born in 37th to 42nd week of gestation, in the Department of Paediatrics of Dr. Vojtech Alexander's hospital in Kežmarok, in 2008 - 2009. The obtained data showed that with increasing mother's age increases also the body weight of newborns. On the other hand, Romany newborns have lower birth weight according to the mother's age than non-Romany newborns.*

Key words: *romany newborns, non-romany newborns, body weight, mother's age*

Birth weight is usually the most affordable and widely used indicator reflecting the whole range of effects of intrauterine environment. Interpretation of the factors that affect growth and development of newborn, requires also to bear in mind also the ethnic aspect.

Some previous studies (Bernasovská et al., 1977, Bernasovská et al., 1998a, Bernasovský et al., 1975a, Bernasovský et al., 1981a) referred significant differences in the birth weight of Romany fullterm newborns with non-Romany.

The aim of this research was to study the somatic development of Romany and non-Romany newborns from Kežmarok district to observe their anthropometric parameters: mean body weight in relation to the mother's age. Another important goal of this study was the comparison of our results with results of Romany and non-Romany newborns from the Prešov Region (Bernasovský et al., 1999).

Materials and methods

The subjects were ethnically diverse groups of 578 Romany and 574 non-Romany fullterm newborns spontaneously up to 37th week of pregnancy. Mothers with the stated

gestose, bleeding, diabetes mellitus or some other pregnancy menace were excluded. We also excluded from the analysis twins, dead born children, children born with the stated developmental defects, children born before 37th week of pregnancy and children who were born pincer childbirth, or section. By this selection we have tried to bring the requirements for a healthy standard. Body weight and sex of newborns and mother´s age were obtained from the medical records of mothers in Department of Paediatrics of Dr. Vojtech Alexander´s hospital in Kežmarok. We studied the relationship between ethnicity, mother's age and sex and body weight of newborns.

The statistic analysis was performed by T-test (MS Excel). We evaluated the significance of differences between the parameters according to the levels of probability of $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

Our results were compared with studies realized in Prešov Region in the years 1968-1972 and 1991-1992 (Bernasovská et al., 1977, Bernasovská et al., 1998a, Bernasovský et al., 1975a, Bernasovský et al., 1981a, Bernasovský et al., 1999).

Results and discussion

Some authors as Pogačnik (1968), Zeman (1968), Malá (1973a, 1975), Malá and Machová (1978), Vopatková et al., (1983) demonstrated the lower birth weight and the lower length of Romany newborns in comparison with majority populations.

In presented study we have analyzed the relationship between the maternal age and the birth weight in Romany and non-Romany newborns from Kežmarok (tab. 1). The average birth weights separately for the boys and the girls are given for the single age groups of the mothers. We found significant difference between the birth weights in Romany and non-Romany newborns in all age groups of mothers ($P < 0.05$, * $P < 0.01$, ** $P < 0.001$, ***). Our results confirmed as the age of mother increase, increase also the birth weight of child. The lowest average values of newborn body weight were in the youngest age group (14 – 16 years old).

Table 2 show the comparison of the birth weight according to age of Romany mothers between Kežmarok District and Prešov Region. The statistics also show the trend of increasing the birth weight of newborns is limited by age of the mother. Comparing the basic somatic parameters in fullterm Romany and non – Romany newborns, born within years 1991 – 1992, with results of basic physical parameters study in East Slovakia newborns within years 1968 – 1972 Bernasovská et al., (1977) noted significantly lower values of birth weight, the length, head and breast circumference.

Studying the relationship between the Romany mother´s age and child birth weight, the highest percentage of Romany mothers (39.5 % in Prešov and 45.7 % in Košice) with high birth weight babies was found in the groups aged 17 – 22 years. In both groups the relatively high presentation was observed in the group of Romany mothers aged 14 – 16 years (6.1 %), three times higher than in Slovak mothers under 17 years (2.1 %) (Štukovský, 1969).

In India, 10.4 % mothers 15 – 19 years old delivered, and the highest presentation was found in 33 % of the mothers aged 25 – 29 years (Sakar, 1968). Among Romany mothers, the highest presentation was found in the group aged 21 – 23 years (36 %).

Our results show a trend towards an increase in birth weight with an increase in the mother´s age. The most conspicuous differences were found between the lowest age group (14 – 16 years old) and the highest one.

Table 1 Romany mothers of Kežmarok and the average values of fullterm newborns

Romany newborns (Kežmarok District)						non - Romany newborns (Kežmarok District)				
Age of mothers	**G**	**N**	**Average values**	**s**	**$s_{\bar{x}}$**	**N**	**Average values**	**s**	**$s_{\bar{x}}$**	**p**
14 - 16	Girls	3	2 890,00	138,92	80,21	0	0,00	0,00	0,00	
	Boys	2	2 755,00	205,06	145,00	0	0,00	0,00	0,00	
17 - 19	Girls	47	3 010,64	363,91	53,08	11	3 005,45	289,46	87,28	0,97
	Boys	40	3 002,25	389,99	61,66	7	3 428,57	608,29	229,91	0,01847*
20 - 22	Girls	64	3 019,53	340,51	42,56	30	3 144,00	402,81	73,54	0,12
	Boys	87	3 042,93	347,15	37,22	29	3 318,62	416,92	77,42	0,00062***
23 - 25	Girls	72	3 051,13	389,00	46,17	63	3 289,68	384,79	48,48	0,00051***
	Boys	59	3 098,47	389,30	50,68	48	3 266,46	372,42	53,75	0,02567*
26 - 28	Girls	40	3 068,75	417,29	65,98	55	3 267,59	412,96	56,20	0,02383*
	Boys	49	3 168,57	366,63	52,38	65	3 455,54	492,28	61,06	0,00085***
29 - 31	Girls	20	3 004,50	281,68	62,98	62	3 206,07	386,74	49,52	0,03481*
	Boys	27	3 109,22	409,24	78,76	52	3 426,92	497,39	68,98	0,00556**
32 - 34	Girls	22	2 942,27	568,34	121,17	43	3 314,42	343,78	52,43	0,00165**
	Boys	18	3 212,78	419,24	98,82	34	3 426,92	396,48	68,00	0,19
35 - 37	Girls	6	3 133,33	420,22	171,55	20	3 298,50	473,16	105,80	0,45
	Boys	9	3 004,44	304,96	101,65	22	3 309,55	373,39	79,61	0,03859*
38 - 40	Girls	6	3 000,00	186,98	76,33	11	3 424,55	374,12	112,80	0,02083*
	Boys	2	3 390,00	410,12	290,00	10	3 538,00	392,76	124,20	0,64
41 -	Girls	1	3 300,00	0,00	0,00	2	3 450,00	707,11	500,00	
	Boys	4	3 160,00	438,71	219,36	10	3 454,00	496,48	157,00	

* statistically significant results (P <0.05, * P <0.01, ** P <0.001, ***, T-test)

Conclusion

The results show that with increasing mother´s age increases also the body weight of newborns and Romany newborns have a lower average values of birth weight in case of mother's age than non-Romany newborns. The obtained lower values in our study may be the cause of external factors acting during the intrauterine development, but mainly of genetic factors.

Table 2 Age comparison of Romany and non-Romany mothers of Kežmarok and Prešov region and the average birth weight of fullterm newborn

		Romany newborns (Kežmarok District)				Romany newborns (Prešov District)			
Age of mothers	G	N	Average values	s	$s_{\bar{x}}$	N	Average values	s	$s_{\bar{x}}$
14 - 16	Girls	3	2 890,00	138,92	80,21	32	2 604,37	377,34	66,78
	Boys	2	2 755,00	205,06	145,00	30	2 860,00	389,86	71,27
17 - 19	Girls	47	3 010,64	363,91	53,08	107	2 872,80	305,94	29,28
	Boys	40	3 002,25	389,99	61,66	94	3 055,31	368,78	38,05
20 - 22	Girls	64	3 019,53	340,51	42,56	90	2 928,88	414,24	43,69
	Boys	87	3 042,93	347,15	37,22	91	3 004,39	420,46	45,20
23 - 25	Girls	72	3 051,13	389,00	46,17	62	3 006,45	542,00	68,86
	Boys	59	3 098,47	389,30	50,68	77	3 159,00	398,48	45,42
26 - 28	Girls	40	3 068,75	417,29	65,98	59	2 906,78	408,40	50,17
	Boys	49	3 168,57	366,63	52,38	38	3 168,43	434,96	75,56
29 - 31	Girls	20	3 004,50	281,68	62,98	32	3 176,87	333,40	58,94
	Boys	27	3 109,22	409,24	78,76	43	3 227,91	402,48	61,38
32 - 34	Girls	22	2 942,27	568,34	121,17	38	3 043,37	478,74	77,66
	Boys	18	3 212,78	419,24	98,82	29	3 158,62	375,20	69,67
35 - 37	Girls	6	3 133,33	420,22	171,55	33	3 006,07	359,44	62,57
	Boys	9	3 004,44	304,96	101,65	35	3 200,00	449,44	75,97
38 - 40	Girls	6	3 000,00	186,98	76,33	22	3 218,18	619,02	131,96
	Boys	2	3 390,00	410,12	290,00	32	3 275,00	424,80	75,11
41 -	Girls	1	3 300,00	0,00	0,00	12	3 266,66	452,00	130,63
	Boys	4	3 160,00	438,71	219,36	10	3 140,00	432,20	136,68

*statistically significant results (P <0.05, * P <0.01, ** P <0.001, ***, T-test)

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EFFECT OF ANDROGEN-DEPRIVATION THERAPY ON THE CHANGE OF BONE DENSITY IN PATIENTS WITH PROSTATE CANCER

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Abstract: *Androgen-deprivation therapy (ADT) is used as the standard treatment for advanced prostate cancer. Its use is associated with several adverse effects. One of the most serious long-term adverse effects is reduction of "bone mass" (bone mineral density – BMD). The aim of this study was to determine the reduction of BMD during ADT, to evaluate the effect of ADT on bone metabolism, to determine the number of complications (fractures) and possibilities for prevention. Study group consisted of 108 patients in an average age of 63.97 years. The BMD of the femoral neck and vertebrae L1-L4 was examined in patients with prostate cancer by X-ray absorptiometry. There were evaluated changes in BMD, the number and type of fracture. Patients with osteoporosis were treated in cooperation with orthopaedic. The same tests were performed on 47 patients in control measurements (average age 66.96 years). In the analyzed group of 108 patients we have found the incidence of osteoporosis in 31% and osteopenia in 37% of patients. In the control measurement was found a significant decrease in BMD (p=0.003). We confirmed the significant dependence of bone density by age (p=0.005). The fractures were found in 38.3% of study group.*

Key words: *osteoporosis, osteopenia, BMD, absorptiometry, fractures*

Prostate carcinoma is the most common malignant disease of the internal organs. In Slovakia are diagnosed 1200 new cases per year.

For the treatment is used hormonal therapy or androgen-deprivation therapy (ADT) that block the production of testosterone. Longstanding low testosterone can cause a decrease in bone mineral density, increasing the risk for osteoporosis.

It has been demonstrated that hormonal therapy significantly reduces growth of the tumor and improves survival. However, there is growing concern regarding the negative effects of ADT on the skeleton. Accelerated bone loss, osteoporosis and potential for increased fracture rates have been reported in men with prostate carcinoma, who are receiving ADT.

Osteoporosis is characterized by reduction in bone mineral density that is associated with bone fragility and increased risk of fracture after minimal trauma.

During the hormonal therapy is important supply of bisphosphonates. Bisphosphonates reduce bone loss, increase bone density and prevent fractures of the hip and spine.

Modification of lifestyle is necessary, particularly no smoking, regular exercise and supplement of calcium and vitamin D.

The risk of skeletal deterioration is increased in elderly patients with prostate carcinoma, by preexisting osteoporosis, vitamin D deficiency, or any of a multitude of medical problems.

The objective of this work was to determine the relationship between hormonal therapy and decrease of bone density.

Materials and methods

Study group consisted of 108 patients in an average age of 63.97 years. Patients are mainly from Western Slovakia. Control group consisted of 47 patients in an average age 66.96 years. Data were collected on the Osteological surgery St. Elizabeth Cancer Institute in Bratislava.

Patients undergo the densitometry examination (DXA), which was determined a bone density (BMD). BMD is considered to be the gold standard for diagnosing and assessing the severity of osteoporosis and fracture risk. The most common is measured a lumbar spine (vertebrae L1-L4) and femoral neck (Figure 1).

The World Health Organization has established criteria for the diagnosis of osteoporosis and osteopenia, which is based on their T score (1 T value representing the standard deviation for BMD of young adult men in general population). Osteopenia at any age is defined by T score between -1 and -2.4 and osteoporosis as a T score great than -2.5.

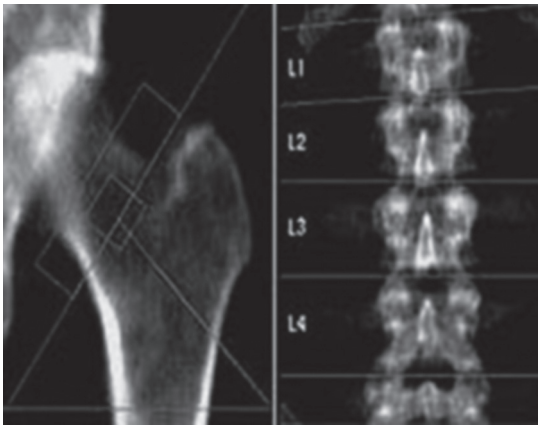

Figure 1 A dexa scan of a hip and spine

Bisphosphonates are a class of drugs that prevent the loss of bone mass, used to treat osteoporosis. They were prescribed of men with low bone density. Bisphosphonates inhibit the digestion of bone by encouraging osteoclasts to undergo apoptosis, or cell death, thereby slowing bone loss (Weinstein et al., 2009).

Probands undergo also laboratory examination. The patients were evaluated levels of testosterone and vitamin D.

