Charles University in Prague

First Faculty of Medicine

PhD thesis - short report

MOLECULAR ANALYSIS IN CASES OF INHERITED DISEAS

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Prague 2008

Doktorské studijní programy v biomedicíně

Univerzita Karlova v Praze
a Akademie věd České republiky

Obor: Molekulární a buněčná biologie, genetika a virologie

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Školicí pracoviště: Ústav dědičných metabolických poruch

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Autoreferát byl rozeslán dne: .....

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Content

1 Introduction

The investigation of inherited diseases is fundamental to medical molecular biology. The understanding of the genetic basis and the mechanisms that lead to the disturbance of complex biological system brings new insights into human biology, as well as assisting in the prognosis and the development of treatments of the diseases.

1.1 Ketone bodies of metabolism

The ketone bodies are: acetoacetate (AcAc), D-β-hydroxybutyrate (βOHB) and acetone. They are inevitable part of the energetic metabolism of the organism during fasting and starvation, mainly important for the brain and heart energy supply. The metabolism of ketone bodies includes ketogenesis (synthesis of ketone bodies) and ketolysis (their utilization). Ketogenesis primarily occurs in liver. AcAc is synthesized from acetyl-CoA and acetoacetyl-CoA, the intermediate of β-oxidation of fatty acids and ketogenic amino acids, principally leucine. It is the final phase of lipid energy metabolism. The enzymes of ketogenesis include acetoacetyl-CoA thiolase (T2), 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS), and 3-hydroxy-3-methylglutaryl-CoA lyase (HMGL). The utilization of ketone bodies occurs in extrahepatic tissues, particularly in brain, kidney, heart and skeletal muscle. The enzymes of ketolysis are succinyl-CoA: 3-ketoacid-CoA transferase (SCOT) and acetoacetyl-CoA thiolase (T2). T2 is active in both ketogenesis and ketolysis.[1] (Fig.1)
Abnormal levels of ketone bodies lead to ketosis or to hypoketotic hypoglycemia.

1.1.1 3-hydroxy-3-methylglutaryl-CoA lyase (HL) deficiency

3-hydroxy-3-methylglutaryl-CoA lyase (HL) deficiency is a rare autosomal recessive disease. The patients usually present in the first year of life or in infancy with acute episodes of vomiting, lethargy, hypotonia, and coma; about 20% of cases are fatal.[2] Laboratory tests reveal metabolic acidosis, and hypoglycemia without ketoaciduria. Urinary organic acid profile includes high levels of 3-hydroxy-3-methylglutaric acid, 3-hydroxy-isovaleric acid, 3-methylglutaconic acid and 3-methylglutaric acid. The treatment is based on protein- and fat-free diet, carnitine supplementation and on avoiding metabolic stress such as fasting and starvation. If appropriately treated without the complications, the illness remit within the aging, and adults are free of symptoms.

3-hydroxy-3-methylglutaryl-CoA Lyase (HL) (EC 4.1.3.4) catalyzes the cleavage of 3-hydroxy-3-methylglutaryl-CoA into acetyl-CoA and acetoacetate, the last step of ketogenesis and of leucine metabolism.
Two isoforms of human HL have been found, mitochondrial and peroxisomal.[3,4]
The mature human mitochondrial HL is a homodimer that requires the presence of divalent cation such as Mg^{2+}, Mn^{2+} for its catalytic activity. The 325aa-residue precursor is cleaved to a mature 298aa-residue 31.5-kDa monomer.[5]
The *HMGCL* gene maps on chromosome 1p35.1-36.1, spans over 24 kb. Nine exons are transcribed into ~1.7 kb mRNA.[5] Up to date 28 mutations in 93 patients have been published. [6]

1.1.2 Mitochondrial acetoacetyl-CoAthiolase (T2) deficiency

Mitochondrial acetoacetyl-CoAthiolase (T2) deficiency (3-ketothiolase deficiency, 3KTD) is an autosomal recessive disorder of isoleucine catabolism and ketone body metabolism.
Patients with 3KTD usually present in neonates with the ketoacidotic episodes accompanied with infections, vomiting, and dehydration. The urinary organic acids profile reveal increased excretion of tiglylglycine, 2-methyl-3-hydroxybutyrate, and 2-methylacetoacetate, 3-hydroxybutyrate, and acetoacetate. The treatment is based on protein-and fat-low diet, carnitine supplementation; and glucose administration during acute episodes. The prognosis depends on early treatment and avoiding massive ketoacidotic episodes.
Mitochondrial acetoacetyl-CoA thiolase (T2) (EC 2.3.1.9) catalyzes the reversible reactions of ketogenesis and ketolysis, and isoleucine catabolism. T2 is the only enzyme of ketone bodies metabolism appeared in ketogenesis as well as in ketolysis. T2 is activated in presence of K^+ ions.[1] T2 catalysis reactions:
AcAc-CoA + CoA ↔ 2Ac-CoA
2MeAcAc-AoA + CoA ↔ Ac-CoA + propionyl-CoA.
Human T2 427aa-residue precursor is cleaved in mitochondria to 394aa-residue 41-kDa subunits forming the mature homotetramer. The T2 gene *ACAT1* is localized on chromosome 11q22.3-q23.1, contains 12 exons and spans over 27 kb. The mRNA is about 1.7 kb.[7] About 30 disease causing mutations in *ACAT1* gene were published up to date.[8]

