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Four Years of Expanded Newborn Screening in Portugal with MS/MS



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Table 1: Decision criteria and confirmatory analysis for disorders detected by expanded neonatal screening with MS/MS

Table 2: Positive cases detected during the screening of 316,243 portuguese newborns

Summary

Introduction: The Portuguese Neonatal Screening Program (PNSP) started in 1979 for phenylketonuria (2,590,700 newborns screened; prevalence 1:11,031) and, shortly after, for congenital hypothyroidism (2,558,455 newborns screened; prevalence 1:3,174). In 2004, the expanded neonatal screening was implemented in the National Laboratory. The program is not mandatory, with 99.8% coverage of the Country (including Madeira and Azores islands).

Material and methods: In the last four years, 316,243 neonates were screened, using the tandem mass spectrometry (MS/MS) to test for selected amino acids and acylcarnitines.

Results: During this time, 132 patients were identified with 24 different inherited metabolic diseases (classic forms and variants). To date, the global frequency for all disorders integrated in PNSP is estimated to be 1:1,380, with 1:2,396 for metabolic disorders. A total of 379 tests (0,12%) were classified as false-positives, yielding an overall specificity of 99.9%. Despite the low frequency of several disorders, the positive predictive value of the overall MS/MS screening was found to be 26%, reflecting high diagnostic specificity of the method. Diagnostic sensitivity of extended screening for the different groups of disorders was 100%. Eight cases of maternal disorders (three glutaric aciduria type I, one carnitine transporter defect and four 3-methylcrotonyl CoA carboxylase deficiency) were also detected through newborn screening.

Conclusions: Our data support the advantage of centralized laboratory in performing an elevated number of samples and making decisions if relying on a clinical network able to provide fast treatment and a good outcome in the screened cases.

Introduction

In the late 70s, the Program for neonatal screening (named Portuguese Neonatal Screening Program designated hereafter as PNSP) was established in Portugal by the Ministry of Health, with phenylketonuria (PKU) and congenital hypothyroidism (CH) screening (Vaz Osorio et al 1999). PNSP is performed in whole Country and in a single laboratory, which processes over 400 samples daily. Recently, development of electrospray tandem mass spectrometry (MS/MS) allowed, using a single test, to screen for multiple inherited metabolic disorders (Wilcken et al 2003). This approach is based on amino acid and acylcarnitine profiling and has been rapidly adopted worldwide (Zytkowicz et al 2001; Bodamer et al 2007).

Based on the analysis of the results of a pilot study in which we screened 100,000 newborns from a restricted area of the Country, we started a nationwide expanded newborn screening by MS/MS, testing 24 treatable biochemical disorders.

Herein, we report the data of a 4-year long PNSP applying MS/MS in a total of 316,243 neonates.

Material and Methods

Study subjects: Over a 48-month period, 316,243 newborns were screened. During the first year the extended newborn screening only included the north and centre regions of Portugal. In entire country, including Madeira and the Azores islands, were studied for 24 conditions in the following years. Blood spots were collected between days 3 and 6 of life on Whatman 903 filter paper.

Methods: Two API 2000 triple quadrupole tandem mass spectrometers (*Applied Biosystems, Sciex*) were used in our laboratory to perform routine MS/MS neonatal screening method, which includes the analysis of aminoacids and acylcarnitines as butyl esters (Rashed et al 1995). Primary and secondary markers as well as ratios and respective cut-offs were defined to identify the conditions of the panel of diseases screened (Table1).

For tyrosinemia type I screening, we set up a “second-tier” testing on the same initial sample. If tyrosine is over the established cut-off (250uM), the succinylacetone (SUAC) quantification is performed. Until now, SUAC quantification has required an independent MS/MS method as described by Allard et al 2004. We use internal quality control materials provided by CDC and participate in external quality control programs from ERNDIM, CDC and NEQAS.

Positive cases were all confirmed by other methods (amino acid analysis in plasma, organic acids in urine and enzymatic or molecular testing), according to standard protocols (Table 1).

Results

One hundred and thirty two cases of inborn errors of metabolism (IEM) were detected and are referred in Table 2. Based on these numbers, the overall frequency of screened disorders in Portugal is approximately 1 case per 2,396 newborns. 379 tests (0,12%) were classified as false-positives (initially elevated values that didn't confirmed on second sample/follow-up), yielding an overall specificity of 99.9%. Despite the low frequency of several disorders, the positive predictive value of the overall MS/MS screening was found to be 26%, reflecting that the programme shows sufficient quality, according to the criteria proposed by Rinaldo et al 2006. Diagnostic sensitivity of extended screening for the different groups of disorders was 100%. In fact, we are not aware of any false negative to date.