The data were statistically processed in Microsoft Office Excel 2003 and SPSS 17.0.

Results and discussion

In the study group we investigated the number of patients with prostate cancer, who had low bone density after androgen-deprivation therapy. In the study group of 108 patients with prostate cancer we observed variations from normal bone density in 68 % of subjects. Results are summarized in Figure 2.

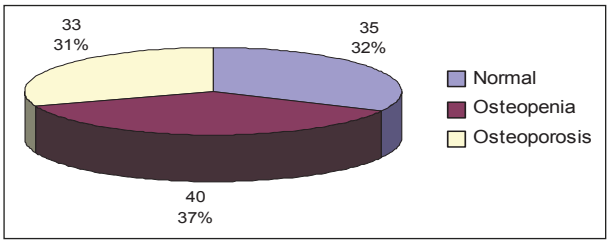

Figure 2 The incidence of osteopenia and osteoporosis in the study group

Bone loss induced by androgen deprivation is clinically significant in patients with long-term ADT (Katolická, 2009). Testosterone is important in the development of bone mass. Testosterone replacement therapy reduces bone resorption and it slows of bone turnover (Szulc, 2011). In the U.S. study, the levels of testosterone were determined in 83 men over the age 65. It was found that 52% of men with low level of testosterone had low BMD, and thus are at increased risk of fractures (Kenny et al., 2011).

In the group of 47 patients we evaluated the level of bone density. As is showed by Figure 3, to 40% of patients had osteopenia. We recorded osteoporosis in 34% of patients.

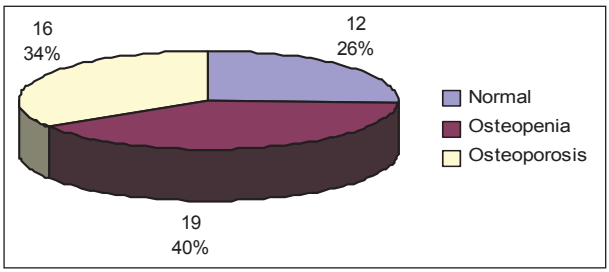

Figure 3 The incidence of osteopenia and osteoporosis in the group of 47 patients

Interesting is the fact that osteoporosis have been diagnosed in the youngest and also in the oldest patients.

It was compared the difference between the values of the bone density of the first and second measurement. Results are summarized in Figure 4. In the control measurements has bone density significantly reduced. It is probably due to the prolonged use of ADT.

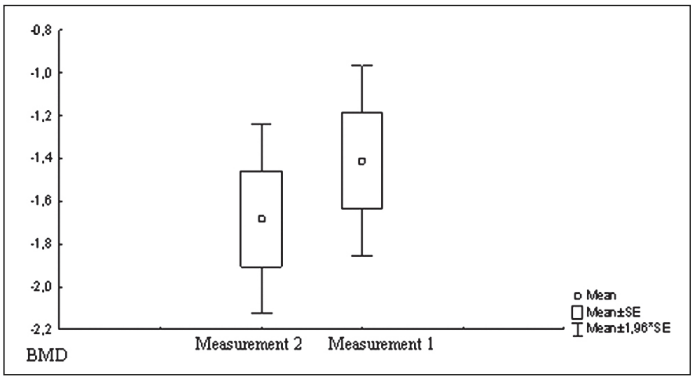

Figure 4 The difference between the values of BMD of the first and second measurement

In men with osteopenia or osteoporosis, who are treated with ADT, advised the National Comprehensive Cancer Network consider giving bisphosphonates (National Comprehensive Cancer Network, 2007). The aim of antiresorptive therapy is prevent further deterioration in bone microarchitecture, stop the bone loss and allow further secondary mineralization of bone (Štěpán, 2011). Therapy by bisphosphonates was deployed 21 patients from a total number of 47. However, in treated patients didn´t occur significant change in bone mineral density.

In 2 retrospective studies, the possible therapeutic approaches have been studied in the treatment bone loss in patients with prostate cancer (Daniell, 2001; Smith, 2002). Early therapy with bisphosphonates may protect bone integrity during disease progression, because it prevents bone loss and reduces the risk of fractures (Saad et al., 2006).

In this study, we evaluated the incidence of osteoporosis and fractures in men with prostate cancer, who are receiving ADT for at least 12 months. Long-term administration of hormone deprivation therapy is associated with progressive bone loss and increasing the risk of fractures. The number of patients with various fractures is presented in Table 1.

The Table 1 shows that over 38% of patients had osteoporotic fracture. Vertebral fracture was recorded only in one patient. 17 individuals had fractures elsewhere in the skeleton (Table 2). The most common fractures occurred at the forearm, wrist and hip.

Table 1 Frequency of patients with fractures

		No. of patients	%
Fracture	non-vertebral	17	36.2
	without fr.	29	61.7
	vertebral	1	2.1
	Total	47	100.0

Table 2 Number and type of non-vertebral fractures

Non-vertebral fractures	Number of fractures
thumb of the left upper extremity	5.9%
forearm	**41.2%**
wrist	29.4%
femoral neck	17.6%
tibia	5.9%

Androgen-deprivation therapy is associated with an increased risk of fracture in older men with prostate cancer (Morote, 2003). Fractures associated with osteoporosis are located at the femoral neck, distal forearm and vertebrae. However, hip fracture is the most serious consequence of osteoporosis. These fractures lead to an overall reduction of 15% survival (Dennison, Coper, 2000). The risk increases with long duration of therapy (Shahinian et al., 2005; Smith et al., 2005). Retrospective analysis (Krupski et al., 2004) points out that ADT increases the risk of osteopenia/osteoporosis (30%), as well as non-pathological and pathological fractures (42% and 16%). Another study (Shahinian et al., 2005) also points to the increased incidence of fractures in patients with prostate cancer treated with deprivation therapy (19.4 %) compared with 12.6 % of men, who were not treated with hormones.

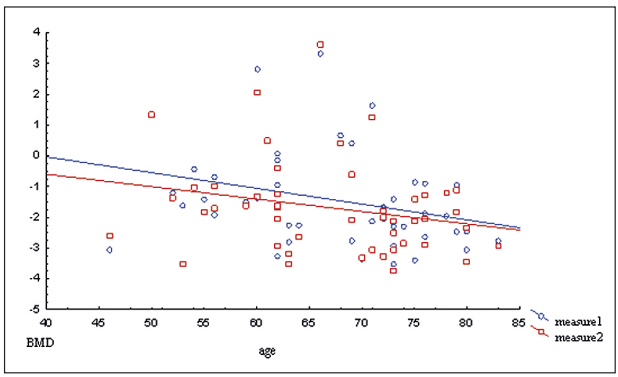

Figure 5 The dependence of bone density and age

Reduction of bone density comes with natural aging of man. We examined this theory to the study group. The dependence of bone density and age in the first and second measurements has a negative trend, it can be seen in Figure 5. It confirms that values decrease with age.

This result is confirmed by several studies. Hormone therapy lowers the level of circulating testosterone that affects the bone resorption and bone formation (Leder et al., 2003). Bone loss is accelerated with age about 1% to 2% per year (Hofbauer, Khola, 1999). In a recent study, the presence of osteoporosis was showed by 42% elderly patients with prostate cancer (Hussain et al., 2003).

Conclusion

The study highlights the importance and meaningfulness of the investigation of bone density in patients with prostate cancer. The risk of osteoporosis in patients with hormone-dependent tumors treated by hormones is much higher than in patients without cancer. We speak about large group of patients who have high curability. This is another reason why it is necessary to pay attention to this disease. Currently are available sensitive and accurate diagnostic capabilities, but also the effective therapy.

In the treatment of male osteoporosis is not available a wide range of drugs as for female. That is why the emphasis is on preventing its occurrence. Construction of bone mass in male is developing already in puberty. During this period it is necessary to ensure proper nutritional composition and optimal physical activity. A prerequisite to the proper development of the male skeleton is mainly the presence of optimal levels of androgens and estrogens. The elderly should follow the principles of osteoporosis prevention.

Patients with prostate cancer have significantly lower BMD than healthy population. ADT leads to a significant decrease in total BMD and BMD of vertebrae L1 – L4. BMD decrease leads to skeletal complications. Therefore the BMD was measured at the beginning of ADT and then periodically during treatment to prevent skeletal complications.

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INHERITED RARE DISEASES IN ROMA POPULATION

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Abstract: *Like other isolated populations with a founder effect, Roma are a valuable resource for research on rare genetic diseases, and the discovery of genes and mutations. Recently, several rare diseases were identified to be caused by a specific mutation of a common origin. Roma often suffer from diseases that are common in the surrounding populations, but in Roma they are caused by a different mutation. Moreover, there are several diseases in this ethnics, that are rare or completely absent in the majority population. Application of this knowledge into medical practice is often difficult. At first sight different gene pool of Roma complicates the diagnosis of patients. But knowledge of diseases and particular mutations specific to certain ethnic group can actually greatly simplify and speed up the diagnostic process. In this study, we review our experience in molecular diagnostics of rare diseases among Roma in Eastern Slovakia. Most telling example is Charcot-Marie--Tooth disease. The most common type of this disease in Slovakia is an autosomal dominant CMT1A caused by PMP22 gene duplication, however, this subtype of disease is not present in Roma population. After excluding of PMP22 gene mutation these conditions often remain without proper diagnosis. Here we present successful diagnostics of specific subtype of this disease in four out of five families using simple protocol based on knowledge of mutations specific for Roma.*

Key words: *analbuminemia, autosomal recessive disease, Charcot-Marie-Tooth, founder population, Gypsy, nephrotic syndrome*

In the field of human genetics, certain populations are playing prominent roles in gene discovery. They are called "founder populations" and they are groups that descend from a small number of ancestors who left one area to settle another with subsequent isolation (geographic, cultural or religious etc.) leading to limited immigration and demographic growth mainly from within. Reduced genetic diversity and founder effect, resulting in a more homogeneous basis of inherited disorders and predispositions, make it possible for genetic studies to treat the whole population as one large family, where individuals affected by a specific condition are likely to share the same ancestral disease-causing DNA variant (Kalaydjieva et al., 2005).

The main recognized founder populations in the world are those of Quebec, Finland, Sardinia, Iceland, Costa Rica, Newfoundland, and several discrete ethic

groups, including the Ashkenazi Jews, Mormones and Amish. All these isolated founder populations have been a precious resource for Mendelian genetics.

Roma/Gypsy ethnic group represents a very unique founder population, formed of socially and culturally heterogeneous and geographically dispersed subisolates. The complex and very specific structure of Roma society has long attracted the attention of cultural anthropologists. It was not always so in the field of human genetics. Unlike other founder populations Roma have been ignored by European medicine for hundreds of years, and their unique genetic heritage is only recently becoming a focus of interest for geneticists and medical practitioners (Kalaydjieva et al., 2005).

The molecular cause of number of diseases in the Roma population has been clarified in the past years (Plasilova et al., 1999; Kalaydjieva et al., 2000; Hantke et al., 2009). Due to founder effect, many of mutations causing these diseases are exclusive for Roma and identical in all affected families of all Roma populations. It is highly expected that these conditions are present in Slovak Roma population as well.

The population of Roma in Slovakia is one of the largest in Europe, outnumbered only by Roma communities in Romania, Bulgaria and Hungary. The estimated number of Roma in Slovakia is 400.000 (Vaňo, 2002).

In this study, we review our experience in molecular diagnostics of rare diseases among Roma in Eastern Slovakia. Families with specific diseases are described and cases of successful molecular diagnostics are depicted.

Material and methods

Families segregating rare disease and lacking proper diagnosis were selected by cooperating general practitioners, clinical geneticists and specialists. Blood samples or buccal swabs were obtained from probands, parents and other available family members. Genomic DNA was isolated from peripheral white blood cells using a blood or tissue DNA isolation kit (MO BIO Laboratories Inc., Carlsbad, USA). Candidate genes or regions were selected based on literature. Several strategies were used to identify causing mutation. In case that mutation private to Roma was known, the single exon was sequenced on an automated sequencer (3500 DNA Analyzer; Applied Biosystems, Foster City, CA) using a terminator kit (BigDye, version 3.1; Applied Biosystems, Foster City, CA). In diseases with several possible mutations and/or genes, microsatellite markers in proximity of suspected loci were genotyped by capillary electrophoresis (3500 DNA Analyzer; Applied Biosystems, Foster City, CA) and association with disease was analyzed confirmed or excluded. In two families with nephrotic syndrome, samples were run on SNP microarray (Affymetrix SNP Genotyping Array 6.0, Affymetrix Inc, Santa Clara, CA) in order to find homozygous region that could be then searched for causing mutation (analysis performed by Atlas Biolabs, Berlin, Germany). All primers were produced by Sigma Aldrich (Bratislava, Slovakia). Primer sequences and reaction conditions are available upon request.

Results

We will demonstrate the results of the study on example of three diseases: analbuminemia, Charcot-Marie-Tooth disease and nephrotic syndrome.

Example no.1: Analbuminemia. Analbuminemia is an autosomal recessive disorder characterized by absence or very low concentration of albumin in the blood. The disease is very rare – so far only 52 cases were described in world. Diagnosis is based on low value of serum albumin (0.01 to 1000 mg/l) together with absence of hepatopathy and gastrointestinal or renal loss of proteins that cause secondary hypoalbuminemia. The disease is caused by mutations in the ALB gene encoding the human serum albumin (Lyon et al., 1998, Albumin 2012). We have examined a newborn girl from Roma family. Examinations showed hypoalbuminemia (9.1 g/l), mild hypoproteinemia and hypercholesterolemia. Although the value of serum albumin was higher than limit for the disease, we assumed diagnosis of analbuminemia because the common causes of hypoalbuminemia were excluded and the patient was from Roma community with a high incidence of consanguinity. Moreover, family with analbuminemia was already described in this area (Campagnoli et al., 2006). Therefore, we searched for mutation delATc228-229 in ALB gene reported in previous study. The mutation was confirmed in homozygous form in patient.