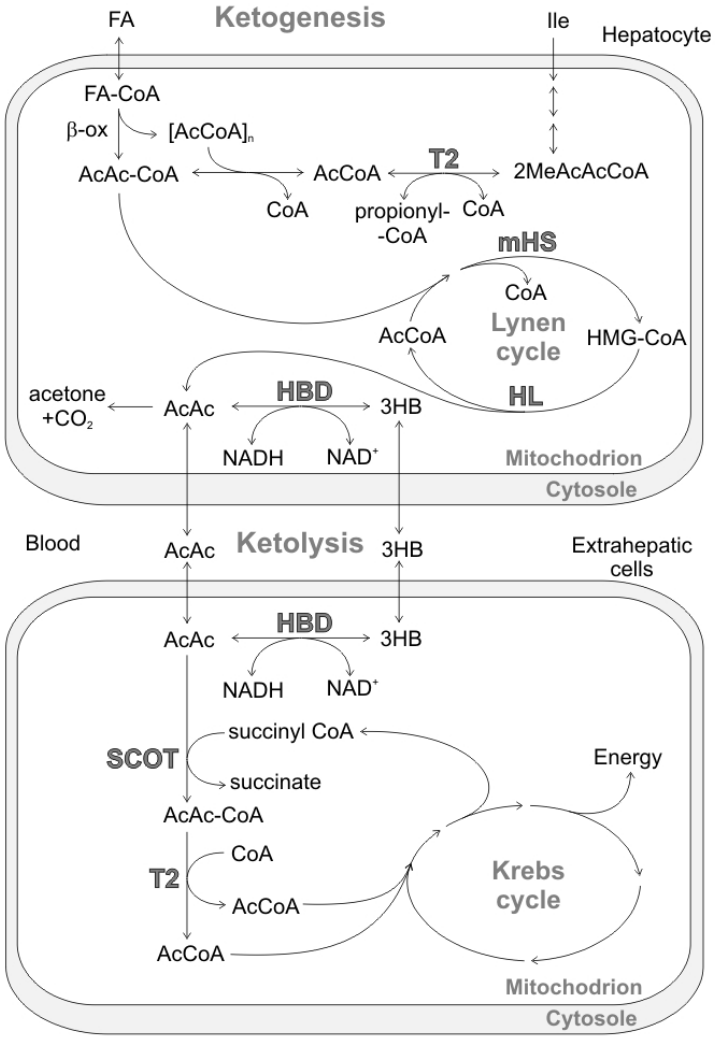

Fig.1: Ketone body metabolism, summarized view. FA = fatty acids, β-ox = β-oxidation, T2 = mitochondrial acetoacetyl-CoA thiolase, mHS = mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase, HL = 3-hydroxy-3-methylglutaryl-CoA lyase, 3HBD = 3-hydroxybutyrate dehydrogenase, SCOT = succinyl-CoA: 3-ketoacid CoA transferase, 3HB = R-3-hydroxybutyrate, Lynen cycle = HMG-CoA pathway.

1.2 Methylmalonic acidemia

Methylmalonic acidemia (MMA) is an autosomal recessive inborn error of metabolism that results from decreased activity of the enzyme methylmalonyl-CoA mutase (MCM, EC 5.4.99.2). MCM catalyzes the isomerization of L-methylmalonyl-CoA to succinyl-CoA in the propionate pathway. In humans it is involved in the catabolism of amino acids valine, isoleucine, methionine and threonine as well as cholesterol and odd-chain fatty acids catabolism. MCM is one of two enzymes which require the derivate of cobalamin (vitamin B_{12}), adenosylcobalamin (AdoCbl), as its cofactor. The other is methionine synthase, with the cofactor, methylcobalamin (MeCbl).[9] According to blocks in the synthesis of these cofactors eight complementation groups (*CblA-CblH*) have been identified through biochemical studies of cell lines from affected patients *in vitro*. Isolated methylmalonic acidemia is caused by a functional defect of the apoenzyme (*mut* forms) or from the insufficiency in three complementation groups occurring in the mitochondrion (*cblA*, *cblB* and *cblH*), which does not affect synthesis of MeCbl.[9] Fig.2

The onset of the disease is early after birth or during infancy. Patients with neonatal form, present with vomiting, lethargy, feeding refusal, muscular hypotonia and seizures. If not appropriately treated, patients progress into acidotic crisis, respiratory distress and coma. Within a few days patients die or develop permanent brain damage. The late onset forms are more variable from chronic symptoms as failure to thrive, anorexia, recurrent vomiting, and behavioral disturbances to life-threatening episodes of metabolic decompensation and severe organ failure.

During acute episodes of metabolic decompensation a massive accumulation of metabolites occurs (e.g. methylmalonic acid, 2-methylcitric acid, 3-hydroxypropionic acid, and other derivates of propionyl-CoA, and ammonia) that lead to intoxication of organism. Homocystinuria is not present. The therapy is based on low-protein, high-energy diet and carnitine and metronidazole supplementation. Vitamin B_{12} is used in B_{12}-responsive patients. Usually very low cases of *mut* patients respond to vitamin B_{12} supplementation, whereas over 90% of *cblA* and 40% *cblB* patients show such a response.[10,11] The most important for the patients is early diagnosis before the metabolic decompensation occurs.

1.2.1 mut form of MMA

Methylmalonyl–CoA mutase (MCM) is the mitochondrial nuclear-encoded enzyme which catalyzes the interconversion of L-methylmalonyl-CoA and succinyl-CoA.

The human *MUT* gene is located on the chromosome 6 (6p12-21.1) and composed of 13 exons spanning more than 35 kb. Transcript is ~2.7 kb in length and encodes propeptide of 750 amino acids. The MCM is targeted

into the mitochondrial matrix, where its 32-residue N-terminal leader sequence is cleaved.[12,13] Human MCM is a homodimer of 78.5 kDa identical subunits (α2).[14] Each subunit contains 1 mol of an adenosylcobalamin cofactor.[15] Two biochemical phenotypes have been distinguished in cultured fibroblasts: *mut*^{0}, mutase activity is completely lost, and *mut*$^-$, residual activity is detectable and/or the activity in *mut*$^-$ cells is increased by the addition of hydroxycobalamin.[16] Over 100 disease causing mutations have been already found in *MUT* gene.[8]

1.2.2 cblA form of MMA

cblA form of MMA is caused by the mutations in *MMAA* gene.[17] The gene product MMAA shares 67% sequence similarity to MeaB, a metallochaperone, member of GTPases subfamily from *Methylobacterium extorquens*.[18] MeaB was hypothesized to have a protective function from irreversible inactivation of MCM, stabilizing the dimer form of the enzyme and/or to protect the bound cofactor from attack by oxygen, water, and highly reactive radical intermediates.[18-20]
The gene *MMAA* was localized to chromosome 4q31.1-2, contains 7 exons, encompassing 17.1 kb and expressing a ~1.2 kb mRNA, predicting a polypeptide of 418 amino acids. The MMAA protein contains a mitochondrial targeting sequence and signal cleavage site.[17] More than 30 mutations have been published in *MMAA* gene up to date.[8]

1.2.3 cblB form of MMA

cblB form of MMA is caused by the functional defect in ATP : cob(I)alamin adenosyltransferase (ATR).[21,22] ATR catalyzes transfer of adenosyl group from ATP to cob(I)alamin to form adenosylcobalamin (AdoCbl)[23].
The human ATR gene *MMAB* maps to chromosome 12q24, contains 9 exons, comprising an open reading frame (ORF) of 750 bp in length. The propeptide of 250 amino acids is targeted by 24 amino acid leader sequence to the mitochondrion, where the protein of a molecular weight of 27.3 kDa is formed.[21,22] About 20 mutations are known in *MMAB* gene.[8]