In our screening program for Tyrosinemia type I, tyrosine level is used as a screening tool. We found about 3 % of neonates with tyrosine levels above *cut-off*, requiring SUAC analysis as second-tier test.

Interestingly, eight cases of maternal disorders (three glutaric aciduria type I, one carnitine transporter defect and four 3-methylcrotonyl CoA carboxylase deficiency) were also detected through newborn screening.

From the 132 true positive cases, two children died. One of them, passed away at the age of 4 months with a diagnosis of 3-hydroxy-3-methylglutaryl-CoA lyase deficiency. The death cause was a severe hypoglycaemia in the sequence of an infectious disease with food refusal. The second neonate, with citrullinemia type I died at 5 days of age.

Discussion

In the past few years, the introduction of MS/MS has unquestionably represented a turning point in the neonatal screening field. Simultaneous diagnosis of several disorders are accomplished by a single test allowing to screen for conditions that otherwise might have been missed and believed extremely rare. This has significantly improved the efficacy of national neonatal screening programs demonstrating the importance of early identification and treatment of infants who have disorders that would otherwise go unrecognised before irreversible clinical damage (Deodato et al 2004).

In our country, the screening was expanded from the initial two disorders to a total of 25 disorders, during the past four years by the National Committee for Newborn Screening. Ethical Committee approved that only potentially treatable diseases would be included in PNSP. Regarding the analytical *cut-off* values, these were initially established based on extensive review of literature and data from our pilot study.

To date, the global frequency for all disorders integrated in the programme is estimated in 1:1,380, with a prevalence of 1:2,396 for metabolic disorders and 1:3,174 for CH. Due to the fact that we perform newborn screening instead of selective screening on clinical grounds, we verify an increase of the detection rate of IEM. This is particularly true for medium chain acyl CoA dehydrogenase deficiency (MCADD), methylcrotonyl CoA carboxylase deficiency (MCCD), or methionine adenosyltransferase deficiency (MATD). Moreover, MS/MS screening opened the possibility to diagnose a number of conditions, including carnitine transporter deficiency (CTD),

very long chain acyl CoA dehydrogenase deficiency (VLCADD), carnitine palmitoyl translocase type I deficiency (CPT1D) and carnitine palmitoyltranslocase type II deficiency (CPT2D) that were unreported in Portugal in the “pre MS/MS era” of neonatal screening.

In our population, MCAD deficiency appears to be the most frequent disorder detected by MS/MS, a figure even higher than PKU, this is in agreement with data presented elsewhere (Wilcken 2008 and Frazier et al 2006).

The inclusion of MCCD deficiency in newborn screening has been questioned in the face of the policies adopted in other countries (Pollitt 2007) where this organic aciduria had been excluded from the panel. However, we believe that this approach would cause further inconvenience because C5OH (3-hydroxyisovalerylcarnitine or its isomers) is also a marker of other diseases such as holocarboxylase synthase deficiency (2 cases identified in PNSP) or even 3-hydroxy-3-methylmethylglutaryl-CoA lyase deficiency (3 cases detected), which represents an important organic aciduria in our country (Vilarinho et al, 1993).

In PNSP we also included the determination of methionine (Met), which allows the screening of remethylation disorders, cystathionine β -synthase (CBS) deficiency as well as other hypermethioninemias such as MATD. In the *MAT1A* gene a common mutation (p.Arg264His) was identified in seven cases with a high Met concentration (range 52-103 $\mu\text{mol/L}$; cut-off 50, percentile 99,95), and all the cases remain asymptomatic, until now. As this mutation is dominant, seven parents (mother or father) were identified, and respective families investigated, due to the elevated risk of vascular and thrombotic diseases (Linnebank et 2005). Although not being a sensitive marker for CBS deficiency, Met quantification allowed an identification of one case with no false negative results known. Regarding the remethylation defects, a diagnosis of CblC case was established initially by a low value of Met (6,8 μM) an increase of C3/Met, and mild increase of C3/C2 ratio. Subsequently, molecular analysis of *MMACHC* confirmed the case (Nogueira et 2008).