Example no.2: Charcot-Marie-Tooth disease (CMT). CMT comprises several different subtypes of demyelinating motor and sensory neuropathies. The most commonly used classification combines clinical findings with the inheritance pattern (autosomal dominant, autosomal recessive, or X linked) and either electrophysiological or anatomical pathology findings (axonal or demyelinating) (Banchs et al., 2009). Unlike autosomal dominant or X-linked forms of CMT, autosomal recessive forms (CMT4) are very rare; they have earlier onset and more severe phenotype.

We carried out molecular diagnosis in five Roma families. Child from consanguineous marriage was affected in four cases; in one case adult woman was affected neglecting consanguinity in family. Although autosomal recessive inheritance and EMG in our patients did not suggest type CMT1A of disease (autosomal dominant), three patients were clinically examined for duplication in PMP22 gene, which is common practice in Slovakia, because this duplication is the most common cause of CMT in Slovak patients. The mutation in this gene was not found and all five patients were diagnosed with suspected CMT of unspecified type.

Recessive forms of disease - CMT4D and CMT4G (so-called HMSN-Lom and HSMN-Russe) were originally found in Bulgarian Roma and they are reported almost exclusively in individuals of Roma origin in Europe (Kalaydjieva et al., 2000, Hantke et al., 2009). Our research confirmed that these forms of CMT are also common among the Roma in Slovakia; one of these types was confirmed in four out of five investigated Roma families with suspected CMT. Sequencing detected single nucleotide substitution R148X in NDRG1 gene in two patients (CMT4D) and AltT2 single nucleotide substitution G> C in an alternative exon of HK1 gene in two other patients (CMT4G).

Example no. 3: Nephrotic syndrome. Nephrotic syndrome (NS) is a heterogeneous group of disorders characterized by heavy proteinuria with hypoalbuminemia, edema and dyslipidemia (Machuca et al., 2009). Two consanguineous Roma families with one and two children affected by nephrotic syndrome were identified. Kinship between families failed to show, however, families have the same surname and live in near villages. After excluding the NPHS2 gene, genome-wide analysis was performed on all three patient samples using Affymetrix SNP Genotyping Array 6.0. Whole-genome analysis of parametric LOD values with reduced set of 20,000 SNPs showed the highest curve on chromosome 14. Subsequently, detailed analysis of the complete set of SNPs was performed for the region, which showed the highest LOD value of 3.31 in two adjacent areas (this is also theoretically the highest possible score for given pedigrees). Literature and database search showed that there is no gene in this region previously associated with any form of nephrotic syndrome.

Discussion

We have shown three examples of performing molecular diagnostics in consanguineous Roma families. In all cases, deep literature search is a must. In first case – analbuminemia – phenotype suggested false diagnosis of hypoanalbuminemia, but knowledge of occurrence of this very rare disease in cases with similar symptoms in Roma families in the same region have led us to predict the same mutation. It is very likely that the mutation is of common origin, which is a phenomenon typical for isolated founder population. Haplotype analysis must be performed to confirm this hypothesis. The second case of CMT4 disease is a typical example of a rare condition caused by a founder mutation present almost exclusively in Roma. Absence of this subtype of disease in non-Roma population in Slovakia has caused that it is not well known and subsequently not properly diagnosed. Last example is that of nephrotic syndrome, were we failed to determine the causal mutation. This case is different from previous ones – there is no mutation private to Roma or at least present in Roma families in Slovakia described in literature. It is clinically and genetically very complex and heterogeneous disorder, were screening the whole genome was the optimal choice to begin search for causal mutation. The homozygosity mapping excluded all genes known so far, which may lead us to two consequences. Either the homozygosity appeared by chance or there is a completely new gene involved in nephrotic syndrome in our families. Considering the second case, we will continue research by analyzing the region on chromosome 14.

Research effort can lead to improvement of medical care. Several mutations specific for Roma population have been revealed in the past years. Application of this knowledge into medical practice is often difficult. At first sight different gene pool of Roma complicates the diagnosis of patients. But knowledge of diseases and particular mutations specific to certain ethnic group can actually greatly simplify and speed up the diagnostic process. It is necessary for clinicians to get a good knowledge of conditions specific for ethnic groups and to bear this in mind when

deciding on diagnostic strategy. It is necessary to know the causes of rare diseases and to focus diagnostic procedure on the ethnically specific mutations. Otherwise an underdiagnosis or wrong diagnosis of these rare conditions may remain a problem.

The Roma families presented here are a good example of fast clinical diagnosis, where disease type was predicted based on ethnic origin of proband and confirmed by genetic testing.

Human rights and socio-economic issues related to the Roma are increasingly becoming the focus of political debate and media coverage throughout Europe, their poor health status is rarely discussed and still awaits the attention of the medical profession (Kalaydjieva, 2001).

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ANTHROPOMETRIC CHARACTERISTICS OF ROMANYAND NON-ROMANY CHILDREN IN PREŠOV REGION

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Abstract: *The goal of this research was to provide actual information about somatic growth and development of Romany children of school age in the Prešov region, identify and characterize the specifics of the physical structures and compare them with non-Romany children with their physical characteristics. The object of the research was a set of Romany and non-Romany children of younger and older school age from two primary schools in Prešov. Both sexes were observed, boys and girls in five age groups. In both studied groups we monitored selected anthropometric parameters - body height, body weight, abdominal circumference, hip circumference, chest circumference and width of the pelvis. The most significant differences between Romany and non-Romany girls were found in the fourth age group, where in Romany girls were statistically significant higher average values of all parameters, except height. In Romany girls were recorded significantly higher average values of abdominal circumference, in the fourth (p=0.001), as well as fifth (p=0.035) age group. When comparing the average values of selected parameters of Romany and non-Romany boys, no statistically significant differences were found. Romany as a specific ethnic group in anthropological view is characterized by several feature differences from non-Romany population arising precisely from their origin, genetic background or culture.*

Key words: *anthropometry, romany population, younger and older school age*

Romany populations from an ethnic point of view belong to the Caucasian (Eurasian) group and by Pospisil et al. (2002) represent the most numerous non-European component of the population of our continent. Their original homeland was probably India, and particularly its northwestern part, came to the European continent in migration flows around the 10th to 11 century. Today Romanies are widespreaded in several European countries and in Slovakia, the number of this population is around 400,000. They belong to an ethnic group, respectively ethnicity, which is defined as a group of people having a common origin, language, and also culture (Ľuptáková, 2004). They form a community of their own with their distinctive features which differs from the Slovak majority population. From the bio-anthropological site specific to them just diversity in several physical characteristics compared to the rest of the population of Slovakia, which is related mainly to their origin.

The aim of this research was to investigate the basic anthropometric characteristics of Romany children of younger and older school age within the selected anthropometric parameters. Compare the find out values with the values of non-Romany children and their physical characteristics.

Materials and methods

Research was conducted in February 2012 at two primary schools in Prešov region. The analyzed group consisted of 62 Romany children, representing 33.51 % and 123 non-Roma children, which represented 66.49 %, aged 6 to 15 years. Both sexes - boys and girls were observed. Children were divided into five age groups according to the grade that attended:

1st class - age category 1
2nd and 3rd class - age category 2
4th and 5th class - age category 3
6th and 7th class - age category 4
8th and 9th class - age category 5

Accurate representation of Romany and non-Romany children by sex in different age groups is shown in Table 1.

Table 1 Representation of Romany and non-Romany children by sex in different age groups

ROMANY PROBANDS			NON-ROMANY PROBANDS		
Age category	Sex		Age category	Sex	
	Boys	Girls		Boys	Girls
1	7	4	1	8	7
2	11	3	2	12	12
3	8	5	3	15	20
4	3	7	4	13	15
5	9	5	5	13	8
TOTAL	38	24	TOTAL	61	62

During the research, we investigated in both groups these selected somatometric parameters: body height, body weight, abdominal circumference, hip circumference, chest circumference and width of the pelvis. Determining the anthropometric data, we stick to the procedures and guidelines as Martin and Saller (1957) and Fetter et al. (1967). Individual measurements were carried out under standard conditions during PE classes, investigating probands were dressed in workout clothes.

When processing the data and interpreting the results, we used IBM SPSS software for Windows. Comparison of average values of selected parameters was

performed by using Student's t-test. For the level of statistical significance was considered p-value <0.05.

Results and discussion

In each of the five age groups, we observed differences in average values and frequency of gain parameters in Romany and non-Romany children of younger and older school age.

In the first, nor the second age group when comparing the average values of monitored parameters between Romany and non-Romany girls, significant differences were not found. The most significant differences between Romany and non--Romany girls were found in the fourth age group, where in Romany girls were seen statistically significantly higher average values for all studied parameters, except for body height (Table 2). In Romany girls were recorded significantly higher average values of abdominal circumference, in the fourth (p = 0.001), as well as in fifth (p = 0.035) age group. We found that at this age (4 and 5 age group) Romany girls have higher values of parameters said above than non-Romany, which agrees with the results of Bernasovský and Bernasovská studies (1999). Higher values of body weight and abdominal circumference in Romany girls may reflect their poor diet and poor eating habits associated with low consumption of fruits and vegetables, and vice versa with increased consumption of fats and sugars - pork, sweets and soft drinks, as well as a comfortable way of life without sport or physical activity. It is a reflection of the social and economic situation of the Romany(2010).

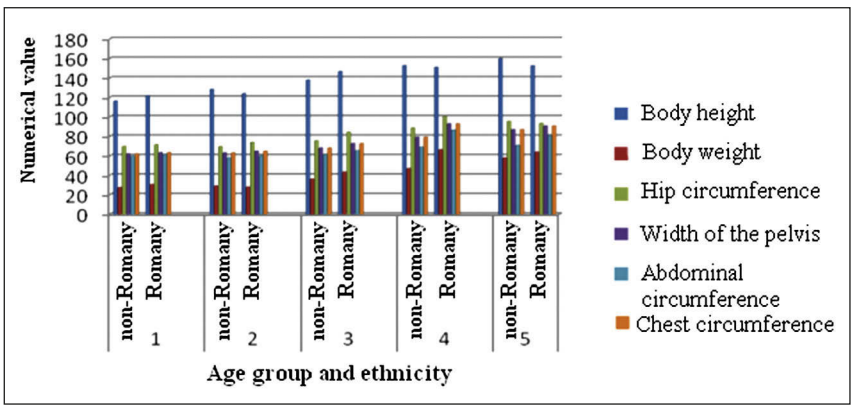

Figure 1 Average values of parameters of Romany and non-Romany girls in different age groups

When comparing the average values of selected parameters of Romany and non-Romany boys statistically significant differences were not found. In the age category 1 non-Romany boys achieved on average higher values of body height and

Table 2 Comparison of average values parameters of Romany and non-Romany girls in different age groups

Age category		Body height (cm)	Body weight (kg)	Hip circumference (cm)	Width of the pelvis (cm)	Abdominal circumference (cm)	Chest circumference (cm)
1 Romany	x	121,50	30,50	71,25	19,25	61,00	63,25
	SD	7,31	4,66	3,95	0,96	5,72	5,25
1 non-Romany	x	116,21	27,29	69,71	19,07	59,43	62,00
	SD	6,20	7,27	5,41	1,17	8,62	5,07
p		0,233	0,452	0,634	0,802	0,754	0,703
2 Romany	x	123,83	28,00	73,67	20,43	59,67	64,67
	SD	3,82	3,46	1,16	1,69	3,51	4,04
2 non-Romany	x	128,13	29,00	69,42	20,25	57,92	62,92
	SD	4,86	4,63	4,32	1,14	4,25	3,87
p		0,181	0,735	0,123	0,822	0,525	0,499
3 Romany	x	146,60	43,20	84,20	22,58	65,20	72,60
	SD	7,21	6,14	6,50	1,86	4,21	5,08
3 non-Romany	x	137,66	36,10	75,30	21,18	61,05	68,00
	SD	7,01	7,31	6,13	1,43	5,84	5,57
p		0,018	0,058	0,009	0,076	0,151	0,107
4 Romany	x	150,79	66,29	100,14	25,86	86,29	92,86
	SD	4,94	14,14	7,88	1,95	12,37	9,65
4 non-Romany	x	152,57	46,87	88,60	23,83	68,80	79,07
	SD	10,61	9,55	10,97	2,14	8,82	9,32
p		0,68	0,001	0,022	0,046	0,001	0,005
5 Romany	x	152,30	63,80	93,20	25,50	80,60	90,60
	SD	3,40	4,15	2,95	1,12	3,13	2,41
5 non-Romany	x	159,69	57,75	95,38	25,00	70,75	86,87
	SD	9,20	13,50	8,28	1,98	8,71	6,47
p		0,117	0,358	0,588	0,620	0,035	0,248

x = average, SD= standard deviation, p = statistical significance($p<0.05$)

weight, with a higher abdominal circumference, but no significant differences in comparison with Romany boys. Also in 5 age group was seen that Romany boys achieve lower average values for all parameters excluding body weight, which was slightly higher. These data moved to the border of significance, but did not reach the statistical significance.

Conclusion

Differences between Romany and non-Romany children were found only in a few parameters. These differences depend on age at a younger age were not significant differences, but in the older age groups, the differences in average values showed off gradually. It should be noted that the results could be influenced by a small number of individuals in the study group, which encourages us in the future to a deeper look at this issue with a larger number of probands or extend the study to a larger number of monitored anthropometric parameters.

