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SELECTIVE SCREENING FOR METABOLIC DISORDERS IN THE SLOVENIAN PEDIATRIC POPULATION

SELEKTIVNI SKRINING METABOLIČKIH POREMEĆAJA KOD DEČIJE POPULACIJE U SLOVENIJI

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Summary

Background: Inborn errors of metabolism (IEM) are disorders with a block in the metabolic pathway caused by a genetic defect of a specific enzyme. Although each of these diseases is quite rare, as a group they account for a significant proportion of newborn and childhood morbidity and mortality. Early diagnosis is important to prevent complications or even death of the child. Selective screening is an important diagnostic tool for the diagnosis of IEM.
Methods: In Slovenia, symptomatic patients with suspected IEM are referred to the University Children's Hospital Ljubljana. Techniques used for selective screening are gas chromatography-mass spectrometry, ion exchange chromatography-post-column derivatization, liquid chromatography-tandem mass spectrometry and isoelectric focusing. Fluorimetric method is used for enzyme activity measurement.
Results: There are 168 patients with amino and organic acidemias, 5 patients with disorders in fatty acids metabolism, 1 patient with a congenital disorder of glycosylation, 42 patients with Fabry disease (of which 37 are adult) and

Kratak sadržaj

Uvod: Urođeni poremećaji metabolizma (*inborn errors of metabolism, IEM*) jesu bolesti kod kojih postoji blokada u metaboličkom putu prouzrokovana nedostatkom specifičnog enzima. Mada se pojedinačno sve ove bolesti prilično retko javljaju, u celosti one značajno doprinose mortalitetu i morbiditetu novorođenčadi i dece. Pravovremena dijagnoza je zato od izuzetnog značaja za sprečavanje komplikacija ili čak smrti dece. Selektivni skrining je važna dijagnostička alatka za dijagnozu IEM.
Metode: Pacijenti kod kojih postoji sumnja na IEM upućeni su u Univerzitetsku dečiju bolnicu u Ljubljani. Za selektivni skrining upotrebljavamo sledeće tehnike: gasnu masenu spektrometriju, jonsko-izmenjivačku postkolonsku derivatizaciju, tečnu hromatografiju-tandemsku masenu spektrometriju i izoelektrično fokusiranje. Aktivnost enzima merimo fluorimetrijskom metodom.
Rezultati: U Registru retkih bolesti Slovenije zabeleženo je 168 pacijenata sa amino i organskim acidemijama, 5 pacijenata s poremećajem u metabolizmu masnih kiselina, 1 pacijent s kongenitalnim poremećajem glikozilacije, 42

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Abbreviations: UMC, University Medical Centre; USLD, Unit for Special Laboratory Diagnostics; IEM, inborn errors of metabolism; GC-MS, gas chromatography-mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; CSF, cerebrospinal fluid; IEF, isoelectric focusing; αGalA, α-galactosidase A; PBS, phosphate buffered saline; PKU, phenylketonuria; CH, congenital hypothyroidism; MCAD, medium-chain acyl-CoA dehydrogenase; OTC, ornithine transcarbamylase deficiency; LCHAD, long-chain 3-hydroxyacyl-CoA dehydrogenase; SCAD, short-chain acyl-CoA dehydrogenase; GA, glutaric aciduria; CDG, congenital disorder of glycosylation.

20 patients with Gaucher disease (of which 18 are adult) in the Slovenian Register for Rare Diseases.
Conclusions: In Slovenia, management of patients with IEM is centralized at the University Children's Hospital, with the exception of adult patients with Fabry and Gaucher disease. The team work is well organized with close cooperation between the laboratory and pediatricians specialized in metabolic disorders. According to the known frequencies of IEM from the literature, we would expect more positive results than obtained. To evaluate these results, we are planning to perform a pilot study on expanded newborn screening.