Worldwide, arginase deficiency is an extremely rare urea cycle disorder, nevertheless, our center has already diagnosed eight symptomatic probandus (Vilarinho et al 1990, Braga et al 1997, Cardoso et al, 1999, Santos Silva et al 2001), a sufficient number to justify the addition of this metabolic disease in our expanded neonatal screening. Until now, only five cases diagnosed by newborn screening are referred (Scaglia et al 2006), all with a high value of arginine (Arg) at screening time. In our case, the value of Arg at day 5 of life was slightly elevated ($50 \mu\text{M}$ – cut-off 50, percentile 99.95) with a clear-cut pathological raise only at the end of first month of life ($>100\mu\text{M}$). The slowly progressive increase of Arg poses the question of false negative values, if a very early screening is performed.

The approach used in the screening for tyrosinemia type 1 has been successful in our screening programme allowing the detection of four cases with tyrosine values of $610\mu\text{M}$, $584\mu\text{M}$, $356\mu\text{M}$ and $659\mu\text{M}$ at day 3, 8, 4 and 4 respectively.

The expanded newborn screening show us that only mild-to-moderate forms of IVA are found with no severe form being identified.

Concerning the pathologies termed “mild” or “benign” and the question of the need to screen for, the PNSP leaves the issue as yet opened. This can be the case of short chain acyl-CoA dehydrogenase deficiency (SCAD) (Waisbren et al 2008), a disorder that is not included in our panel.

In addition, eight cases of maternal disorders were also detected through newborn screening. The first three instances of maternal GA I were identified through the low free carnitine in neonates and two of them already reported by our group (Garcia et al 2008). One case of CTD and four MCCD were also picked in the mothers, but these were already known occurrences (Schimmenti et al 2007 and Gibson et al 1998). In all cases, a definitive diagnosis was obtained upon detailed confirmatory analyses, including enzymatic and or mutational studies. This illustrates that

Neonatal Screening not only allows neonatal diagnosis, but also contributes to uncover unknown maternal medical conditions.

The strategy used in newborn screening varies among different countries, many centres screen only for conditions with a good evidence of clinical benefit such as MCAD deficiency (Seymour et al 1997 and Dionisi-Vici et al 2006), whereas others even include diseases with ineffective treatment. In Europe, there is a need for more consensus and harmonic programs (Loebler 2007) although, the organisation of health care be different in the various countries.

Conclusion

Our data highlights the importance to expand the PNSP as early detection of many inborn errors of metabolism, and support the advantage of centralized laboratory in performing an elevated number of samples and making decisions if relying on a clinical network able to provide fast treatment and a good outcome in the screened cases.

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Portuguese Neonatal Screening Homepage: www.diagnosticoprecoce.org

International Society for Neonatal Screening: www.isns-neoscreening.org



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TABLES

Table 1: Decision criteria and confirmatory analysis for disorders detected by expanded neonatal screening with MS/MS

Disorder	Positive screening criteria	Confirmatory analysis
Phenylketonuria-PKU / hyperphenylalaninemia-HPA	Phe ($>150 \mu\text{M}$) and Phe/Tyr (>1.5)	PKU: Phe $> 360 \mu\text{M}$; HPA: Phe $> 150 \mu\text{M}$ and $<360 \mu\text{M}$
Maple Syrup Urine Disease	XLeu ($>342 \mu\text{M}$) and Val ($>350 \mu\text{M}$)	Plasma aminoacids and presence of alloisoleucine; molecular analysis.
Tyrosinemia type I	Tyr ($>250 \mu\text{M}$) and positive succinylacetone test	Succinylacetone in urine and molecular analysis.
Tyrosinemia type II	Tyr ($>450 \mu\text{M}$)	Molecular analysis.
Homocystinuria (CBS deficiency)	Met ($>50 \mu\text{M}$)	Total homocysteine in plasma elevated; molecular analysis.
Methionine adenosyltransferase deficiency	Met ($>50 \mu\text{M}$)	Molecular analysis
Citrullinemia type I	Cit ($>46 \mu\text{M}$)	Plasma ammonia and citrulline; orotic acid in urine; molecular analysis.
Argininosuccinate lyase deficiency	Asa ($>1 \mu\text{M}$)	Asa in urine; molecular analysis
Arginase deficiency	Arg ($>50 \mu\text{M}$)	Plasma ammonia and arginine; orotic acid in urine; molecular analysis.
3-Methyl crotonyl-CoA carboxylase deficiency	C5OH ($>1 \mu\text{M}$)	Increased 3-OH-isovaleric acid and 3-methylcrotonylglycine in urine; molecular analysis.
Isovaleric acidemia	C5 ($>1 \mu\text{M}$)	Increased isovalerylglutamine and 3-hydroxy-isovaleric acid in urine; molecular analysis.
Holocarboxylase synthetase deficiency	C5OH ($>1 \mu\text{M}$)	Organic acid profile compatible with multiple carboxylase deficiency; molecular analysis.
Propionic acidemia	C3 ($>6.23 \mu\text{M}$) and C3/C2 (>0.3)	Increased 3-hydroxy-propionic acid, propionylglycine, tiglylglycine and methylcitrate in urine; molecular analysis
Methylmalonic acidemia (mutase)	C3 ($>6.23 \mu\text{M}$) and C3/C2 (>0.3)	Increased methylmalonic acid and methylcitrate in urine; molecular analysis.
Glutaric acidemia type I	C5DC ($>0.2 \mu\text{M}$)	Glutaric and 3-hydroxy-glutaric acids in urine; molecular analysis or enzymatic activity in fibroblasts
Methylmalonic acidemia (Cbl C,D)	C3 ($>6.23 \mu\text{M}$), Met ($<12 \mu\text{M}$) and C3/Met (>0.4)	Total homocysteine in plasma elevated; increased methylmalonic acid in urine; molecular analysis.
3-hydroxy-3-methylglutaryl CoA lyase deficiency	C5OH ($>1 \mu\text{M}$) and C6DC ($>0.07 \mu\text{M}$)	Increased 3-hydroxy-3-methylglutamic, methylglutamic and 3-methylglutaconic acids in urine; molecular analysis.
Medium-chain acyl-CoA dehydrogenase deficiency	C8 ($>0.3 \mu\text{M}$) and C8/C10 (> 2.5)	Molecular analysis or enzymatic activity in fibroblasts/lymphocytes
Long-chain 3-OH acyl-CoA dehydrogenase deficiency	C16OH ($>0.10 \mu\text{M}$), C18:1OH ($>0.07 \mu\text{M}$), C18OH ($>0.06 \mu\text{M}$) and C16OH/C16 (>0.04)	Molecular analysis or enzymatic activity in fibroblasts/lymphocytes
Multiple acyl-CoA dehydrogenase deficiency	Multiple elevations from C4 to C18	Molecular analysis or enzymatic activity in fibroblasts