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EFFECT OF OCCUPATIONALLY PHTHALATE EXPOSURE ON PULMONARY FUNCTIONS IN SLOVAKIAN PLASTIC INDUSTRY

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Abstract: *Phthalates have adverse effect on human endocrine or reproduction system, but there is still lot of questions about their potential activity in human physiological functions. Number of papers indicates respiratory symptoms associated with possible phthalate exposure. Especially presence of MBP in human urine has been associated with decrease of FVC and FEV1 values. The aim of this study was to assess, by biological monitoring, worker's exposure to phthalates in the flexible--PVC industry in Slovakia to provide additional occupational exposure data, which are particularly scarce. Additionally, parameters of pulmonary functions and anthropometric values were obtained and analysed with exposure data. In response to determine human exposure to phthalates, we used high performance liquid chromatography (HPLC) and tandem mass spectrometry (MS/MS) analysis to quantitate trace levels of four phthalate metabolites, monobutyl phthalate (MBP), monoethylhexyl phthalate (MEHP), mono-n-octyl phthalate (MnOP) and monoisononyl phthalate (MiNP) in human urine. Urine samples, somatometric measures and spirometric values were collected from group of workers in plastic manufacture (n = 15; average age 44.8 ± 11.34). Lower values of FEV1 and FEV1/FVC point to potential airways obstruction in 13.3 % of probands (n = 2, average p/y = 12.5). We also observe overweight in 53.3 % of probands (n=8) indicated by BMI ≥ 25. Phthalate metabolites were detected in all urine samples. We suppose that occupationally increased exposure to phthalates has potential adverse effect on observed pulmonary parameters. However, to prove this assumption, we need more data to be analyzed.*

Key words*: anthropometry, HPLC/MS/MS, occupational health, phthalates, spirometry*

Advances in materials science and engineering in recent decades have led to the widespread and diverse use of plastics to provide cheaper, lighter, stronger, safer, more durable and versatile products and consumer goods that serve to improve our quality of life. (Andrady et al.,2009; Thompson et al., 2009). Whereas every inhabitant in Europe and North America used 6.5 kg plastic each year in1980, the use had increased to around100kg in 2006 and continues to increase. In year 2005, the worldwide production amounted to 245 million tonnes a year and the industry employed more than 1.6million people (Andrady et al.,2009; Thompson et al., 2009).

Components used in plastics, such as phthalates, bisphenol A (BPA) and others are released from plastic products and are also known as endocrine-disrupting compounds (EDCs) owing to their ability to modulate the human health (Stanley et al., 2003). The esters of 1,2-benzene dicarboxylic acid, commonly called phthalates, are group of man-made chemicals used in large variety of industrial applications. As plasticizers, phthalates are additives which improve the flexibility, processability and softness of vinyl. (Blount et al., 2000) Phthalates can be also used for nonpolymeric application as fixatives, detergents, lubricating oils, and solvents (Ntp-Cerhr, 2003). It is proved that phthalates exhibit endocrine disrupting activity and they can affect reproductive system a cause developmental anomalies (Stanley et al.,2003; Ntp-Cerhr, 2003; Swan, 2008; Øie et al.,1999). But there are lot of suggestions that they also play role in development of respiratory system disorders associated with obstructions of airways. In studies (Jaakkola et al., 2004; Kolarik et al., 2008; Polakoff et al., 1975) based on questionnaire data about plastic materials in homes was potential exposure to the phthalates associated with respiratory symptoms in young children. In adult studies (Nielsen et al., 1989; Norback et al., 2000; Bornehag et al., 2005; Jaakkola et al.) was assessed relationship between risks of respiratory disorders related to asthma and occupational exposure to PVC degradation products – potentially to phthalates. Urinary phthalate monoesters are considered good biomarkers for assessing phthalate exposure because of low contamination risk during processing and analysing samples (Ntp-Cerhr, 2003). Despite of available analytical methods to assess human exposure via urinary levels of phthalate metabolites, there is lack of studies, which are using these methods in association with pulmonary function tests. There are basically three ways of exposure to these chemicals: by ingestion, inhalation and dermal contact (Øie et al., 1999). Because of presence of phthalates in big amount of materials and products of common use, there is high opportunity of exposure in everyday life. This risk is much higher in the process of production because workers are exposed to the fumes of the processed materials. Hence, we focused on assessment of phthalate exposure of workers in plastic factory by urine analysis and evaluation of pulmonary function of these workers.

Materials and methods

Study was conducted on workers of plastic factory (n=15), in which were used plastic injection molding machine to produce variety of products from PVC, PP and PET material. We obtained data about age, height, weight, pulmonary function parameters, smoking status and urine specimens. Control group consisted from probands of common population working outside of factory (n=25). All participants were physically healthy, without any acute symptoms. Probands' participation in this study was entirely voluntary and also had the possibility to withdraw their participation at any time during the study. Informed consent was required to be interviewed by the researcher, to provide samples of urine, complete questionnaires and allow the researchers to take measurements and also to process their medical and personal records and data. The anthropometric data was collected using standard anthropo-

logical methods; Body height was estimated by anthropometer (A 319 TRYSTOM, Ltd., Olomouc, Czech Republic). Body weight was estimated by digital weighting scale Omron BF510 (Kyoto, Japan) and body-mass index was classified by WHO (Samandar et al., 2009). Spirometry was performed by Spirolab II (MIR S.r.l, Via Del Maggiolino, Rome, Italy) and Winspiro PRO 4.1 software. The European Respiratory Society predicted values (ERS) were used to calculate "normal" spirometry values. The best test result was determined following the ERS and ATS standards (American Thoracic Society), and FEV_1, FVC and PEF parameter reproducibility was also calculated.

For the phthalate analysis, urine samples were obtain from all volunteers and stored in transport box at 2-6 °C. In laboratory were all samples stored in deep freeze at the temperature -73°C until analysis (Silvia, 2004). Four phthalate metabolites, monobutyl phthalate (MBP), monoethylhexyl phthalate (MEHP), monooctyl phthalate (MnOP), monoisononyl phthalate (MiNP) were measured in urine specimens by high performance liquid chromatograpy (HPLC) and tandem mass spectrometry (MS/MS). Urine analysis was made according to analytical method described by Silva (Meeker et al., 2009) with use of manual solid phase extraction (SPE). Analytical standards were purchased from Cambrindge isotope laboratories (MA, USA). Briefly, 1ml of urine was thawed buffered with ammonium acetate and spiked with isotope labelled phthalate standards, β-glucuronidase enzyme (Roche, Germany) and incubated (37°C). After deconjugation were samples diluted with phosphate buffer (NaH_2PO_4 in H_3PO_4) and loaded on SPE cartridges (ABS Elut Nexus, Agilent). Cartridges were conditioned with acetonitrile followed by phosphate buffer before extraction. To remove hydrophilic compound were SPE cartridges flushed by formic acid and HPLC grade water. Elution of analytes was performed by acetonitrile and ethylacetate. Eluate was dried by nitrogen gas and reconstituted with 200µl of H_2O. For HPLC purposed was used Agilent 1260 liquid chromatograph equipped with ZORBAX Eclipse plus phenyl-hexyl column. Separation was done using non-linear gradient program (Table 1). Agilent 6410 triplequad with electro-spray ionization was used for mass specific detection of phthalate metabolites. Instrumental settings were as follows: spray ion voltage (−3800 V), nitrogen nebulizer gas pressure (8 psi), nitrogen curtain gas pressure (7 psi), capillary temperature (430°C), and collision gas (nitrogen) pressure (1.5 mTorr). Precursor and product ions, collision energies, retention times and limits of detection (LOD) are showed in Table 2.

Table 1 Gradient program for HPLC separation

Time, min	0	4	6	8
A%	80	60	40	10
B%	20	40	60	90

Flow rate (0.3 $ml.min^{-1}$); mobile phase A (0.1% acetic acid in HPLC grade water) and mobile phase B (0.1% acetic acid in acetonitrile)

Table 2 Characterization of precursor, product ions, collision energies and retention times of phthalate metabolite and their labelled standards

Compound Name	Precursor Ion	Product Ion	Fragmentor (V)	Collision Energy (V)	RT, min	LOD, $ng.ml^{-1}$
MiNP	291,2	141,2	95	13	13,1	8.12
MEHP-C4	281,1	137,1	90	14	12,7	
MEHP	277,1	133,9	90	14	12,7	10.2
MnOP	277,1	127,2	90	10	12,9	8.52
MBP-C4	225,1	78,8	90	10	8,7	
MBP	221,1	76,9	90	10	8,7	3.47

Results

For evaluation of pulmonary functions, we obtained anthropometric and spirometric data from 15 workers of plastic factory, three males and 12 females. Control group consisted of five males and 20 females approximately same age as target group. Values of anthropometric measurements, pulmonary function tests, age of both groups are showed in Table 3. Pack/year index (p/y) was calculated as a number of cigarettes smoked per day × number of years smoked/20 for participants with smoking history. We observed increased values of BMI in plastic factory workers and decrement of spiromteric parameters in plastic factory workers.

Table 3 Mean±SD values of anthropometric and spirometric parameters

	Case		Control	
	♂ n=3	♀ n=12	♂ n=5	♀ n=20
Age	51.67±10.12	43.04±11.36	43.00±21.43	39.1±14.09
BMI	26.27±1.36	27.44±6.27	26.33±4.27	24.13±4.06
FVC	4.02±0.34	3.17±0.4	4.92± 0.78	3.53±0.52
FEV_1	3.66±0.10	2.85±0.45	4.16 ±1.09	2.89±0.58
FEV_1/FVC	78.7±3.62	78.17±7.72	79.9± 9.23	81.51±7.62
MEF 25-75	3.33±0.43	2.87±0.97	4.03± 1.86	3.65±0.49
VC	3.52±0.5	3.7±0.59	5.03±0.98	3.45±0.45
p/y	1.67±2.89	2.42±4.98	9.09±21.27	5.65±24.79

FEV_1/FVC value is one of crucial parameters for diagnosis obstructive disorders of airways. In 13.3% probands of target group and 8% of control was determined decrement of this parameter under value 0.7. Workers in plastic factory (46.6%) had

decreased this value under 0.8 as compared with 36% of control group. Also other parameters of pulmonary functions were decreased in case group.

Table 4 Effect of smoking status on reduced FEV_1/FVC values

		Case				Control			
		♂ n=3		♀ n=12		♂ n=5		♀ n=20	
		n	%	n	%	n	%	n	%
$FEV_1/FVC < 0.7$	Smoker	0	0	2	16.6	0	0	0	0
	Ex-smoker	0	0	0	0	1	20	0	0
	Non-smoker	0	0	0	0	0	0	1	5
	All	**0**	**0**	**2**	**16.6**	**1**	**20**	**1**	**5**
$0.7 \leq FEV_1/FVC < 0.8$	Smoker	0	0	1	8.4	0	0	4	20
	Ex-smoker	0	0	2	16.6	0	0	1	5
	Non-smoker	2	66.6	2	16.6	2	40	2	10
	All	**2**	**66.6**	**5**	**41.6**	**2**	**40**	**7**	**35**
$0.8 \leq FEV_1/FVC$	Smoker	1	33.3	0	0	1	0	0	0
	Ex-smoker	0	0	0	0	0	0	1	5
	Non-smoker	0	0	5	38.4	1	0	11	55
	All	**1**	**33.3**	**5**	**41.6**	**2**	**40**	**12**	**60**

We analysed urine samples of all plastic factory workers (n=15) and part of control group (n=10). Unfortunately, optimization of analytical procedure is not still over, hence we have no relevant concentration data about levels of phthalates in collected urine samples. Recoveries of phthalate metabolites are not satisfactory and vary from 32.4% to 68.5%. We were able to detect trace levels of phthalate metabolites in all urine samples of plastic factory workers (Figure 1). Qualitative analysis confirmed presence of MBP and MEHP in all urine samples. MiNP was determined in 66.6% of plastic factory workers in contrast with 28% of control group and MnOP in 40% of target group but in none of control.

Discussion

Phthalates used in plastics for property enhancement are emerging environmental contaminants of concern. The limited human data, and in certain instances inconsistent data across studies, highlight the need for further epidemiological research on these classes of chemicals (Meeker et al., 2009). In our study, we monitored the environmental health status of workers in plastic industry. As shown by the results, lung volumes and flow rates were decreased in plastic factory workers as compared with control group. In accordance with GOLD criteria (Gold, 2006), we determi-

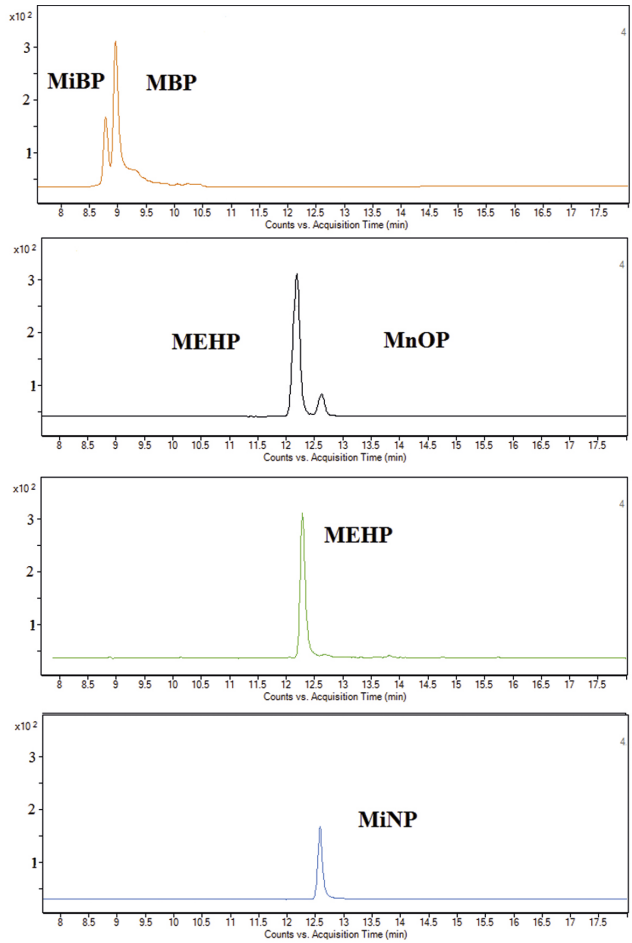

Figure 1 HPLC-MS/MS chromatogram of human urine sample with detection of all phthalate metabolites, retention times are showed in tab. 2

ned values of FEV_1/FVC and FEV_1 that can be associated with chronic obstructive pulmonary disease (COPD) in 13.3% probands of target group and 8% of control. Symptoms such as chronic cough, expectoration and normal spirometry ($FEV_1/FVC \leq 0.7 – 0.8$; $FEV_1 \geq 80$ %) are typical for cancelled 0 stage of COPD, which was reclassified (Gold, 2006) as simple and mucopuruluent chronic bronchitis with a higher risk of developing COPD. We identified this potential risk in 46.6% of plastic factory workers and in 36% of control group. We have also observed decrement of MEF_{25-75} parameters in plastic factor workers. This decline is associated with obstruction in small airways (Gold, 2006). As shown in table 3, risk of these respiratory disorders was higher in a group of plastic factory workers.