1.2.4 Correlation of genotype and phenotype

The typical neonatal form of the disease with an early onset, high mortality frequency, and poor neurological outcome occurs in *mut*^{0} patients. *mut*$^-$ and *cblB* patients phenotypes vary from severe neonatal forms to milder forms with a later onset. B_{12}-nonresponsive *cblB* patients (about 60%) have the same phenotype as *mut*^{0} patients contrary to *mut*$^-$ patients, where the B_{12}-responsivity is not critical to determination of the outcome. The less severe forms show *cblA* patients, who became symptomatic later in infancy and have a better survival outcome.[10,11]

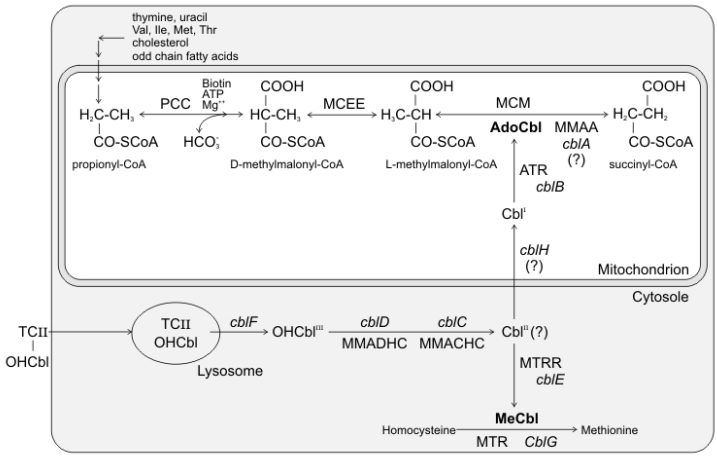

Fig.2: The summarized view of Propionate, Methylmalonate and Cobalamin metabolism.

The major catabolic pathway of propionyl-CoA and methylmalonyl-CoA. Propionyl-CoA carboxylase = PCC (requires biotin as its cofactor and ATP and Mg^{++} ions), DL-methylmalonyl-CoA racemase = MCEE, methylmalonyl-CoA mutase = MCM (requires AdoCbl as its cofactor).

Cobalamin pathway. Cobalamin = Cbl, transcobalamin II = TCII, methylcobalamin = MeCbl, adenosylcobalamin = AdoCbl. Complementation groups *cblA*-*cblH* (in italic) - defects in the synthesis of AdoCbl and MeCbl. *cblA* ~ MMAA protein, *cblB* ~ ATR (ATP : cob(I)alamin adenosyltransferase), *cblC* ~ MMACHC (predicted protein), *cblD* ~ MMADHC (predicted protein), *cblE* ~ MTRR (methionine synthase reductase), *cblG* ~ MTR (methionine synthase).

1.3 Contiguous X-linked deletion syndrome

Contiguous X-chromosome deletion syndrome encompassing the *BTK* and *TIMM8A* genes leads to atypical clinical course of X-linked agammaglobulinemia with neurological impairment, typical for Mohr-Tranebjaerg syndrome.

X-linked agammaglobulinemia (XLA) is an inherited immunodeficiency caused by a B-cell specific maturation defect[24]. The affected gene *BTK* (Bruton tyrosin kinase) is located on chromosome Xq22.1, composed of 19 exons and spanning more than 37.5 kb.[25,26]

Mohr-Tranebjaerg syndrome (MTS; dystonia-deafness syndrome, DDS, DDP) presents with progressive sensorineural deafness and dystonia.[27-29] The disease gene *TIMM8A* (originally named *DDP1*) maps to chromosome Xq22 and contains of 2 exons.[30]

The first exon of the cDNA sequence of *TIMM8A* begins 770 bp downstream from the polyadenylation signal of *BTK*.[31]
Chromosome X is rich on interspersed repetitive sequences; especially on retrotransposones class LINEs, the L1 family (29 % of chromosome sequence)[32]. The high homology of repetitive sequences may lead to unequal homologous recombination resulting in gross deletions.[32,33]

1.4 Mucopolysaccharidosis type IIIC

Mucopolysaccharidosis type IIIC (MPS IIIC) is a lysosomal storage disease caused by deficiency of enzyme heparin acetyl-CoA: α-glucosaminide N-acetyltransferase (HGSNAT, EC 2.3.1.78).[34] MPSIIIC is a rare autosomal recessive disease, which manifests during the childhood with progressive neurological degeneration. Patients present first with hyperactivity, sleep disorders, behavioral abnormalities, that proceeds to mental retardation, hearing loss and convulsions. The visceral manifestation, as hepatosplenomegaly and delayed growth, is relatively mild. Most patients die before adulthood. The glycosaminoglycans are present in the urine.[34]
HGSNAT is the lysosomal membrane enzyme catalysis the N-acetylation of the terminal glucosamine residues of heparin sulfate. It is the only non-hydrolase in the degradation of glycosaminoglycans. Fig.3
The *HGSNAT* gene was independently discovered by Hrebicek[35] and Fan[36] in 2006. The gene maps on chromosome 8p11.1 and contains 18 exons, comprising an ORF of 1,992 bp in length, which encodes a 73-kDa protein.[35] About 43 mutations among over 70 probands have been found in *HGSNAT* gene up to date.[36-38]

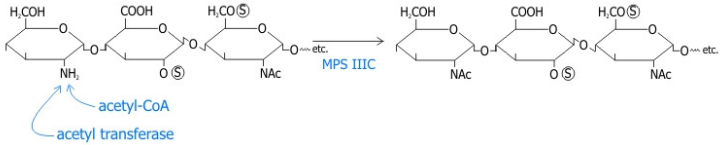

Fig.3: Reaction catalyzed by heparin acetyl-CoA: α-glucosaminide N-acetyltransferase (HGSNAT, EC 2.3.1.78) the step of degradation of heparin sulfate. The deficiency in HGSNAT corresponds to mucopolysaccharidosis type IIIC.

2 Aims

The main goal of the PhD thesis "**Molecular analysis in cases of inherited diseases**" is to describe various methods, which lead to identification and characterization of the mutations in nuclear genes.