Carnitine transport defect	C0 (<7 μ M)	Molecular analysis or carnitine uptake in fibroblasts
Very-long-chain acyl-CoA dehydrogenase deficiency	C14:1 (>0.46 μ M) and C14:2 (>0.17 μ M)	Molecular analysis or enzymatic activity in fibroblasts/lymphocytes
Carnitine palmitoyl-transferase Ia deficiency	C0/(C16+C18) (>30)	Molecular analysis or enzymatic activity in fibroblasts
Carnitine palmitoyl-transferase II deficiency	C0/(C16+C18) (<3)	Molecular analysis or enzymatic activity in fibroblasts


Table 2: Positive cases detected during the screening of 316,243 portuguese newborns

Disorders	Positive cases	Frequency
Amino acid disorders	54	1:5 856
Phenylketonuria (PKU)	26	1:12 163
Hyperphenylalaninemia	12	1:26 354
Maple Syrup Urine Disease (MSUD)	3	1:105 141
Tyrosinemia type I	4	1:79 061
Tyrosinemia type II	1	1:316 243
Homocystinuria (CBS deficiency)	1	1:316 243
Methionine adenosyltransferase deficiency (MATI/III)	7	1:45 178
Urea Cycle Disorders	4	1:79 061
Citrullinemia type I	2	1:158 122
Argininosuccinate lyase deficiency	1	1:316 243
Arginase deficiency	1	1:316 243
Organic Acid Disorders	24	1:13 177
3-Methyl crotonyl-CoA carboxylase deficiency	7	1:45 178
Isovaleric acidemia	3	1:105 141
Holocarboxylase synthetase deficiency	2	1:158 122
Propionic acidemia	1	1:316 243
Methylmalonic acidemia (mutase)	1	1:316 243
Glutaric acidemia type I	6	1:52 707
Methylmalonic acidemia (Cbl C,D)	1	1:316 243
3-hydroxy-3-methylglutaryl CoA lyase deficiency	3	1:105 141
Fatty acid oxidation disorders	50	1:6 325
Medium-chain acyl-CoA dehydrogenase deficiency	35	1:9 036
Long-chain 3-OH acyl-CoA dehydrogenase deficiency	3	1:105 141
Multiple acyl-CoA dehydrogenase deficiency	3	1:105 141
Carnitine transport defect	3	1:105 141
Very-long-chain acyl-CoA dehydrogenase deficiency	3	1:105 141
Carnitine palmitoyl-transferase Ia deficiency	1	1:316 243
Carnitine palmitoyl-transferase II deficiency	2	1:158 122
Total	132	1:2 396