Smoking is considered to be most important risk factor in development of obstructive disorders of airways (Gold, 2006). Due to the small number of participants, we had to include to our study also active or ex-smokers. So decreased pulmonary function parameters might by partially due to the effect of smoking. However changes in pulmonary functions are also observed in non-smokers. Comparison of decreased values of FEV_1/FVC in both groups in dependence on sex and smoking status are shown in table 4.

The combined effects of smoking and work environment on the development of chronic respiratory disease were highlighted by the high percentage of subjects with symptoms of simple and mucopurulent chronic bronchitis in target group of our study.

Qualitative urine analysis confirmed presence of phthalate metabolites in all samples and there was no difference in detection of MBP and MEHP between case and control group. Parent phthalate compound of these two metabolites are one of the most common phthalates (Meeker, 2009) and general population might be easily exposed to them. These findings correlate with other biomonitoring studies of phthalate exposure (Blount et al., 2000;Kato et al., 2005;Silvia et al., 2003). Silva (Kato et al.,2005) and Blount (Blount et al.,2000) assessed presence of MnOP and MiNP in less than 20% of general population urine samples and Kato (Silvia et al., 2003) did not even detect these metabolites. Their findings are in correspondence with our results for control group; nevertheless in urine of plastic factory workers was detected increased presence of these two phthalate metabolites. We suppose that this fact is affected by the work environment and not only presence, but also concentration levels of phthalates should be higher in plastic factory workers.

In addition, we detected levels of monoisobutyl phthalate (MiBP), which is isomeric form of MBP. As we see at the figure 1, there are two not fully separated compounds with fragmentation characteristics of MBP. According to Silva (Silvia,2004) MBP and MiBP have very similar fragmentation pattern, same precursor and product ions; hence they have to be qualified by chromatographic separation. Because of small side alkyl chain, retention times of these two analytes are shorter and they elute too close. For this reason, our gradient program needs to be modified to achieve better separation.

Exposure to MBP was associated with decrement of FVC, FEV1 and PEF in males (Hoppin et al., 2004). We determined presence of MBP in all urine samples. Unfortunately, without assessment of concentration of phthalate metabolites in urine, we cannot verify association between exposure to phthalates and impairment of pulmonary functions. Although there was contrast in presence MnOP and MiNP between groups, association between presence of these phthalates and spirometric measurements was not found. In studies based on presence of plastic material in homes (Jaakkola et al.,2004; Kolarik et al.,2008;Polakoff et al., 1975) and workplace (Nielsen et al., 1989; Norback et al., 2000; Bornehag et al., 2005; Jaakkola et al.) were found similar obstructive disorders in association with potential exposure to phthalates.

Conclusion

We identified reduction of lung volumes and flow rates in plastic factory workers and detection of phthalate metabolites in urine samples confirmed exposure to unusual phthalates as in compare with control group. Presence of MBP and MEHP in all of analysed samples support assumption, that risk of exposure to phthalate is part of our everyday life. Following presented studies and our results, we suppose that exposure to phthalates play role in development of obstructive disorders of airways. However this assumption needs to be proved by quantitative analysis of phthalate.

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RISK FACTORS OF OBESITY

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Abstract: *Nowadays obesity is considered to be the greatest threat to health. Analysis of International Obesity Task Force (IOTF) and the World Health Organization (WHO) concluded that in the world there is 1.1 billion adults who are overweighed and with obesity. Over the last twenty years there has been a sharp increase in the incidence of obesity and obesity has reached epidemic proportions. It is a growing health problem that can support the creation and development of serious and very often life-threatening diseases in terms of cardiovascular and respiratory diseases such as heart disease, high blood pressure, diabetes second type, hyperlipidemia, degenerative joint disease, obstructive sleep apnoea and disorders of the spine. It affects not only the excessive rate of adults, but in the last decade it has had also a global increase in the prevalence of obesity in children and adolescents. Nowadays children eat differently than a few years ago and this is considered to be a significant factor in the high prevalence of obesity. The aim of this research work was through a questionnaire survey estimate the prevalence of obesity and weight gain among Romany and non-Romany students in primary schools and to determine whether differences are present not only in the socio-economic sphere, but whether these two ethnic groups differ in the way of eating and sport activity. When comparing the values of anthropometric indicators such as body height and body weight, we found that Romany children achieve compared to non-Romany lower values of these parameters. Statistically significant differences in the prevalence of obesity and weight gain between these two ethnic groups were found. In Romany students we were able to demonstrate a higher incidence of irregular meals during the day, increased consumption of sausages and a significantly lower consumption of milk, dairy products, fruits and vegetables.*

Key words: *obesity, risk factors, nutrition, sports activities, prevalence*

Obesity is a multifactorial disorder characterized by proliferation of the subject of body fat. It is the result of genetic predispositions interacting with environmental factors. The worldwide increase of the prevalence of obesity is given by changes in eating habits, especially increased consumption of foods with a high energy, high fat and simple carbohydrates and also a decrease of physical activity (Hainerová, 2009). Reduced physical activity and excessive energy intake leads to uncontrolled weight gain. Unhealthy lifestyle is not crucial for the development of obesity, it is important to keep in mind other factors leading to its development. Garaulet et al. (Garaulet et

al., 2011) assigned to these factors in addition a lack of physical activity, lack of sleep and psychosocial factors. It should be noted that the word "obesity" is not overweight, but excessive accumulation of adipose tissue (Mrosková et al., 2012). In childhood, there is obviously a continuous weight gain, not only due to fat tissue, but also the development of the skeleton and muscles. The proportion of these components varies in different age periods and by gender (Lisa et al., 1990). The problem of childhood obesity is nowadays increasingly come to the forefront of scientific centres; with up to 80% of obese children remain obese as adults, while the percentage of obese individuals in children is still increasing (Bláha et al., 2001). Lifestyles of most Roma is differs from the majority population. A large part of the Roma living in marginalized settlements not normally practiced sports and active rest only during childhood. To the wrong lifestyle is necessary to add also consumption of unhealthy, fast food. This situation is caused by traditional customs and especially by price unavailability of healthy food. The aim of this research was to precisely investigate eating habits and sports activities of Roma and non-Roma children of school age.

Materials and methods

The analyzed group consisted of students from two primary schools, Romany students from primary school in Chminianske Jakubovany in the number of 192 (60.75%) and non-Romany students from primary school in Medzany in the number of 124 (39.25%) students, a total number of respondents 316 (100%).At the beginning of the research the informed consent was obtained from the parents to include their children in this study. Into the research were involved students of first grade except first classes and all students of second grade.

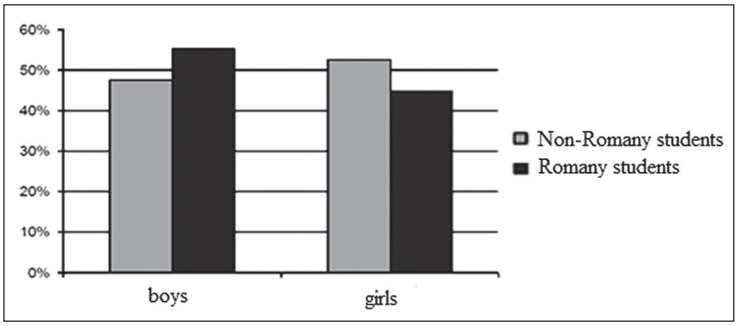

Figure 1 Representation of girls and boys in each analysed group

In determining the anthropometric data such as body height and body weight, we adhere the methodological rules according to Martin and Saller (Martin et al., 1957). We also calculate the BMI index and then we determined the respondents by age we determine the percentile values, which were used for classification of students into different weight categories. A survey of eating habits and physical activity of the cur-

rent population of Romany and non-Romany students was realised by questionnaire research method in the month of September and October 2011. The questionnaire consisted of 32 questions about the frequency of eating, diet composition and quality, related to sports and sports activities of children apart from the PE lessons. Before we distributed questionnaires to students, the goal of our work was explained as well as the importance of accuracy in completing the answers. The return of distributed questionnaires was 100%. To the statistical analysis of the data was used statistical program IBM SPSS for Windows, version 19.0. In the individual files we tested the hypothesis of equality of mean values of variables by unpaired Student's t-test or ANOVA test. The statistical significance of categorical data where tested by chi-square test. For all statistical tests was used as the significance level of p-value <0.05.

Results and Discussion

In the analyzed group, we observed average values of selected parameters such as body height, body weight, average values of BMI index, and were statistically evaluated. When comparing anthropometric data, we found higher average values of body height and body weight in respondents of majority population. Statistically significant difference between the two groups of students was found in any investigation of anthropometric parameters (Table 1). Results of our anthropometric research therefore show significant differences in body growth of Romany children as Bernasovský and Bernasovská indicate in their ethno-anthropological specificity (Bernasovský et al., 1996).

Table 1 Analysis of anthropometric parameters

	Group	n	Average	SD	p
Body weight (kg)	Romany students	192	35,98	11,58	< 0,001
	Non-Romany students	124	42,02	11,90	
Body height (cm)	Romany students	192	136,29	14,58	< 0,001
	Non-Romany students	124	151,76	12,80	
BMI	Romany students	192	18,81	2,68	0,009
	Non-Romany students	124	17,99	2,80	

SD = the standard deviation, n = number of respondents, p = statistical significance

Based on the obtained parameters we then determined percentiles and by usage of percentile charts we set seven weight groups. Since the study is mainly concerned with the incidence of overweight and obesity, we set aside all the respondents on the basis of our calculations did not reach a healthy weight and achieve weight below the

25 percentile. Analysed set thus consisted of 267 students, of which 168 of Romany and 99 of Non-Romany students (Table 2).

Table 2 Selected weight categories in both analyzed groups

Designation category	Number of Romany students	%	Number of Non-Romany students	%	p
K3	124	73,81	66	66,67	0,555
K4	17	10,12	15	15,15	0,317
K5	18	10,71	9	9,09	0,654
K6	9	5,36	9	9,09	0,285
Total	168	100 %	99	100 %	

p = statistical significance

The normal weight of the non-Romany students in the analysis set us occurred in 66.67% and 15.15% for overweight respondents. Excess weight and also obesity occurred in 9.09% of non-Romany students. In Romany children normal weight was observed in 73.81%, 10.12% for overweight, excess weight in 10.71% and obesity in 5.36% of respondents. Statistically significant difference between Romany and non-Romany students was not found in either weight category. Obesity occurs due to several factors, the most important of them is an imbalance between energy intake and expenditure. Energy intake depends on the quantity and quality of food and energy expenditure depends on the amount of physical activity spent. Obesity is a metabolic disease that affects not only adults, but also increasingly in children population. In our work we deal with the eating habits of Romany and non-Romany students and their sports activity in relation to their weight. Collecting data on how diet and physical activity was conducted through a questionnaire, which consisted of a set of questions relating to the catering during the day, the composition of the diet as well as issues related to the intensity of physical activity of observed respondents.

From our survey, we found that their free time spent by sports activity only 21% of Romany students and 23% of non-Romany students (p = 0.762). Most of the free time spent children outdoors with their friends. Popper in his research indicates that the majority of the adult Romany population regularly do not play Sports (Popper et al., 2009). In our study, we reached the similar results. We found that 73% of parents of Romany children do not sport. In contrast, parents of non-Romany children do sports on a regular basis. Parents and their value system can greatly affect or not whether their children will perform sports activities. Unflattering are data documenting interest in sports activities that Romany students cannot perform because of financial and material conditions. A relatively high percentage of alcoholism and delinquency often both parents, as well as unemployment and often even less ability to effectively deal with money cause financing problems of the family (Ďurošová, 2005). In addition to physical activity from the point of preventing obesity in children is also important the

question of the correct diet. One of the questions in the questionnaire was a question that was given to the frequency of meals during the day, by which we wanted to find out how many times a day children eat and if there is a presence of difference between the weight categories. We found that the 5 times a day, consumes only 25% of Romany children and vice versa 57% of non-Romany students ($p < 0.001$). In Romany children less than 3-times daily diets of children in each weight category, while non-Romany have seen this option in only one category, namely the category of normal weight. The most common deficiency in catering mode in children and adolescents belong completely irregular eating breakfast or skipping breakfast. The research work aimed at detecting adverse effects of irregular consuming breakfast show the importance of regular consumption. Children who eat breakfast regularly compared with children who skipped breakfast found better nutritional composition balanced diet and nutrient intake. In contrast, children who do not eat breakfast or eat irregularly found in several studies a higher prevalence of the risk of increased weight and obesity (Babinská et al., 2007). In our questionnaire survey, we also investigated the frequency of eating breakfast. We found that breakfast eats daily 52% of Romany children, 43% of Romany children eat breakfast sometimes and 5% do not eat at all. In non-Romany children, regularly eat breakfast 64% children ($P = 0.265$), 29% eat breakfast sometimes ($p = 0.098$) and 7% of non-Romany do not eat breakfast at all ($p = 0.563$). Statistically significant differences between Romany and non-Romany students in the frequency of consumption of breakfast were not found. The traditional Romany cuisine, when it is possible to use this term, in terms of a diet is very unhealthy. Lifestyle of Romany thus can be described as unhealthy. According to the expert of monitoring the health status of the population Emil Ginter, their way of life is characterized by a high intake of fats and sweets, smoking, alcohol consumption, low consumption of fresh vegetables and fruits. They prefer fat food, meat of lower quality and farinaceous food (Ginter et al., 2001).