Keywords: metabolic disorders, inborn errors of metabolism, pediatric population, selective screening

pacijenta s Fabrijevom bolešću (37 su odrasli pacijenti) i 20 pacijenata s Gaucherovom bolešću (37 su odrasli pacijenti).
Zaključak: Medicinska pomoć za pacijente sa IEM centralizovana je na jednom mestu, u Univerzitetskoj dečijoj bolnici, osim odraslih pacijenata s Fabrijevom i Gaucherovom bolešću. Radi se o dobro organizovanom timskom radu, sa tesnom saradnjom između laboratorije i specijaliste za metabolizam. S obzirom na učestalost pojavljivanja IEM poznatu iz literature, očekivali smo više pozitivnih rezultata nego što ih dobijamo. Za procenu naših rezultata planiramo izvođenje pilotske studije sa proširenim skriningom novorođenčadi.

Ključne reči: metabolički poremećaj, urođeni poremećaji metabolizma, dečija populacija, selektivni skrining

Introduction

Selective screening is an important diagnostic tool for the diagnosis of various types of IEM. IEM are individually rare, heterogeneous genetic disorders, but collectively numerous. They mostly occur in early infancy and childhood, with a diverse clinical spectrum. The incidence within different racial and ethnic groups varies, with predominance of certain IEM within particular groups. Late diagnosis and treatment of some metabolic disorders can cause irreversible mental retardation ranging from mild to severe, neurological damage, physical disability and even fatality (1, 2). Early diagnosis is important not only for treatment but also for genetic counseling. In previous years, an increase in recognized IEM has been observed, possibly reflecting the improved diagnostic facilities, better coverage, increased medical awareness and newly discovered diseases (2, 3). Selective screening is performed for patients with clinical signs, findings of routine laboratory tests or family history indicating a metabolic disorder. Basic tests measure metabolites that are elevated due to a block at some point in the metabolic pathway. The techniques used most commonly for metabolites detection are gas chromatography-mass spectrometry (GC-MS) for organic acids determination, ion exchange chromatography-post-column derivatization for amino acids measurement (5) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) for acylcarnitine analysis (6). Isoelectric focusing of serum transferrin is a widely used method to screen for congenital disorders of N-glycosylation (7). Additionally, the approach is to screen at the enzyme level, as for galactosemia or Fabry disease (8, 9). An abnormal result from a screening test is followed by a subsequent definitive test to confirm the suspected diagnosis, namely enzyme activity measurement or DNA testing.

In 2000, we initiated selective screening for inborn errors of metabolism in Slovenia by analyzing amino acids and organic acids. Other methods were introduced later-on. In the present work we discuss our experiences from the past 13 years.

Materials and Methods

Patients

Symptomatic patients with suspected IEM from all parts of Slovenia were referred to the University Children's Hospital in Ljubljana or their samples were sent from other hospitals. Vomiting, convulsion, slow development, mental retardation, abnormal muscular tone, jaundice and hepatomegaly, coma of unexplained etiology, cardiomyopathy, dysmorphic features were the most often signs in patients. Laboratory tests, which indicated the referral, showed metabolic acidosis, hypoglycemia, hyperammonemia, lactic acidemia and positive ketone bodies in urine. Patients' clinical information was obtained from the request forms.

Organic acids analysis

Random urine samples without addition of preservative were collected; creatinine was measured and urine was stored at –20 °C prior to analysis. Briefly, a urine sample was oximated with hydroxylamine (O-Ethyl hydroxylamine hydrochloride, Aldrich, Germany). An internal standard, 2-phenylbutyric acid (Aldrich, Germany), was added in a concentration of 100 mmol/mol creatinine. Urine was acidified and saturated with NaCl and organic acids were extracted using ethyl-acetate. Organic layer was evaporated under the steam of nitrogen. The dried residue was dissolved in pyridine (Fluka, Germany), derivatized with BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide, Aldrich, Germany) and injected into a GC-MS system (Agilent Technologies, USA). The obtained spectra were compared with the known library spectra.