The study is divided into three sections with specific aims:

Clinical, biochemical and molecular biological analysis in cases of inherited diseases of organic acids metabolism

- to establish system of differential diagnostic methods for the patients with the erorrs of ketone body metabolism and methylmalonic acidemia
- to identify pathogenic mutations in the known disease genes (*HMGCL*, *ACAT1*, *MUT*, *MMAA*, *MMAB*) in the cohorts of patients
- to perform phenotype - genotype correlation and summarize clinical and biochemical symptoms in patients

Characterization of X-linked contiguous syndrome

- to set up the molecular biological method, which leads to detection of gross deletions in hemizygotes

The molecular study of mucopolysaccharidosis type IIIC

- to map and identify disease gene *HGSNAT* of monogenic disorder MPS IIIC
- to characterize *HGSNAT* gene and the protein product acetyl-CoA: α-glucosaminide N-acetyltransferase

to identify pathogenic mutations of *HGSNAT* gene in the cohort of patients

3 Material and Methods

3.1 Biochemical and enzymatic studies

Metabolite measurements: The metabolites were determined using GC-MS for urinary and plasma organic acids [39], MS/MS for acylcarnitines in a dry blood spot[40], ion exchange liquid chromatography for amino acids in blood[41], spectrophotometric method for ammonia in plasma[42], HPLC with fluorescence method for total homocystein in plasma and serum[43], radiochemical method for carnitine in plasma and serum[44] and spectrophotometric method for glycosaminoglycans in urine.[45]

Enzyme assays: The enzyme activity of 3-hydroxy-3-methylglutaryl-CoA lyase was determined by spectrophotometric[46] analysis or by HPLC method[47] in lymphocytes and/or cultured fibroblastes using 3-hydroxy-3-methylglutaryl-CoA as a substrate and measuring the elevation of acetoacetyl-CoA and acetoacetate, respectively. The activity of β-ketothiolase was measured as decreasing level of the substrate acetoacetyl-CoA with and without K^+ ions by spectrophotometric method[1] in cultured fibroblasts. Methylmalonyl-CoA mutase activity was measured in the presence of 5µmol/l adenosylcobalamin, using L-methylmalonyl-CoA as a substrate and detecting the formation of succinyl-CoA by HPLC method with UV detection[48] in lymphocytes and/or cultured fibroblasts. The enzyme activity of N-acetyltransferase was measured using the substrate 4-methylumbelliferyl β-D-glucosaminide by the fluorogenic method[49] in lymphocytes and/or cultured fibroblasts.

3.2 Mutation analysis

Nucleic acid isolation and RT-PCR: Genomic DNA (gDNA) and total RNA was isolated from cultured skin fibroblast and/or venous blood using the standard protocols.[50,51] The total RNA was used for preparation of complementary DNA (cDNA) by reverse transcription with SuperScript II and Oligo$(dT)_{18}$ primer (Invitrogen).

PCR amplification and DNA sequencing: The entire coding sequences and all exons with their intron boundaries of the investigated genes (except exons of *ACAT1* gene) were PCR amplified employing cDNA and gDNA, respectively, as a template. The PCR products were directly sequencing on an ALF Express DNA sequencer (Pharmacia) or on an ABI 3100-Avant DNA Analyzer (Applied Biosystems).

Confirmation of mutation and cis/trans position: The presence of some mutations was verified by ARMS method or PCR/RFLP method using cDNA and/or gDNA as a template. The trans position of most mutations was

verified by investigation of parents samples or by dividing the allels by cloning the PCR products into the pCR4-TOPO TA vector (Invitrogen).

3.3 Analysis of gross deletion using PCR probes

The extent of the deletions were analyzed by inspection of presence or absence of PCR products in hemizygote patients and control genomic DNA. Sixteen PCR probes were designed in the region of *BTK* gene, *TIMM8A* gene and *TAF7L* gene according to the reference sequence in GenBank (accession no. NC_000023.9). Specificity of the probes was verified by direct sequencing.

3.4 Genotyping and linkage analyses

The samples of five patients from four unrelated families and their 49 relatives were genotyped for 18 microsatellite markers in an 18.7-cM region. The region on chromosome 8 included previously published 8.3-cM candidate region.[52] The genotyping was performed on LI-COR IR2 sequencer by use of Saga genotyping software (LiCor).[53] Genotypes were screened for errors by use of the Ped Check Program. The linkage analysis was performed using a codominant model based on the measurement of enzyme activity of N-acetyltransferase. The values of enzyme activities distinguished between the affected probands, carriers and the health individuals. For the multipoint linkage analysis was used program Allegro 1.2c.[54]

4 Results and discussion

4.1 Clinical, biochemical and molecular biological analysis in cases of inherited diseases of organic acids metabolism

4.1.1 Metabolism of ketone bodies

From the cohort of 39 children with ketosis, who were differentially diagnosed on biochemical and enzymological level, only one patient was revealed with the defect in ketolysis; the deficit of 3-ketothiolase (3KTD). Three patients with hypoketotic hypoglycemia were diagnosed with a defect in ketogenesis, 3-hydroxy-3-methylglutaryl-CoA lyase (HL) deficiency. In these four cases (one 3KTD deficiency and three HL deficiency) molecular analysis was performed. The molecular analysis was further performed for two patients from Austria and one patient from Germany with HL deficiency.

4.1.1.1 Molecular analysis in patients with HL deficiency

Molecular analysis revealed all patients as carriers of mutations in *HMGCL* gene. Summarized results of all patients with HL deficiency examined in our laboratory are in Table. 1

Two missense mutations p.R41Q and p.H233R and one novel deletion p.R10fs (originally named p.P9fs(-1)) were found in Czech patients. Another missense mutation p.D42E and one deletion p.F305fs were found in two Austrian patients.

The German patient is heterozygous for a novel mutation: The substitution c.252G>A does not alter the amino acid residue p.Q84Q, but affects the donor splice-site in exon 3 (AG/gt). The latter mutation was not detected in any exon and exon/intron boundaries of *HMGCL* gene. The analysis of RT-PCR products suggests presence of two splicing mutations: c.252G>A, which lead to skipping of exon 3, and predicted intronic mutation, which leads to the skipping exon 2 and probably skipping of exons 2 plus exon 3.

A novel mutation p.R10fs (c. 27delG) in the first exon, leads to a frame shift and premature stop codon after 32 amino acid residues. We predict the mutation as disease-causing, because it affects the HL ORF. This frame shift mutation does not lead to nonsense mediated decay (NMD) - the degradation of mRNA

Tab. 1 Mutations in HMGCL gene in patients with HL deficiency

Family no.	Origin	Allele 1	(predicted effect on protein)	Allele 2	(predicted effect on protein)
1	Czech	c.698A>G	p.H233R	c.698A>G	p.H233R
2	Czech	c.698A>G	p.H233R	c.27delG	p.R10GfsX24[#]
3	Czech	c.122G>A	p.R41Q	c.122G>A	p.R41Q
4	Austria	c.126G>T	p.D42E	c.126G>T	p.D42E
5	Austria	c.913_915delTT	p.F305YfsX10[##]	c.913_915delTT	p.F305YfsX10[##]
6	Germany	c.252G>A; r.145_252del[$]	p.N49_Q84del	r.61_144del; r.61_252del[$$]	p.V21_K48del; p.V21_Q48del