Analysis of the results of the questionnaire revealed us statistically significant difference between Romany and non-Romany students in the daily consumption of dairy products ($p = 0.007$). From responses to the question of how many children consume dairy products 4-6 times a week, we found that only 8% of Romany children consume dairy products such amount until in non-Romany, it was 20% ($p = 0.023$). Consumption of energy-rich and less nutritionally valuable foods like sweets and fast-food type of food can disrupt the regularity of diet and regular full-fledged food intake (Babinská, 2007). Also in our set, we observed frequent consumption of sweets. In the group of Romany students most sweets are consumed daily in children obesity category up to 63% and at least these children consume sweets in the overweight category 13% of which responded that eating sweets only a few times a month. In the group of non-Romany children consume sweets the most in the category of overweight and at least 53% in the category of obesity, where 22% of children answered they eat sweets a few times in a month.

Even though fruits and vegetables in human nutrition is irreplaceable due to its high content of vitamins, minerals, fiber and other important substances positively affect physiological processes in our bodies, in the Slovak Republic for the past 10 years

adversely reduced its consumption (Beňo, 2003). In our research work, we have also noticed less power consumption of these important components of nutrition in school age children. In evaluating such issues often eat fruit; we found a statistically significant difference between Romany and non-Romany students. According to our results, up to 39.40% of non-Romany students consume fruit daily. In the group of Romany children 2 times a day eat fruit only 18.83% of respondents, what is in comparison to the non-Romany population 20.57% fewer consumers. We also found a statistically significant difference in eating fruit less than once a week, when Romany students identified this answer in 20.78% while the non-Romany in 7.07% ($p < 0.001$) (Table 3).

Table 3 Consumption of fruit

		2 times a day and more	once a day	4-6 times in a week	1-3 times a week	less than once a week	total
Romany students	number	29	20	31	42	32	154
	%	18,83	12,99	20,13	27,27	20,78	100 %
Non-Romany students	number	39	24	17	12	7	99
	%	39,40	24,24	17,17	12,12	7,07	100 %
	p	0,225	0,546	0,043	< 0,001	< 0,001	

p = statistical significance

When evaluating issues related to the consumption of vegetables, we can conclude similar results to the question of fruit and that the non-Roma pupils eat more vegetables than Roma pupils.

Conclusion

Since obesity is alarming not only in adults, but increasingly also the children population, it is necessary to start with prevention of obesity as soon as possible at early childhood. It is necessary to change the current perception of obesity, which is often seen only as an aesthetic problem and not a health risk bringing the amount of comorbidities and risk of premature death. When comparing the values of anthropometric indicators such as body height and weight, we found that Romany children achieve in comparison to non-Romany, achieve lower values of these parameters. Distinctive growth and development of the Romany population is due to ethnic specificity, by a significant endogamy, socio-economic and natural conditions in which they live. The assessment of obesity in respondents in our study, we used the so-called percentile charts of BMI. Since the majority of respondents in our study had a physiological body weight so we did not find statistically significant differences between Romany and non-Romany students in the prevalence of obesity and weight

gain. From the results we were able to find a higher prevalence of irregular eating in Romany in the diet during the day, we also found in Romany students higher consumption of smoked meat and significantly lower consumption of milk, dairy products, fruits and vegetables.

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DRUG ADDICTION IN THE ROMA POPULATION

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Abstract: *Drugs and drug addiction problem is now a widely discussed topic in our country as well as in the world. Young people often become the victims of abuse. One of the reasons is the easy availability of drugs, already in childhood. A large part of the Roma population is characterized by an inappropriate way of life which is related to the lack of economic security. It is expected that children who grow up in such circumstances will result in the same life as their parents. Based on this assumption, we decided to compare the lifestyle of Roma and non-Roma children, focusing on the consumption of addictive substances. The research was conducted through the questionnaire among Roma children from Chminianske Jakubovany and non-Roma children from the village Medzany. The aim of our research was to determine to what extent the Roma and non-Roma pupils come into contact with the commercially available addictive substances and to identify the impact of the families on incidence of drug addiction in both groups. The analysis results showed that non-Roma children have first experience with alcohol at a younger age compared to Roma children, especially boys at the secondary level (p= 0.036). A significant difference was found in the frequency of smoking, especially among Roma and non-Roma girls at the secondary level (p=0.037). The overall results of the research showed that parents of Roma children increasingly take addictive substances, which is likely related to the results of our analysis in the context of alcohol and smoking. Our research has confirmed the need to continue in paying attention to this issue, intensive work in education and prevention not only in the Roma population.*

Keywords*: Drug addiction, Addictive substance, Roma children, Non-Roma children*

Drug addiction is a serious, genetically and environmentally influenced worldwide problem. It is chronic relapsing disease in which the individual is compulsively searching for drugs and the habit of taking the addictive substances persists in spite of the significant negative consequences (Nestler, 2001). A family, in which the individual grows up has a major influence on developing his or her personality. Through family an individual gets to know society, takes over its habits and behaviour, but also the values. If the protective and educational functions of family fail, if the parents are too strict, or on the contrary too benevolent towards their children, if the parents have frequent conflicts with each other, or themselves have a problem with the use of addictive substances, the child gets on a dangerous path of addiction

much faster (Trávničková, 1998). Roma children as well as non-Roma use their free time to have fun with friends and spend their free time outside the home. The Roma population is, however, characterized by the fact that children live and grow up in a different environment, often socially and financially insecure, with lack of basic hygiene and health care. That was the reason why we have focused on this minority.

The aim of our research was to determine to which extent a family, in which children grow up, is affecting their lives. We compared the life of Roma and non-Roma children and we surveyed whether the environment in which they live, has an impact on increased risk of substance abuse, and we compared the extent to which Roma and non-Roma children come into contact with addictive substances.

Materials and methods

Our research was carried out in May and September 2011 with the participation of 214 Roma pupils from United elementary school in Chminianske Jakubovany aged 8 - 16 years and 124 non-Roma pupils from Elementary school in Medzany aged 7 - 15 years.

Table 1 Comparison of the number of Roma and non-Roma pupils from I. and II. level in the chosen elementary schools

	ROMA		NON-ROMA		Total
	Boys	Girls	Boys	Girls	
I. level	64	51	23	25	163
II. level	51	48	37	39	175
Total	115	99	60	64	338

Due to the fastest and most comprehensive survey we have chosen the method of questionnaire survey. The questionnaire consisted of 26 questions focused on respondents experience with the use of addictive substances, tobacco, alcohol, understanding of their harmful effects and drug use of parents and friends. For the statistical analysis of the data we used statistical software SPSS for Windows, version 20.0. We used chi-square test for the evaluation of categorical data. The criterion for statistical significance was $p < 0.05$. To simplify orientation and facilitate overview, we present the results in the form of tables and graphical data processing.

Results and discussion

Responses to the questionnaires were evaluated and processed using tables and graphs, which served to compare the two schools together. For statistical analysis we compared the responses of girls and boys on a given level of the school. We also compared the answers of the respondents of the same sex at the same level between the two schools.

Table 2 The first alcohol consumption in Roma and non-Roma from I. and II. level of school

Sex	ROMA								NON-ROMA							
	I. level				II. level				I. level				II. level			
	Boys		Girls		Boys		Girls		Boys		Girls		Boys		Girls	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
No experience	26	40	32	62	18	35	26	54	12	53	15	60	9	24	17	44
5-10 years	22	34	9	18	8	16	3	6	10	43	8	32	13	35	7	18
11-15 years	3	5	1	2	15	29	12	26	0	0	1	4	14	38	15	38
15 and more	1	2	1	2	7	14	3	6	0	0	0	0	0	0	0	0
No response	12	19	8	16	3	6	4	8	1	4	1	4	1	3	0	0
p	0,166				0,221				0,688				0,155			

n- number; p- statistical significance

The most frequent answer to the question „At what age have you consumed alcohol for the first time?“ was option „I have not tried yet“. 34% of Roma and 43% of non-Roma boys and 18% of Roma and 32% of non-Roma girls from I. level of the school have tried alcohol between 5-10 years. We observed a statistically significant difference between the Roma and non-Roma pupils from II. level of school. The difference between Roma and non-Roma boys ($p = 0.036$) and between Roma and non-Roma girls ($p = 0.041$) from II. level of the school (data shown in table 2) implies that more non-Roma pupils consumed alcohol before 15. year of age than Roma pupils. The research on the use of addictive substances between Roma and non-Roma children was conducted during the years 2009 - 2010. Regarding use of alcoholic beverages, it was found out that 0% of Roma and 5% of non-Roma pupils up to 11 years of age have tried drinking alcoholic beverages; in the group up to 15 years

Table 3 Frequency of smoking in Roma and non-Roma from I. and II. level of school

Sex	ROMA								NON-ROMA							
	I. level				II. level				I. level				II. level			
	Boys		Girls		Boys		Girls		Boys		Girls		Boys		Girls	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Every day	2	3	1	2	2	4	3	6	0	0	0	0	1	3	0	0
Sometimes	1	2	5	10	14	27	9	19	2	9	0	0	9	24	2	5
Never	61	95	45	88	35	69	36	75	21	91	25	100	27	73	37	95
p	0,136				0,546				0,132				0,031			

n- number; p- statistical significance

of age it was 28% of Roma and 22% of non-Roma pupils (Kollarčík et al., 2001). Our results suggest that the Roma children had more experience with the use of addictive substances, however it is interesting that Roma children up to 11 years did not have any experience with alcohol according to our results.

We did not find any significant difference in responses between Roma and non-Roma pupils from I. level of school after evaluation of the results regarding smoking. As shown in table 3, 5% of Roma respondents from I. level of school said that they smoke every day. This option was not selected by any of the non-Roma pupils. However, after evaluation of the questionnaires we have found a statistically significant difference in responses between Roma and non-Roma girls from II. level of school ($p = 0.037$).

A study from the year 2011 regarding smoking in the group of children under 15 years of age presents that 11% boys and 4% girls under 11 years of age smoke. In the group of children up to 15 years it was 35% of boys and 29% of girls. When comparing these results, it is clear that older boys and girls have greater experience with smoking cigarettes (Baška, 2011).

Table 4 Smoking of Roma and non-Roma parents of pupils from I. and II. level

Sex	ROMA								NON-ROMA							
	I. level				II. level				I. level				II. level			
	Boys		Girls		Boys		Girls		Boys		Girls		Boys		Girls	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Yes	40	63	30	58	14	27	19	40	1	4	2	8	3	8	2	5
No	7	11	6	12	12	24	9	19	10	43	13	52	22	59	29	75
Only mother	0	0	1	2	7	13	5	10	1	4	0	0	2	5	0	0
Only father	4	6	2	4	6	12	5	10	0	0	3	12	6	17	4	10
Both parents	6	9	5	10	10	20	8	17	0	0	1	4	1	3	1	3
No response	7	14	7	14	2	4	2	4	11	49	6	24	3	8	3	8
p	0,820				0,883				0,212				0,622			

n- number; p- statistical significance

In Table 4, we compared the answers focused on smoking habits of parents of Roma and non-Roma pupils from I. level of school. The results showed that 63% of Roma boys have parents who smoke cigarettes, 6% said that only father smokes and 9% said that both parents smoke. The situation is significantly different among non-Roma boys from I.level of school, only 4% indicated that parents smoke. Based on these results, we found a statistically significant difference in responses between Roma and non-Roma boys from I.level of school ($p < 0.001$). A significant difference was also observed between the Roma and non-Roma girls ($p < 0.001$), as much as 58% of Roma girls and only 8% of the non-Roma girls reported that parents are

smokers. We have found interesting facts, in the case of Roma children we noticed 103 positive responses, this means that almost half of the pupils live in a family where the the parents are taking some of the addictive drugs. Only 23 respondents (less than 76%) from Elementary school in Medzany answered positively regarding their parent´s smoking habit.

Ostrihoňová and Bérešová (2008) observed very similar results, they found out that on average 70% of the adult Roma population smoke (Ostrihoňová and Bérešová, 2008). We suppose that if a child from a young age has the opportunity to watch parents smoke, drink alcohol or use other addictive substances, he or she may consider such behavior as right one, and under the influence of various life events he or she also become drug dependent.

Conclusion

Mankind has always been in contact with unknown things. The desire for new knowledge and experiences motivates a person to constantly try and discover. It‘s the human curiosity that constitutes first impulse to try the drug. Children are much more curious than adults and often mindlessly throw themselves into danger, not realizing the risks and consequences of their actions. Their first experiences with drugs are often the result of the curiosity satisfaction. There is no guarantee that even one experience does not cause addiction and there is a probability that a child in order to experience feelings of euphoria and carelessness will repeat the attempt to take a drug. It is therefore important to map these factors and perform precautions, particularly by parents, schools and social workers to avoid the development of addiction, and today‘s generation of children would grew up healthy, which will benefit the society.

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CASE REPORT: ATYPICAL CRANIAL FINDING OF INDIVIDUAL FROM ST. EMMERAM CATHEDRAL, NITRA, SLOVAKIA (14TH – 18TH CENTURY AD)

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Abstract: *This article presented osteological finding of skeletal remains from the St. Emmeram Cathedral, where cranial trauma was detected. Osteological remains were excavated during archaeological examination of the site Nitra - Castle hill, especially Nitra - Cathedral in 2008, where bishops, canons and children (probably from aristocratic families), were buried since 14th till 18th century AD. Therefore more accurate dating of the skeletal remains is not possible. The aim of our study was investigating the process of formation of this wound with available methods. Skeletal remains were anthropomorphological and paleopathological determined and diagnosed by RTG and CT scans to ascertain etiology of aperture of parietal bones. Mentioned skeletal remains of an individual from grave no. 211 entirely consist of the calvarium (probably male, adultus II/ maturus I) which came from rubble, therefore there are no other remains of this individual presented. The presented calvarium showed trauma of likely man-made aperture. Aperture is situated on parietal bones in biconvex shape of approximately length 100 mm and thickness in the middle 40 mm. Based on anthropological examination we conclude on accidental damage during exhumation of skeletal remains.*

Key words: *cranial finding, palaeoanthropology, Post-Medieval Period, radio-diagnostic examination*

Implementation of new examination methods in biology is very important. Nowadays osteoanthropology uses for basic analysis of human skeletal remains radio-diagnostic examination, computed tomography (CT scan). In this study we were able to ascertain our analysis using these techniques as well.