Amino acids analysis

Blood samples (2 mL) were collected into a heparinized vacutainer and centrifuged. Plasma was separated, proteins from plasma were precipitated (Seraprep, Pickering Laboratories, USA), supernatant was filtered through a 0.2 μm syringe filter (Sartorious

Minisart, Germany) and stored at –20 °C till the analysis. Urine and cerebrospinal fluid (CSF) samples were treated with Uriprep (Pickering Laboratories, USA), supernatants filtered and stored at –20 °C. The plasma, urine and CSF amino acids concentrations were analyzed by ion exchange chromatography with ninhydrin detection (HPLC pump Series 1200 from Agilent Technologies, and Pinnacle PCX from Pickering Laboratories, USA). All buffers and ninhydrin reagents were obtained from Pickering Laboratories (USA). Amino acids standard solution used for calibration was purchased from Sigma (Germany) and prepared according to the manufacturer's protocol.

Acylcarnitine analysis

Capillary blood was spotted directly on filter paper (Whatman 903) for the MS/MS analysis. Blood samples were allowed to dry at room temperature for at least 4 hours. They were stored at 8 °C prior to analysis. For the determination of acylcarnitines, Chromsystems MassChrom® Aminoacid and Acylcarnitines from dried blood spot reagent kit was used. The sample preparation was based on extraction followed by derivatization to butyric esters. The dried blood spot controls level I and level II were included in every analytical batch to monitor accuracy and precision within the system. The MS/MS measurements were performed using 3200 QTrap AB SCIEX (USA) and the Perkin Elmer Series 200 HPLC system (USA). ESI-positive ionization and MRM mode were used. The application did not require an HPLC column.

Isoelectric focusing (IEF) of transferrin

Serum was collected and stored at –20 °C prior to analysis. Total iron saturation is necessary prior to IEF. Hydrated Immobiline Dry gel pH 4.0–7.0 (GE Healthcare, Sweden) and Pharmalyte pH 2.5–5 and pH 5–8 (GE Healthcare, Sweden) on a Multiphor II (Pharmacia Biotech, Sweden) system were used; immunofixation was done by exposure to anti-transferrin antibody (Dako, Denmark). Detection was performed by Bomasie Blue solution. Positive controls from CDG type I and CDG type II patients were included in each batch.

Enzyme activity of α-galactosidase A (α-GalA) and glucocerebrosidase in leukocytes

Peripheral venous blood samples (5 mL) were obtained by venepuncture into EDTA tubes. Leukocytes were isolated from whole blood by the ammonium chloride method. The leukocyte pellet was washed with phosphate buffered saline (PBS) and resuspended in 0.25% triton X-1000T. The suspension was sonicated on ice. After centrifugation, the supernatants were used as the cell lysate. Supernatants were stored at –20 °C prior to analysis.

The activity of leukocyte α-GalA and glucocerebrosidase was determined by fluorimetric measurement of the amount of 4-methylumbelliferone released by hydrolysis of a synthetic substrate 4-methylumbelliferyl-α-D-galactopyranoside and 4-methylumbelliferyl-β-D-glucopyranoside, respectively.

Quality control

We are participating in the ERNDIM (European Research Network for Evaluation and Improvement of Screening, Diagnosis and Treatment of Inherited Disorders of Metabolism) schemes for qualitative organic acids, quantitative amino acids, qualitative blood spot acylcarnitine, congenital disorders of glycosylation (http://cms.erndimqa.nl/) and the INSTAND (Institute for Standardization and Documentation in the Medical Laboratory) scheme for Neonatal Screening – Inborn Errors of Metabolism (http://www.instandev.de/).