$ the exonic mutation c.252G>A does not change the amino acid residue p.Q84Q, but leads to skipping of exon 3 r.145_252del (p.N49_Q84del)
$$ the predicted intronic mutation leading to skipping of exon 2 r.61_144del (p.V21_K48del) and probably to skipping of exon 2 plus exon 3 (p.V21_Q48del)
mutations p.R10GfsX24 and **##** p.F305YfsX10, were originally named p.P9fs(-1) and p.F305fs (-2), respectively

4.1.1.2 Molecular analysis in patient with 3KTD

The first case of, 3-ketothiolase deficiency, an error in ketolysis was revealed by differential diagnosis in Czech Republic.(ref) The molecular analysis revealed compound heterozygosity for two novel mutations p.M193T (c.578T>C) and p.I347T (c.1040T>C). The expression analysis of both novel mutations was performed by Fukao and confirmed the disease causing effect of these mutations. Both amino acids residues are well conserved. Mutation p.M193T in exon 6 probably affects the CoA binding site, important for the liver specific T2 thiolase. Even exonic mutation c.578T>C occurs at the splice-site of exon 6 (TG/gt), it likely does not alter the correct splicing of mRNA. Mutation p.I347T in exon 11 probably influences the catalytic site of the enzyme.

4.1.2 Methylmalonic acidemia

The cohort of 14 patients from 13 unrelated families (two patients were twins) from the region of former Czechoslovakia were diagnosed with methylmalonic acidemia (MMA) in The Institute of Inherited Metabolic Diseases.
Patients were admitted with the typical symptoms of MMA and the routine biochemical testing revealed high levels of methylmalonic acid, methylcitric

Tab.2 Biochemical, enzymological and molecular biological results in

patient no.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
complementation group	*cblA*	*cblA*	*cblB*	*cblB*	*cblB*	*mut*	*mut*
Clinical presentation							
present age [years]	7 y	3 y	9 y	6 y	8 m	15 y	7†
age of onset	6 m	5 m	2 d	5 m	3 d	1 d	3 w
sex	F	F	F	F	M	M	F
B_{12} responsivity	yes	yes	no	no	no	no	no
Metabolites in urine *							
methylmalonic acid [mmol/mol creat.]	4 401	↑↑↑	1 914	↑↑↑	5800	↑↑↑	9 568
3-hydroxypropionic acid [mmol/mol creat.]	148	↑	271	↑↑	327	↑↑	203
methylcitric acid [mmol/mol creat.]	84	±	43	↑↑	217	↑↑	210
Metabolites in plasma *							
methylmalonic acid [µmol/l]	177	not done	356	not done	1328	not done	881
ammonia [µmol/l]	122	79	224	117	896	216	236
total homocysteine [µmol/l]	7.6	not done	6.3	3.8	11.9	not done	3.3
Enzyme assay**							
total mutase in lymphocytes	2.29	not done	2.36	not done	not done	nd	nd
total mutase in cultured fibroblasts	3.61	5.55	3.09	5.82	2.53	not done	nd
Molecular analysis							
gene	*MMAA*	*MMAA*	*MMAB*	*MMAB*	*MMAB*	*MUT*	*MUT*
allele 1	c.551dupG	c.592_595 delACTG	c.556C>T	c.556C>T	c.556C>T	c.655A>T	c.1106G>A
(predicted effect on protein)	(C184YfsX3)	(T198SfsX6)	(R186W)	(R186W)	(R186W)	(N219Y)	(R369H)
exon	3	4	7	7	7	3	6
allele 2	c.551dupG	c.592_595 delACTG	c.557_559 delGGinsC	c.556C>T	c.577G>A	c.2179C>T	c.1105C>T
(predicted effect on protein)	(C184YfsX3)	(T198SfsX6)	(R186PfsX28)	(R186W)	(E193K)	(R727X)	(R369C)
exon	3	4	7	7	7	13	6

nd - not detectable, # the twin brother of patient no.11, we suggest the same molecular results
* laboratory findings during the first attack of disorder, $ except patients 12 and 13 who were diagnosed at age of 13 and 8 months, respectively

patients with MMA

8	9	10[#]	11	12	13	14	controls
mut	*mut*	*mut*	*mut*	*mut*	*mut*	*mut*	
5 y †	9 m †	16 d †	6.5 y	15.5 y	20 y	2 y	
3 d	2 d	2 d	2 d	1 - 2 m	3 m	5 d	
F	F	M	M	M	M	F	
no	no	no	no	no	?	no	
17 414	6 219	6 219	7 463	↑↑↑[$]	↑↑↑[$]	1832	< 14
251	371	293	212	↑↑[$]	↑↑[$]	117	< 19
61	64	147	65	↑↑[$]	↑↑[$]	144	< 8
1 788	975	750	898	not done	↑↑[$]	2149	nd
500	2 400	279	218	215[$]	not done	4600	< 80
9.6	4.2	5.6	5.3	not done	not done	3.8	< 12.0
nd	not done	not done	nd	not done	not done	not done	1.32 - 2.62
nd	nd	not done	not done	nd	not done	nd	2.31 - 6.18
MUT	*MUT*	*MUT*	*MUT*	*MUT*	*MUT*	*MUT*	
c.655A>T	c.607G>A	#	c.655A>T	c.1105C>T	c. 2080 C>T	c.655A>T	
(N219Y)	(G203R)	#	(N219Y)	(R369C)	(R694W)	(N219Y)	
3	3	#	3	6	12	3	
c.1881T>A	r.546_911del; r.754_911del	#	c.1881T>A	c.1156C>A	c. 2080 C>T	r.546_911del; r.754_911del	
(H627Q)	(p.S183_R304del; p.H252VfsX22)	#	(H627Q)	(H386N)	(R694W)	(p.S183_R304del; p.H252VfsX22)	
11		#	11	6	12		

** total methylmalonyl Co-A mutase activity estimated in the presence of 5 µmol/l adenosylcobalamin in nmol of created succinyl-CoA in min on mg of protein

acid, and 3-hydroxypropionic acid in urine, and normal concentration of homocysteine in plasma.
The nonradioactive enzyme assay distinguished patients with *mut* form MMA and patients suspected of the *cbl* forms of MMA. Patients with undetectable MCM activity were examined for the mutations in MCM apoenzyme *MUT* gene, whereas patients with MCM activity restored in the presence of AdoCbl were examined for mutations in *MMAA* and/or *MMAB* gene, respectively. On the basis of molecular analysis we were able to perform prenatal diagnosis in affected families. The results of biochemical, enzymological and molecular biological analysis are summarized in Table 2.