In the years 2008 – 2009, there was archaeological research focused on the St. Emmeram Cathedral in Nitra. It was the last archaeological research of the site Nitra - Castle hill, included locality Nitra - Cathedral and Nitra - Castle. Particular researches of the site Nitra - Castle hill, were taken in the years 1930 – 1931 (led by Mencel and Böhm), in the years 1988 – 1992 (led by Bednár, Fusek and Chropovský) and in the years 1992 – 2009 (directed by Bednár, Samuel and Poláková) (Bednár and Staník, 1993; Ruttkay, 2005).

The latest recovery research in the years 2008 – 2009 was focused on the interior of the church of St. Emmeram. There were detected about 30 graves dated to the Modern Period, 14th – 18th century AD (Bednár and Poláková, 2011).

Within the research there were most of skeletal remains of the individuals significantly damaged by environmental condition, especially by humid environments; as well in the crypt they were deposited under very shallow layer of soil. After the exhumation, human skeletal material was improperly transferred and stored in plastic bags, which resulted in crack on surface and were attacked and infested by mold. Because the archaeologists neglected scientific procedures during exhumation, there were some of the individuals unfortunately mixed together in the boxes. Using anthropological methods, we were able to ascertain the human skeletal remains as well as possible.

For the aim of this study we focused on individual from grave no. 211, where paleopathological cranial finding was observed. The etiology of the cranial wound on the parietal bones of the individual is unknown. Therefore we tried to investigate the process of formation and timing of this wound with available methods.

Anthropologists in context of traumas generally use terms antemortem, perimortem and postmortem trauma. Timing of the bone injury is very important issue of the anthropological analysis. Antemortem trauma occurs before death, perimortem occurs around the time of death and postmortem after death. Antemortem trauma is associated with an active healing (partial or complete) of the injured bone. On the other hand, postmortem trauma is usually considered to be related with taphonomic processes (Wieberg and Wescott, 2008). When this happens soon after death and the bone is still fresh, it takes characteristics of perimortem trauma. Specific features emerge when the bone is dry (Byers, 2011).

Antemortem trauma is associated with three features usually presented on the bone. The first is the porosity near the breaks, indicating bone activity and resorption. The second feature is the rounding of the edges of the break, because of remodelling the bone tissues (Sauer, 1998). The third feature is the presence of callus.

Perimortem trauma is characterized with the sharp edges, hinging, that occurs when section of the bone is bent away from the direction of the blow. Additional features of perimortem trauma are formation of fracture lines, angled and jagged surfaces of the broken bone and staining after the hematoma (Sauer, 1984).

Postmortem damage can also make significant changes to the skeleton. Breaks in dry bone seldom show radiating fracture lines. For dry bones it is more common to snap like a dry twig because of strong postmortem damage (Byers, 2011).

Trauma determining is facilitated by colour of the bones and the edge characteristics of the broken surfaces. Fractured surfaces display light coloration that contrasts with the dark staining presented on those surfaces which have been exposed for most or the whole postmortem interval (Ubelaker and Adams, 1995).

Materials and methods

This study focuses on the analysis of the skeletal remains of an individual from grave no. 211, from the interior of the church of Saint Emmeram from the locality

Nitra - Cathedral. Skeletal remains consists from calvarium which came from the rubble, therefore there are no other remains of this individual presented.

Available methods for the formation process of the mentioned wound of the calvarium, were used. For diagnosing of supposed pathology we used methods of Ortner and Putschar (1981) Ortner (2003) and Horáčková, Strouhal and Vargová (2004).

While processing of the skeletal material we used the standard morphoscopic and morphometric methods by Martin and Saller (1957) and Knusmann (1988). Life expectancy of the individual was estimated using the method of Meindl and Lovejoy (1985). For sex determination were used methods of Ascádi and Nemeskéri (1970) and Ferembach, Schwidetzky and Stloukal (1980).

For purpose of radio-diagnostic examination, computed tomography (CT scan) we cooperate with Jessenius - diagnostic centre, JSC and work with software Tomo-Con Lite.

Results and discussion

Skeletal remains of the individual from grave no. 211 consist of the calvarium (Fig. 1, Fig. 2). Based on the anthropological analysis, we can assume that the calvarium probably belongs to a man with age adultus II/ maturus I. The calvarium is normal shaped, with no signs of craniostenosis or other pathological deformations. Preserved is the skeleton of neurocranium and the right part of the splanchnocranium (part of the corpus maxillae dx. with preservation of facies orbitalis, processus frontalis maxillae dx., medial wall of the orbit, part of the os nasalis, part of the processus zygomaticus dx.). Based on the morphometric analysis cranial index showed that the skull represents the mesocranial head, length-height cranial index on hypsicranial head and breadth-height cranial index showed that the skull belongs to acrocranial.

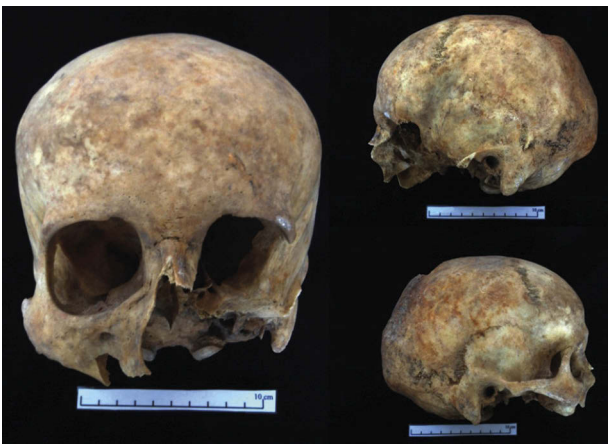

Figure 1 Skull of individual from grave no. 211 (frontal and lateral views)
(photo: author, 2012)

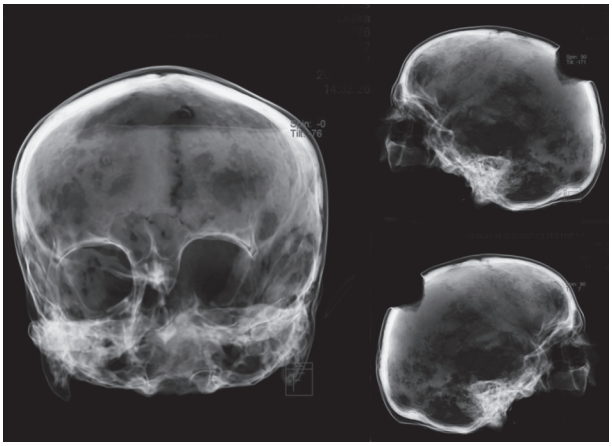

Figure 2 CT scan of the skull of individual from grave no. 211
(frontal and lateral views)

Cranial capacity of the skull no. 211 showed on aristencephal (1518 cm^3 by Welcker method; 1622 cm^3 by Manouvrier method.)

On the calvarium we detected large aperture of the both parietal bones, about 34 mm above the suture lambdoidea. Shape of aperture is fusiform up to oval, or biconvex, with approximately length 100 mm and thickness in the middle 40 mm (Fig. 3).

In the paleopathological analysis it is critical to identify perimortem and postmortem trauma. Process of formation of the wound, size of the perceived violence and the extent of damage are the main criteria by which we assess the wounds.

Mentioned wound of the individual no.211, shows that the edges of the aperture are irregular, slightly jagged, few skewed and rather blunt-ended.

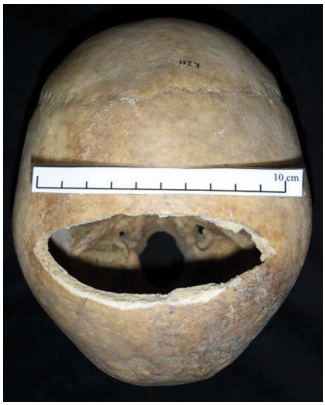

Figure 3 Aperture of the parietal bones, grave no. 211
(photo: author, 2012)

Based on the information from Sauer (1998) who state that bony healing around the fracture is typical for antemortem trauma we conclude that this could be postmortem trauma, because signs of bone remodelling around the wound were not presented.

The thickness of the bone between the lamina interna and lamina externa shows in the middle approximate 8 mm of anterior direction, 7 mm in the middle of posterior direction and about 5 mm in the lateral edges of the aperture. On the basis of radio-diagnostic examination other pathological finding, underlying changes in the thickness of the cranial bones was not detected.

After CT scan examination we could assume that there is no evidence of microfractures. Our assessment confirms the argument, that based on Sauer (Sauer, 1984, 1998) and Byers (2011), this wound could be associated with antemortem damage. This contention would be refuted with the fact that, if the bone had been stored in a humid environment, postmortem damage would not have left other traces of fractures on the surface of the wound. Therefore this statement confirms our theory of the postmortem damage of the calvarium.

Fracture surface is colorated differently. It is lighter in unfosilized bone than the adjacent unbroken surface is. Within the information from Ubelaker, Adams (1995) and White, Folkens (2000) we could assume that it is result of postmortem damage.

If the calvarium, from grave no. 211, belongs to recent individual, based on clinical analysis, radio-diagnostic examination, CT scan, it should be case of biparietal osteoclastic craniectomy. Osteoporotic destruction of the right contours craniectomy, irregular contour of lamina interna and lamina externa indicate that the individual did not die immediately after craniectomy implementation, but in a longer time range after its realization.

In historical context, we can conclude that large aperture on parietal bones of the individual from grave no. 211 originated by the accidental damage probably during the exhumation of skeletal remains.

A similar finding from our territory was found at Bučany, burial site Vinohrady (dated to the 10th century AD). A younger adult man (maturus I) from grave no. 82, showed evidence of an extensive survived neurocranial trauma with antemortem loss of 88 cm^2 of parietal bones. The man had survived the injury by at least one year (Thurzo et al., 1991).

Another similar finding from Western part of Slovakia was found at the site Ducové, district Piešťany (dated to the beginning of the 12th century AD). An adult male individual (adultus II/ maturus I) from grave no. 479, probably suffered fatal injury, when a piece of his skull was cut off. The size of aperture on the skull was 7x5 cm, situated on the left parietal bone (Hanáková et al., 1984; Ruttkay, 2003).

We supose that implementation of new methods into the osteoanthropological analyses could help us to understand some of the paleopathological findings in atypical or contentious situations. Use of techniques like CT scans and RTG in our study, allowed us to identify paleopathological contexts of atypical cranial finding on cranium from grave no 211 from St. Emmeram Cathedral, Nitra, Slovakia (14th – 18th century AD).

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MONITORING CHANGES IN ANTHROPOMETRIC DIMENSIONS OF SLOVAK PATIENTS' HEADS WITH DIAGNOSED CRANIOSTENOSIS BEFORE AND AFTER SURGERY

Eva VALACHOVIČOVÁ – Dana DÚBRAVOVÁ – Eva NEŠČÁKOVÁ – František HORN – Ján TRNKA

Abstract: *The present research deals with specific issues of dimensions in craniofacial changes Slovak patients diagnosed with craniostenosis. We deal with changes in various types of synostosis and changes before and after surgery and in no operated patients. We evaluate the pathological growth of patients' cranium and deviations from the healthy population. Anthropometric examinations are carried according to the methodology of Martin and Saller (1957) and its modifications. We use standard anthropometric instrumentations with donation of Grant Comenius University (UK/77/2012). In our group of patients (n = 43) prevailed in 44 % synostosis of sagittal suture, in 24 % synostosis of metopic suture, in 12 % synostosis of coronal suture, in 3 % synostosis of squamosal suture and in 17 % complex synostosis. We found that patients, who were operated on, had values of anthropometric dimensions approaching to the standard, as those who were not operated on. In these patients we are dealing with pathologies in length, head circumference, in the width of the head and face width. Trend growth of cranium is parallel with the standard in progress 3 months after surgery. Cephalic index is suitable identifier due to rapid and non--invasive determination of pathology growth of cranium. Long-term monitoring of patients allows us to record trend growth of cranium in craniostenosis and disproportionality of observed anthropometric dimensions.*

Key words: *longitudinal study, cephalic index, pathological growth, craniofacial dimensions.*

Craniosynostosis, and related skull deformities are pathologies associated with premature synostosis of cranial sutures. These are etiologically and patho-genetically very heterogeneous clinical entity, now considered a risk factor for worsening central nervous system, the blood supply of the brain and social adaptation. Changes in the blood supply of brain tissue due to constriction of the brain in premature fusion of the neurocranium are an indication of the operating solution not only for cosmetic reasons, but also for the prevention of brain injury- intracranial hypertension (Šnajdauf et al., 2005). They are one of the most common physical deformations in newborns and occur in about one in 2,000 births (Wood et al., 2005). According to the etiology

craniosynostosis divided into primary, caused developmental disorder of unknown etiology, and secondary - in breach of brain development (microcephaly) and after drainage operation for hydrocephalus. Craniosynostosis may have prenatal or perinatal start and may appear in infancy or later in childhood. Shape of the cranium depends not only on the fact which suture has premature synostosis as well as the order and the time at which seams prematurely concluded. Early fusion of one suture cranium let up to grow perpendicular to this suture and growth compensate in other sutures and in the direction of the suture obliteration. Deformations of the cranium then respond to those cranial sutures which were closed (Náhlovský et al., 2006). Monosynostosis with premature synostosis of one, possibly several sutures (*s. sagittal* and *s. metopic*) comprise up 90 %. The remaining 10 % are complete craniofacial syndromes, where craniosynostosis is one of the larger spectrums of symptoms of the disease, in which already known molecular etiology, but still not clear up pathogenesis (Tichý et al., 2002). One of the diagnostic methods of cranial dysmorphia is direct cephalometrics. This is a set of methods for objective assessment of the size of the direct, arc and circumference dimensions of the cranium, which are detected between defined craniometrical points. The human cranium is oriented most frequently in Frankfurt horizontal line (Martin et al., 1957). Other diagnostic methods are CT/MRI of cranium/brain and 3D reconstruction, also ultrasonography of the brain through a large fontanel. Goal of anthropometric methods is to measure size and rate of neurocranium growth to detect defects and diseases that are manifested in changes in speed. The first organized a wide-ranging approach to search hidden defects and diseases in our country were a screening method for determining the development of children from 4 weeks to 18 months, which developed Bargárová et al. (1969). Detailed monitoring of a large number of children produced accurate growth curves of children cranium. The majority of the child population growth cranium more or less in agreement with the developmental curve, or is it only deviates slightly. These deviations from the developmental curve are due to the individual differences in the growth of cranium, but are considered normal. A small number of children deviations beyond the normal growth of the head and evaluated as pathological (Brozmanová et al., 1997). In this study, we focus on the monitoring and measurement of differences in the types of premature synostosis of one or more sutures, as well as to determine which craniofacial dimensions are patients with craniosynostosis differ among themselves depending on type of carniosynostosis and in which craniofacial dimensions are patients different from a healthy population.