Results

The number of measured samples has increased since the beginning of implementation of individual methods and has stabilized in the past years. In the year 2013, around 800 samples were measured for organic acids, 1100 samples for amino acids, 350 samples for acylcarnitines, 50 samples for glycosylation disorders, 13 for Fabry disease and 2 for Gaucher disease. Confirmation of positive results was primarily performed in a different specialized laboratory elsewhere in the EU, a few being confirmed in USLD (mostly genetic tests). Until April 2014, 168 patients with amino and organic acidemias (of which 140 were PKU patients, diagnosed on newborn screening), 5 patients with disorders in fatty acid metabolism, 1 patient with a congenital disorder of glycosylation, 42 patients with Fabry disease (of which 37 are adults) and 20 patients with Gaucher disease (of which 18 are adults) were identified, as presented in *Table I*. Some of them were diagnosed prior to introducing the abovementioned methods. A list of the DNA tests currently available for confirmation of metabolic disorders in USLD can be found in *Table II*.

Discussion

The number of live births in Slovenia between 2000 and 2012 was on average 19.634 per year, as reported by the Statistical Office of the Republic of Slovenia (www.stat.si). Regarding the number of births and 2 millions of inhabitants in Slovenia, management of the patients with IEM is reasonably centralized in one metabolic centre at the University

Table I Part of the Slovenian Register of Rare Disorders.

	Amino acid and protein metabolism	No. of patients
1	Phenylketonuria	140
2	BH4 metabolism – PTPS deficiency	2
3	Propionic acidemia	2
4	Methylmalonic acidemia	1
5	Glutaric aciduria type I	2
6	Glycerol kinase deficiency	1
7	Nonketotic hyperglycinemia	3
8	Urea cycle disorder – OTC deficiency	7
9	Homocystinuria	7
10	Canavan disease	1
11	Alkaptonuria	1
12	Barth syndrome	1
	Lysosomal diseases	
13	Fabry disease	5 (+37 adults)
14	Gaucher disease type I	2 (+18 adults)
	Fatty acids metabolism	
15	LCHAD deficiency	3
16	SCAD deficiency	1
17	GA II	1
	Congenital disorders of glycosylation	
18	CDG type II	1

Table II Genetic tests available in USLD for confirmation of metabolic disorders.

Gene	Disease
PHA	Phenylketonuria
GLA	Fabry disease
CBS	Homocystinuria
ASPA	Canavan disease
ACADS	SCAD
SH2D1A	X-linked adrenoleukodystrophy
TAZ	Barth syndrome
MVK	Mevalonic aciduria

Children's Hospital to ensure a sufficient level of experience; the exception are adult patients with Fabry and Gaucher disease.

The introduction of selective screening in Slovenia enabled the measurement of more patient samples, leading to an increased number of identified patients with metabolic disorders. Some of the samples came from acutely ill patients, but the majority came from patients with nonspecific clinical signs. Most of the patients were diagnosed within the first years of life. Marked exceptions were present, for example, a case of propionic acidemia, where the patient was diagnosed at the age of 15 with mild symptoms and only slightly increased metabolites and a case of late-onset OTC deficiency diagnosed at the age of 13. The most common lysosomal storage diseases are Fabry and Gaucher disease. Fabry patients were mostly diagnosed as adults in a specialized centre for Fabry disease in Slovenj Gradec Hospital. Diagnosis of Fabry disease is frequently delayed due to uncharacteristic symptoms (10–12). Adult Gaucher patients are managed at the Hematology Department, UMC.