4.1.2.1 Molecular analysis

Mutations in MUT gene
Seven missense mutations, one nonsense mutation and one splicing mutation were found in *MUT* gene in eight patients with *mut* form of MMA. Four missense mutations p.G203R, p.N219Y, p.R369H and p.R369C, and nonsense mutation p.R727X have been previously reported and associated with mut^0 biochemical phenotype.[55-58] Another missense mutation p.R694W was associated with mut^- biochemical phenotype.[58,59] Mutation p.N219Y was found in 4 alleles from 16 alleles investigated. The high occurrence (25% of alleles) in Czech and Slovak patients corresponds to the high frequency of this mutation in Caucasian population, where represents about 20% of tested alleles.[55]
Two novel missense mutations p.H386N (c.1156C>A) and p.H627Q (c.1881T>A) were found. The prediction program SIFT[60] does not allow any alternative amino acid at position 386. Two different mutations were previously found in codon 627: p.H627R and p.H627N, respectively. [56,61] Mutation p.H627R was associated with mut^0 biochemical phenotype.[56]
A novel heterozygous splicing mutation was detected at the RNA level in two cases. Mutations were not detected in exons 2-13 and exon/intron boundaries in the *MUT* gene. The predicted intronic mutation leads to two truncated transcripts; skipping of exon 4, and skipping part of exon 3 plus exon 4.
The very close proximity of some mutations in *MUT* gene and their occurrence often in patients of very different backgrounds, (namely mutations p.R369H, p.R369C, p.R727X, p.H627Q and p. G203R) suggest the presence of mutational hotspots at these locations.[57]
Mutations in MMAA gene
Two frame shift mutations p.C184fs and p.T198fs were found in two *cbl* patients in *MMAA* gene. Both mutations were homozygous and lead to a frame shift. Mutation p.T198fs was already published.[17] Mutation p.C184fs

(c.551dupG) is novel and lead to a premature stop codon at amino acid position 186. The transcripts are not sensitive to NMD.

Mutations in MMAB gene

Three patients were diagnosed as *cblB*. All mutations found in *MMAB* gene are localized in exon 7. Two missense mutations p.R186W and p.E193K were already published.[21,62] The missense mutation p.R186W is the most common mutation in population of European descent (about 30%). Dobson et al. first reported p.R186W as potential rare polymorphism, because it was present in 4 of 240 alleles in control samples.[21] None of 300 control alleles examined in Czech samples, did not carry this mutation. The high occurrence in different ethnic backgrounds can be explained by the presence of CpG dinucleotide, a mutational hotspot. A novel indel mutation p.R186fs (c.557_559delGGinsC) is the first insertional-deletional mutation found in *MMAB* gene. Mutation leads to a frame shift and premature stop codon at position 213.

Phenotype - genotype correlation

Seven of nine patients with MCM apoenzyme deficit presented in the first days or weeks of life. Four are deceased and two patients in which the disease had severely progressed. All these patients were heterozygous for at least one previously reported mutation associated with mut^0 biochemical phenotype. Two patients, a deceased female (no.8) and the twin brother (no.11) of the deceased male (no.10) were heterozygous for the same mutations p.N219Y/p.H627Q; we assume the same genotype in both twin brothers. Regarding the severity, progression of the disease, as well as nature and position of a novel mutation, we predict the mutation p.H627Q is assigned with a mut^0 class.

The heterozygous patient p.R369H/p.R369C died at five years of age, which correlates with the published mut^0 biochemical phenotype of the mutations.

Two patients, a deceased female and a patient with a serious progression of the disease, were heterozygous for a novel splicing mutation and described mut^0 mutations p.G203R and p.N219Y, respectively. The splicing mutation was assigned to the mut^0 class.

The heterozygous patient p.N219Y/p.R727X (no. 6) with an early onset of the disease, in the first day of life, is now 15 years old. Both mutations are biochemically associated with mut^0 phenotype.[57] Despite this, the patient's condition is mild.

The heterozygous patient for known mutation p.R369C (mut^0 phenotype) and a novel mutation p.H386N is 15 years old with the mild progression of the disease. We predict the novel mutation p.H386N to be assigned with mut^- class.

The oldest patient in our cohort, a 20 years old male, presents with a relatively mild clinical phenotype. It correlates with the known homozygous mutation p.R694W that is associated with mut^- biochemical phenotype.[58,59]

Both *cblA* patients present with a mild clinical phenotype and progress well. On the contrary, all three *cblB* patients presented with a serious course of the disease. The nature of all mutations found in *cblB* patients, suggest a poor prognosis.

Cobalamin response was observed in both *cblA* patients; in one *mut*$^-$ case (no.13) we have observed marked improvement with therapy, however, we were not able to unambiguously connect these findings with B_{12}-medication or with general metabolic compensation. None of *mut*^{0} and *cblB* patients and one *mut*$^-$ patient (no.12) were B_{12}-responsive. This correlates with previously reported data about B_{12}-response in patients with MMA.[10,11]

4.2 Characterization of X-linked contiguous syndrome

The gross deletion on X-chromosome encompassing *BTK* gene and *TIMM8A* gene were examined in six cases with X-linked agammaglobulinemia with neurological problems.

Sixteen PCR probes were used to determine the extent of deletion in five cases. The deletion in all five patients included part of *BTK* gene and whole *TIMM8A* gene; *TAF7L* gene, which lies 93 kb centromerically of the first exon of the *BTK* gene, was presented. The results are in Fig.4.

The extent of deletion in 6th patient was performed in USA by ligation-mediated PCR; deletion was 196 kb in length and included genes *BTK*, *TIMM8A*, *TAF7L* and *DRP2*.

Eighty percent of the *BTK* and *TIMM8A* genes consist of interspersed repeats, mainly Alu and L1 family. The high sequence identity between repetitive sequences may lead to unequal homologous recombination[33,63,64], resulting in gross deletions, duplications or inversions. We suggest that the gross deletion in patients with X-contiguous syndrome encompassing *BTK* and *TIMM8A* genes is caused by unequal homologous recombination between repetitive sequences.

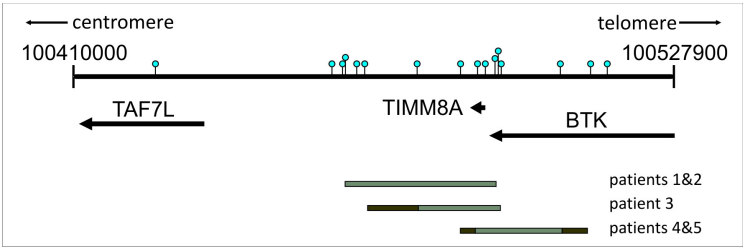

Fig.4 Extent of deletion in patients 1-5. Pinheads depict sixteen PCR probes, Arrows show position and direction of transcription of genes *BTK*, *TIMM8A* and *TAF7L*. The lines depict the extent of deletion (patients 1&2 - 30 kb, patient 3 - 16-25 kb, patients 4&5 - 15-18 kb), a dark part of lines in patients 3-4 shows boundaries of deletion.