Patients and methods

Anthropometric examinations were carried out on patients in Department of Children Surgery (Children Pediatric and Teaching Hospital) in Bratislava. All anthropometric examination from October 2010 to July 2012 by patients with Q75 (craniosynostosis) were statistical analysed. The group of patients consisted of 43 patients, including 30 boys and 13 girls. The group was divided into groups accor-

ding to age, sex and type of premature synostosis. In this work we used standards from Bláha et al. (Bláha et al., 2010). Anthropometric measurements took place by standard anthropological technique according to Martin and Saller (Martin et al., 1957) and its modifications. We studied eight dimensions – transversal headband (t-v-t), sagittal headband (n-v-i), head circumference, length of the head (g-op), width of the head (eu-eu), width of the forehead (ft-ft) width skull base (t-t) and morphological facial height (n-gn). In this time, we measure 31 craniofacial dimensions in patients with craniosynostosis and the basic characteristics of the body measurements. We calculated the index cephalicus (ICE index), which we evaluate by percentage before and after surgery in group of patients with craniosynostosis and also in no operated patients. We assess in our longitudinal study of patients with craniosynostosis the observed variations in selected craniofacial dimensions due to the physiological standards by Bláha et al. (Bláha et al., 2010). We use standard metod called "z-score or normalized index" which indicates in how many standard deviations the comparison value is different from the reference values – standards. We have compiled bar charts patients before and after surgery on the basis of the calculated normalized index. We can capture the pathology and the subsequent success of surgery treatment. We evaluated types and number of craniosynostosis by percentage in the study group of patients, also the index cephalicus before and after surgery. We statistically evaluated group of patients before and after surgery in the watched craniofacial anthropometric measurements.

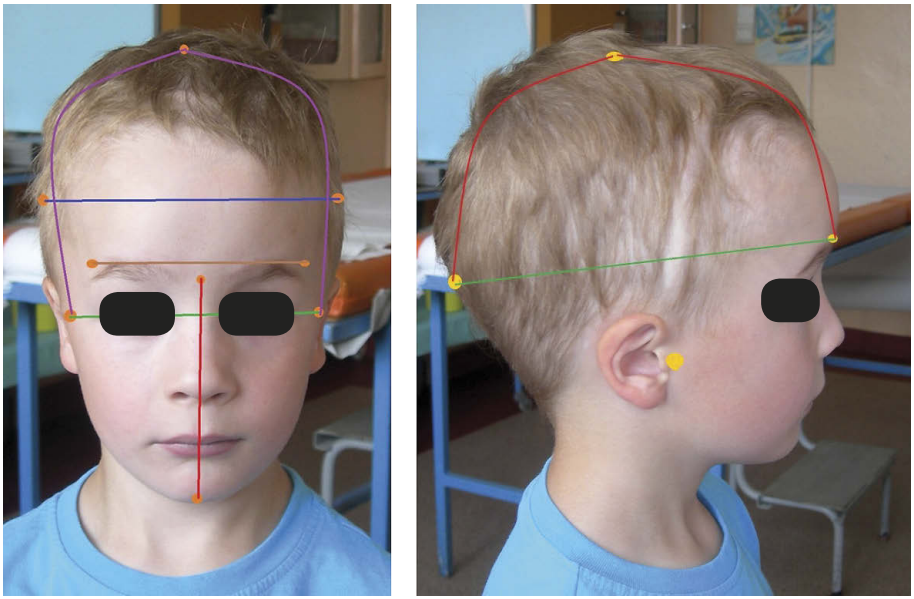

Figure 1 Initial anthropometry, norma frontalis and norma lateralis
(Valachovičová 2011)

Results

The most common types of premature cranial synostosis were sagittal synostosis in 44 % and metopic synostosis in 24 %. Complex synostosis of two or more sutures occurred in 17 %, while 12 % were coronal synostosis.

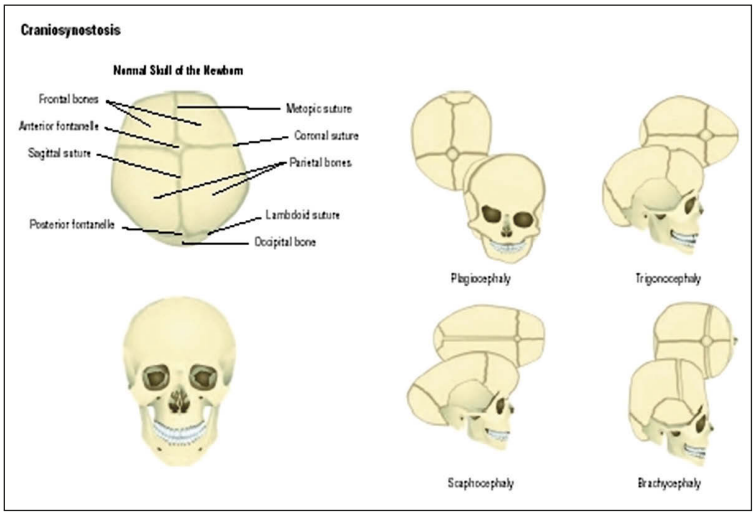

Figure 2 Types of craniosynostosis (http://www.healthofchildren.com)

We recorded compensation growth in the anterodorsal direction in *sagittal suture synostosis (scaphocephaly).* Head is markedly extended. We assess craniofacial dimensions in this synostosis that may indicate possible pathology: the head circumference and length of the head. In observed group of patients were standard deviations ranging from +1.5 SD and more (over 75. percentile and more). Indeed dimensions of the transversal headband and the width of the head varied from -1

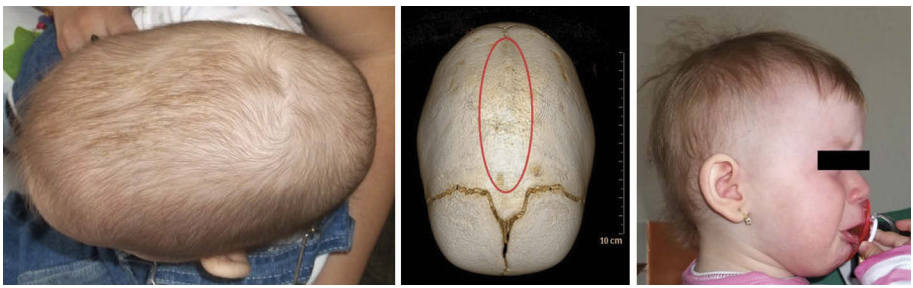

Figure 3 Patients with scaphocephaly before surgery treatment, CT scan, after surgery treatment (Valachovičová, 2011)

SD and below (under 25. percentile and below) standard deviations from the normal range. It depends of extension in premature synostosis, in some cases we evaluated standard deviation from standard in width of head as -2,0 SD (pathology) and in length of head as +2,5 SD. The most common category of cephalic index was dolichocephaly and hyperdolichocefaly. In nonoperated patients pathology persists in length and head circumference. Increase intracranial pressure occurs exceptionally in this type of craniosynostosis.

Metopic suture synostosis (trigonocephaly) is characterized by a compensatory increase in the sides and length of the head. This type of craniosynostosis involves fusion of the metopic suture that runs from the top of the head toward the nose, which can create a ridge running down the forehead and gives the front of the head a wedge-shaped effect. The eyes also may be close together. The craniofacial dimensions important in this case are width of the forehead, head width and head length. Standard deviations from the standards ranged below 10th. percentile and over 90th percentile. Based on the cephalic index prevailed hyperbrachycefaly, ultrabrachycefaly and brachycephaly. In nonoperated patients persists pathology in the width of the head and in cephalic index dominated categories from hyperbrachycefaly to dolichocephaly.

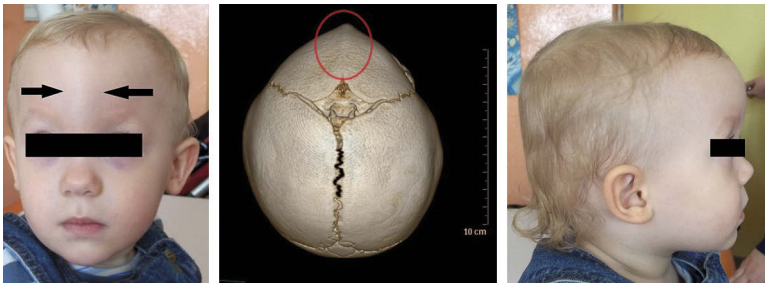

Figure 4 Patients with trigonocephaly before surgery treatment, CT scan (Valachovičová, 2011)

In synostosis of *coronal suture* (both sides) we observed strong brachycephaly. In partial synostosis compensates growth contralateral. The craniofacial dimensions that may indicate possible pathology is the head circumference (-1.5 SD or more), head width (-1.2 SD or more), transverse headband (-1 SD or more). Categories of ICE index are from dolichocephaly to hyperdolichocefaly.

Complete synostosis resulting of premature cranial synostosis of two or more sutures give rise to oxycephaly, turicephaly and towering skull. For all studied craniofacial dimensions were found possible pathology (-2 SD or more) in these cases. Cephalic index was variable.

The calculated indices proved most cephalic index, which recorded the best change in trend growth of the cranium. Other indices, such frontoparietal index, parietobasial index and index of transverse headband showed on the standard devia-

tions no significant from the standard before surgical treatment. It can be concluded that patients who have been early seizures and have recommendation for surgical treatment have approaching to the norm in craniofacial dimensions in the postoperative period.

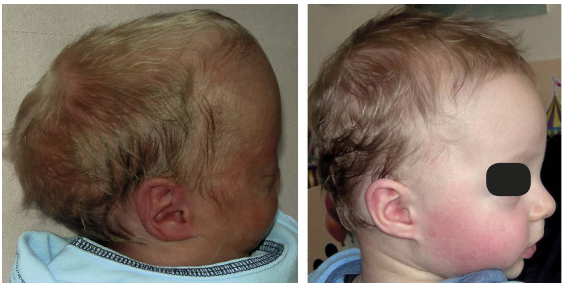

Figure 5 Patient with complex synostosis befor and after surgical treatment (Valachovičová, 2011)

Discussion

A successful approach to craniosynostosis requires timely and accurate diagnosis (already in the neonatal and infancy), selection of appropriate surgical methods and long-term anthropometric follow-up of patient (up to about 6 years). Kolar and Salter (Kolar et al., 1997) have years of experience in craniofacial surgery and cephalometry. The results of our research agree with the results of research Kolar and Salter. They denoted in coronal suture synostosis abnormally low value of the length of the head, a very high value for the width of the head and abnormally large size in width of forehead and cephalic index was usually brachycephalic. They focused as we on measuring length, width and arch dimensions of cranium and craniofacial size selection depends on the type of deformation. We found that patients who have been diagnosed premature synostosis and have no surgical treatment, pathology persisted in the growth of cranium and categories of cephalic index were hyperdolichocephaly, hyperbrachycephaly and dolichocephaly. We recorded in metopic synostosis either abnormally low value of head circumference or very high value of the width of the head. Craniofacial dimensions had mostly average values. Pathology of sagittal synostosis persisted in length and head circumference (abnormally high values). Other synostosis showed no significant deviations from the standard. Varga et al. (Varga et al., 2008) focused on the seven dimensions of cranium and three indexes. They measured length of the head, the width of the head, base width of cranium, face width, largest head circumference and transverse headbands. They confirmed as we that the best proportion for all growth anomalies of the cranium are sagittal and transverse headband. In the group of patients (n = 120) diagnosed with premature synostosis occurred in 45 % sagittal suture synostosis, in 22 % plagiocephaly, in

16 % trigonocephaly and in 13 % brachycephaly as mentioned (Ferreira et al ., 2006). In our group of patients (n = 43) prevailed sagittalis suture synostosis in 44 %, in 24 % metopic suture synostosis, in 12 % coronal suture synostosis, in 3 % squamosal suture synostosis and in 17% a complex synostosis. Other study (Kane, 2004) stated in 64 % sagittal synostosis of all cases, then in 28 % coronal synostosis, in 5 % metopic synostosis and in 3 % lambdoid suture synostosis. Direct cephalometric is fast, reliable, completely non-invasive, repeatable method that can be used for diagnosis, at the indication for surgery, reoperation for postoperative monitoring and development of the skull in craniosynostosis. Long-term monitoring of patients trend to record growth of the cranium and the disproportionality of observed proportions. Growth and shape changes in neonatal cranium not only accurately reflect changes in intracranial pressure, but they are often a valuable indication for detection of skeletal dysplasias, metabolic and hematologic diseases, chromosomal abnormalities or intracranial pathology.

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