The most patients were diagnosed with PKU through the newborn screening program at the Department of Nuclear Medicine, UMC (0–6 confirmed cases per year). The incidence of PKU in Slovenia is around 1:6,000 (13), while in Europe it is between 1:3,000 and 1:30,000 (14, 15). Homocystinuria was diagnosed in 7 patients from 5 unrelated non-consanguineous families with the calculated prevalence estimated to 1 in 300,000. This is similar to the report from former Czechoslovakia, with the calculated frequency of detected homocystinuria of 1 in 349,000 (16). Based on data from countries that consistently screen newborns, the estimated worldwide frequency of homocystinuria ranges from 1 case per 58,000 to 1 case per 1,000,000. Significant variability in the frequency of homocystinuria has been observed (17). OTC deficiency as the most common urea cycle defect was detected in 7 patients. Therefore, the prevalence was similar as for homocystinuria and less than estimated by Testai and Gorelick (15). They estimated the prevalence of OTC to range from 1 in 40,000 to 1 in 80,000. Based on the conditions mentioned in *Table I* (not taking PKU into account), 1–2 cases were confirmed per year. According to the reported frequencies of IEM, we would expect more identified cases of IEM in the Slovenian population. In particular, more cases of congenital disorders of glycosylation (18) and MCAD deficiency disorders (19). MCAD deficiency is the most common disorder of mitochondrial β-oxidation and has been part of many screening programs for the past ten years (20–22). Reports from newborn screening programs stated that the prevalence of MCAD deficiency was higher than expected (23). Harms and Olgemöller (24) showed that expansion of a screening program resulted in a 57% increase in the overall number of detected cases. Early detection of IEM improves the outcomes of the patients (25). Introduction of MS/MS enables the detection of several diseases in a single run and is nowadays widely used for newborn screening. Many countries have incorporated newborn screening in their public health programs (26–28). In Slovenia, only PKU and CH have been screened for since 1979 and 1982, respectively (29, 30). Based on the positive experiences in selective screening and newborn screening for PKU and CH, expanded screening should be implemented into the Slovenian public health program. To facilitate this, we are planning to perform a pilot study to estimate the incidence of newborn errors of metabolism in Slovenia.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

References

1. Saudubray JM. Clinical Approach to Inborn Errors of Metabolism in Pediatrics. In: Saudubray JM, van den Berghe G, Walter JH, editors. Inborn Metabolic Diseases. Diagnosis and Treatment. Springer, 2012: 3–52.
2. Burton BK. Inborn errors of metabolism in infancy: a guide to diagnosis. Pediatrics 1998; 102: E69.
3. Applegarth DA, Toone JR, Lowry RB. Incidence of inborn errors of metabolism in British Columbia, 1969–1996. Pediatrics 2000; 105(1): e10.
4. Dionisi-Vici C, Rizzo C, Burlina AB, Caruso U, Sabetta G, Uziel G, et al. Inborn errors of metabolism in the Italian pediatric population: a national retrospective survey. J Pediatr 2002; 140(3): 321–7.
5. Woontner M, Goodman SI. Chromatographic analysis of amino and organic acids in physiological fluids to detect inborn errors of metabolism. Curr Protoc Hum Genet 2006; 51: 17.2.1–17.2.19.
6. Chace DH. Mass spectrometry in newborn and metabolic screening: historical perspective and future directions. J Mass Spectrom 2009; 44: 163–70.
7. Marklova E, Albahri Z. Screening and diagnosis of congenital disorders of glycosylation. Clinica Chimica Acta 2007; 385: 6–20.
8. Rhode H, Elei E, Taube I, Podskarbi T, Horn A. Newborn screening for galactosemia: ultramicro assay for galactose-1-phosphate-uridyltransferase activity. Clin Chim Acta 1998; 274: 71–87.
9. Van der Tol L, Smid BE, Poorthuis BJ, Biegstraaten M, Deprez RH, Linthorst GE, et al. A systematic review on screening for Fabry disease: prevalence of individuals with genetic variants of unknown significance. J Med Genet 2014; 51(1): 1–9.
10. Desnick RJ1, Brady RO. Fabry disease in childhood. J Pediatr 2004 May; 144(5 Suppl): S20–6.
11. Mehta A, Ricci R, Widmer U, Dehout F, Garcia de Lorenzo A, Kampmann C, et al. Fabry disease defined: baseline clinical manifestations of 366 patients in the Fabry Outcome Survey. Eur J Clin Invest 2004; 34: 236–42.
12. Fumić K, Bilić K, Rogić D. Integrative algorithms in the diagnostics of lysosomal storage diseases. J Med Biochem 2014; 33: 82–7.
13. Grošelj U, Žerjav Tanšek M, Trebušak Podkrajšek K, Battelino T. Genetic and clinical characteristics of patients with phenylketonuria in Slovenia. Zdrav Vest 2013; 82: 767–77.
14. Loeber JG. Neonatal screening in Europe; the situation in 2004. J Inherit Met Dis 2007; 30: 430–8.
15. Stojiljković-Petrović M, Klaassen K, Pavlović S. Molecular Characteristics, phenotypic diversity and genotype-esti-

mated therapeutic responsiveness of Serbian patients with phenylketonuria. J Med Biochem 2014; 33: 97–107.