4.3 The molecular study of mucopolysaccharidosis type IIIC

Mucopolysaccharidosis type IIIC (MPS IIIC) is caused by the mutations in *HGSNAT* gene.[35,36] To the identification of this gene lead three complementary approaches: narrowing locus for MPS IIIC by linkage analysis, bioinformatic search and gene-expression analysis.

4.3.1 Linkage analysis

The linkage analysis in four Czech families affected by MPS IIIC was performed by use of an autosomal codominant model based on biochemical factors. The enzyme assay, measurement of acetyl-CoA: α-glucosaminide N-acetyltransferase (HGSNAT) activity, distinguished between affected carriers and unaffected individuals in the pedigrees. The enzyme activity increased in the informative cohort from 5 to 49 individuals (four measurements were not informative). Eighteen STR (short tandem repeats) markers were used to narrowed interval on chromosome 8 to 2.6-cM, between markers D8S1831 and D8S1051. The maximum LOD score was 7.8 at marker D8S531. The candidate region contains 32 known or predicted genes and ORFs.

4.3.2 Identification of HGSNAT gene

The *HGSNAT* gene, originally named *TMEM76*, was identified on the basis of biochemical properties of N-acetyltransferase. Ausseil suggested N-acetyltransferase as an oligomeric transmembrane glycoprotein, ~ 100-kDa polypeptide with an enzyme active site.[65] It corresponded to a predicted *TMEM76* gene product, a ~73-kDa protein with multiple transmembrane domains and glycosylation sites. The gene-expression analysis of all 32 genes located in the candidate region was performed using oligonucleotide-based microarray assay in two patients with MPS IIIC and four controls. Only *TMEM76* revealed statistically significant decreased level of transcript in patients' samples compared to controls. The molecular analysis of all five Czech patients with MPS IIIC revealed pathogenic mutations in *TMEM76* gene. The further functional expression studies of human *TMEM76* and the murine ortholog *Hgsnat* in the fibroblast cell line from a patient with MPS IIIC, verified *TMEM76* as the disease causing gene of MPS IIIC.

Tab.3 Mutations in *HGSNAT* in patients with MPS IIIC

family. patient	Origin	allele 1	predicted effect on protein	exon/ *intron*	allele 2	predicted effect on protein	exon/ *intron*
1.1	Czech	c.1118-1133del16	p.I373SfsX5	11	c.1600C>T	p.R534X	15
2.1	Czech	c.1046T>G	p.L349X	10	c.1529T>A	p.M510K	14
3.1	Czech	c.935+5G>A	p.F313XfsX1	*9*	c.1234C>T	p.R412X	12
3.2	Czech	c.935+5G>A	p.F313XfsX1	*9*	c.1234C>T	p.R412X	12
4.1	Czech	c.1115G>A	p.R372H	11	c.1796C>T	p.P599L	17
5.1	Belorussia	c.311G>T	p.C104F	2	?	?	
6.1	Turkey	c.1810+1G>A	p.S567NfsX14	*17*	c.1810+1G>A	p.S567NfsX14	*17*
6.2	Turkey	c.1810+1G>A	p.S567NfsX14	*17*	c.1810+1G>A	p.S567NfsX14	*17*
7.1	Turkey	g.124_2462del[$]	del promotor_exon1		g.124_2462del[$]	del promotor_exon1	
8.1	Turkey	g.124_2462del[$]	del promotor_exon1		g.124_2462del[$]	del promotor_exon1	
9.1	USA	c.1706C>T	p.S569L	17	c.202+1G>A	#	*1*
9.2	USA	c.1706C>T	p.S569L	17	c.202+1G>A	#	*1*
9.3	USA	c.1706C>T	p.S569L	17	c.202+1G>A	#	*1*
10.1	Germany/Turkey	c.1334+1G>A	#	*12*	c.1334+1G>A	#	*12*
11.1	Germany/Turkey	c.1234C>T	p.R412X	12	c.1234C>T	p.R412X	12
12.1	Germany	c.1114C>T	p.R372C	11	c.1114C>T	p.R372C	11
13.1	Germany	c.1541G>A	p.G514E	14	c.1541G>A	p.G514E	14
14.1	Germany/Turkey	c.932C>T	p.P311L	9	c.932C>T	p.P311L	9
15.1	Germany	c.494T>C	p.L165P	4	c.1637C>T	p.S546F	16
16.1	Greece	c.1706C>T	p.S569L	17	c.1351_1355dupG	p.I453HfsX45	13
17.1	Greece	c.1495G>A	p.E499K	14	c.1351_1355dupG	p.I453HfsX45	13
18.1	GB	c.1626+4dupA	#	*15*	c.1758C>G	Y586X	17
19.1	GB/Pakistan	c.828-2A>G	#	*7*	c.828-2A>G	#	*7*

$ the boundaries of gross deletion 2339 bp were assigned according to the gDNA sequence from GenBank accession number NC_000008.9 (CON 03_MAR_2008) Region: 43112749_43177127

the predicted splicing mutation; cDNA sequencing was not done

4.3.3 Molecular analysis of the patients with MPS IIIC

Twenty three patients with MPS IIIC from 19 unrelated families were diagnosed on the molecular level in The Institute of Inherited Metabolic diseases up to date. Summary of mutations found is in Table3.

All Czech patients and both Greek patients were compound heterozygotes, verified by examination of parents' samples. In the other cases the parents' samples were not available; we cannot exclude the gross deletion in homozygote patients on one allele. In one case only one heterozygous mutation was found in gDNA.

Four nonsense, 11 missense, two predicted frame shift mutations, 6 intronic splice-site mutations and one gross deletion were identified in *HGSNAT* gene.

The nonsense mutation p.R412X was found in 15 alleles by now in patients of various ethnic backgrounds.[37,38] The Arg 412 involves CpG dinucleotide, the known mutational hotspot. We suggest that this mutation has occurred more than once in human history.

All eleven missense mutations affect highly conserved residues.

Two mutations, one 16 bp deletion and one duplication, lead to the frame shift and premature codon.

Six intronic mutations were found in the donor or acceptor splice sites. In two cases the examination of cDNA showed the presence of the abnormal transcript. In other four cases, the cDNA was not available, despite this, character and location of the nucleotide changes and according to prediction programs,[66,67] we suggest these mutations affect the splice site and lead to the abnormal transcripts.

One gross deletion was found in two homozygous patients. The deletion is 2339 bp in length and includes the first exon and the predicted promotor region. However, the RT-PCR product, including exons 2 - 18, was amplified in both cases. We suggest existence of alternative promotor in intron 1.[68,69]

4.3.4 Characterization of HGSNAT gene and HGSNAT protein

The *HGSNAT* gene contains 18 exons and spans over 62 kb. The RT-PCR of the 5'-UTR of the transcript in control samples extended the beginning of the transcript at least by 55 nucleotides, compared to GenBank reference sequence NM_152419.2. It extends the ORF by 84 nucleotides to 1,992 bp in length. The translated predicted polypeptide is thus 28 amino acids longer than in GenBank reference sequence NP_689632.2.

The RT-PCR of total RNA from lymphocytes, cultured skin fibroblasts and skeletal muscle revealed three transcripts, one containing the full ORF sequence and two shorter; in one case skipping of exons 9 and 10, which lead to in-frame deletion of 64 aa residues, and in the second case exons 3, 9 and 10 were spliced out. We suggest both transcripts do not encode the

active enzyme. The predicted protein HGSNAT contains 11 transmembrane domains, four glycosylation sites and 47 residues long signal peptide. The only confirmed ortholog is murine Hgsnat; but it was predicted also in four other species (rat, bovine, dog and chimpanzee), HGSNAT is highly conserved. However, the HGSNAT protein does not show homology to any known N-acetyltransferase, even the predicted domains do not show any similarity to any known functional domains. We suggest, the predicted protein HGSNAT is a member of a new enzyme family.

At the same time as Hrebicek et al.[35] released these data; another group published the same results based on proteomic analyses.[36]

5 Conclusions

1) **The complex system of biochemical, enzyme assays, and molecular methods for differential diagnostics was implemented for the patients with inborn errors of ketone bodies and methylmalonic acidemia.**
The correct early diagnosis leads to setting the appropriate therapy and thereby prevents the progression of patient's condition.
The development of molecular methods to confirm the biochemical and enzymological results assisted in a course of suitable therapy and prognosis in each individual patient. Last but not least, the molecular analysis assisted in the early prenatal diagnosis in affected families.

2) **The molecular analysis of patients with X-linked agammaglobulinemia showed the gross deletion on chromosome X encompassing *BTK* and *TIMM8A* genes** in five cases and another two genes *TAF7L* and *DRP2* in one case. The genetic mutation corresponds to clinical course of the disease. The investigation of the locus revealed that the possible cause of the deletion may be by unequal homologous recombination between interspersed repetitive sequences.
The method of PCR probes is simple and inexpensive and was developed to detect the gross deletions in hemizygotes and homozygotes.

3) **The novel gene *HGSNAT* was identified** using three complementary approaches, linkage analyses, bioinformatic methods and gene-expression studies. The mutations in *HGSNAT* gene lead to mucopolysaccharidosis type IIIC, the lysosomal storage disease. MPS IIIC was the last of the lysosomal enzymopathies, in which the disease causing gene was not known.
The molecular analysis and deduced amino acid sequence showed that the protein HGSNAT (lysosomal N-acetyltransferase) most likely belongs to a new structural class of proteins.

6 List of author's publications and presentations

6.1 Publications related to the thesis

1) Pospisilova E, Mrazova L, Hrda J, Martincova O, Zeman J. (2003) Biochemical and molecular analyses in three patients with 3-hydroxy-3-methylglutaric aciduria. *J Inherit Metab Dis* **26**, 433-41. ***IF 1.722***
2) Mrazova L, Fukao T, Halova K, Gregova E, Kohut V, Pribyl D, Chrastina P, Kondo N, Pospisilova E (2005) Two novel mutations in mitochondrial acetoacetyl-CoA thiolase deficiency. *J Inherit Metab Dis* **28**, 235-6. ***IF 1.722***
3) Pospíšilová E, Mrázová L, Klement P, Martincová O, Hrubá E, Chrastina P, Přibyl D, Vobruba V, Hálová K, Zeman J. (2006) Methylmalonová acidémie: klinická, biochemická a molekulárně biologická studie. *Čes.-slov. Pediat.* **61**, 190-198.
4) Sediva A, Smith CI, Asplund AC, Hadac J, Janda A, Zeman J, Hansikova H, Dvorakova L, Mrazova L, Velbri S, Koehler C, Roesch K, Sullivan KE, Futatani T, Ochs HD (2007) Contiguous X-chromosome deletion syndrome encompassing the BTK, TIMM8A, TAF7L, and DRP2 genes. *J Clin Immunol* **27**, 640-6. ***IF 2.638***
5) Hrebicek M, Mrazova L, Seyrantepe V, Durand S, Roslin NM, Noskova L, Hartmannova H, Ivanek R, Cizkova A, Poupetova H, Sikora J, Urinovska J, Stranecky V, Zeman J, Lepage P, Roquis D, Verner A, Ausseil J, Beesley CE, Maire I, Poorthuis BJ, van de Kamp J, van Diggelen OP, Wevers RA, Hudson TJ, Fujiwara TM, Majewski J, Morgan K, Kmoch S, Pshezhetsky AV (2006) Mutations in TMEM76* cause mucopolysaccharidosis IIIC (Sanfilippo C syndrome). *Am J Hum Genet* **79**, 807-19. ***IF 12.649***

6.2 Other publications

6) Hodanova K, Hrebicek M, Cervenkova M, Mrazova L, Veprekova L, Zemen J (1999) Analysis of the beta-glucocerebrosidase gene in Czech and Slovak Gaucher patients: mutation profile and description of six novel mutant alleles. *Blood Cells Mol Dis* **25**, 287-98. ***IF 2.427***
7) Matej R, Dvorakova L, Mrazova L, Houst'kova H, Elleder M (2008) Early onset Alexander disease: a case report with evidence for manifestation of the disorder in neurohypophyseal pituicytes. *Clin Neuropathol* **27**, 64-71. ***IF 0.918***

7 Abbreviations

aa	amino acid
3KTD	3-ketothiolase deficiency
AcAc	acetoacetate
AdoCbl	adenosylcobalamin
ARMS	amplification refractory mutation system
ATR	ATP : cob(I)alamin adenosyltransferase
DDP, DDS	dystonia-deafness syndrome
FA	fatty acids
HGSNAT	heparin acetyl-CoA: α-glucosaminide N-acetyltransferase
HL	3-hydroxy-3-methylglutaryl-CoA lyase
LINEs	long interspersed elements
MCM	methylmalonyl-CoA mutase
MeCbl	methylcobalamin
MMA	methylmalonic acidemia/aciduria
MPS IIIC	mucopolysaccharidosis type IIIC
MTS	Mohr-Tranebjaerg syndrome
NMD	nonsense mediated decay
ORF	open reading frame
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
RT-PCR	reverse transcriptase PCR
STR	short tandem repeats
T2	mitochondrial acetoacetyl-CoA thiolase
XLA	X-linked agammaglobulinemia

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