16. Janosik M, Oliveriusova J, Janosikova B, Sokolova J, Kraus E, Kraus JP, et al. Impaired heme binding and aggregation of mutant cystathionine beta-synthase subunits in homocystinuria. Am J Hum Genet 2001; 68: 1506–13.
17. Testai FD, Gorelick PB. Inherited metabolic disorders and stroke part 2: homocystinuria, organic acidurias, and urea cycle disorders. Arch Neurol 2010; 67: 148–53.
18. Jaeken J, Matthijs G. Congenital disorders of glycosylation. Annu Rev Genomics Hum Genet 2001; 2: 129–51.
19. Matern D, Rinaldo P. Medium-Chain Acyl-Coenzyme A Dehydrogenase Deficiency. In: Pagon RA, Adam MP, Bird TD, Dolan CR, Fong CT, Smith RJH, Stephens K, editors. GeneReviews®. Seattle (WA): University of Washington, Seattle; 2000: 1993–2014.
20. Ziadeh R, Hoffman EP, Finegold DN, Hoop RC, Brackett JC, Strauss AW, et al. Medium chain acyl-CoA dehydrogenase deficiency in Pennsylvania: neonatal screening shows high incidence and unexpected mutation frequencies. Pediatr Res 1995; 37: 675–8.
21. Andresen BS, Bross P, Udvari S, Kirk J, Gray G, Kmoch S, et al. The molecular basis of medium-chain acyl-CoA dehydrogenase (MCAD) deficiency in compound heterozygous patients: is there correlation between genotype and phenotype? Hum Mol Genet 1997; 6: 695–707.
22. Arnold GL, Saavedra-Matiz CA, Galvin-Parton PA, Erbe R, Devincentis E, Kronn D, et al. Lack of genotype-phenotype correlations and outcome in MCAD deficiency diagnosed by newborn screening in New York State. Mol Genet Metab 2010; 99: 263–8.
23. Derks TG1, Boer TS, van Assen A, Bos T, Ruiter J, Waterham HR, et al. Neonatal screening for medium-chain acyl-CoA dehydrogenase (MCAD) deficiency in The Netherlands: the importance of enzyme analysis to ascertain true MCAD deficiency. J Inherit Metab Dis 2008; 31: 88–96.
24. Harms E, Olgemöller B. Neonatal screening for metabolic and endocrine disorders. Dtch Arztebl Int 2011; 108: 11–22.
25. Wilcken B, Haas M, Joy P, Wiley V, Bowling F, Carpenter K, et al. Expanded newborn screening: outcome in screened and unscreened patients at age 6 years. Pediatrics 2009; 124: 241–8.
26. Therrell BL, Adams J. Newborn screening in North America. J Inherit Metab Dis 2007; 30: 447–65.
27. Burgard P, Cornel M, Filippo FD. Report on the practices of newborn screening for rare disorders implemented in Member States of the European Union, candidate, Potential Candidate and EFTA Countries. 2012.
28. Padila CD, Therrell BL. Newborn screening in the Asia Pacific Region. J Inherit Metab Dis 2007; 30: 490–506.
29. Battelino T, Kržišnik C, Pavlin K. Early detection and follow up of children with phenylketonuria in Slovenia. Zdrav vest 1994; 63: Suppl 1: s25-8.
30. Kržišnik C, Battelino T, Bratanič N, Hojker S, Pavlin K, Žerjav-Tanjšek M, et al. Results of screening for congenital hypothyroidism during the ten-year period (1981–1991) in Slovenia. Zdrav vest 1994; 63: Suppl 1: s29–31.

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