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GC/MS as a One-Step Metabolomics Analysis for Symptomatic Screening of Inherited Metabolic Disorders: A Regional Experience in Northern Spain

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Abstract: ***Background: New-born screening (NBS) using MS/MS technology started in northern Spain region with a restricted disease panel for four Inborn Errors of Metabolism (IEM) (phenylketonuria [PKU], glutaric aciduria type-1 [GA-1], medium-chain acyl-CoA dehydrogenase [MCAD] and long-chain 3-hydroxyacyl-CoA dehydrogenase [LCHAD] deficiencies) within the last years. We report the results of the two strategies for a three-year period in a developed region of northern-Spain and present the incidence and clinical presentation features of IEM in our Hospital. Methods: From January 2015 to December 2017 dried blood spots (DBSs) specimens from 12,915 new-borns were included in the regional NBS program. In the same period, 134 urine samples collected from patients who were highly suspected of having IEM or with a positive result in the NBS, were evaluated using GC/MS. Results: During the period, just one patient (PKU) was diagnosed by the NBS program, and other seven studies were positives, being negatives in the second-tier GC/MS-urine analysis. By GC/MS-urine analysis of highly suspected IEM, eight cases of IEMs were diagnosed, plus three other patients with secondary, but symptomatic, methylmalonic aciduria (MMA) due to maternal B12 deficiency. Symptoms were neurological in 7 of 11 (63%) patients, followed by failure to thrive in 6 of 11 (54%) patients and vomiting in 5 of 11 (45%) patients. Conclusions: The present study highlights the feasibility of using GC/MS as a one-step analysis for symptomatic metabolic screening, which is particularly useful for regional hospitals. The incidence of treatable IEM is at least 1 in 1,435 new-borns, or 1 in 1,076 new-borns considering secondary MMA.***

Keywords: new-born screening, metabolomics, inherited metabolic disorders, gas chromatography, tandem mass spectrometry

1. Introduction

The field of inherited metabolic disorders (IMDs) has changed from a limited group of rare, untreatable, often fatal disorders to important causes of acutely life-threatening but increasingly treatable illnesses, mainly based on the access to new laboratory technologies for diagnosis and screening. Early screening and diagnosis are critical for these patients, as the clinical consequences are often severe and are important causes of morbidity and mortality in clinical practice, particularly in paediatrics. Although each disorder is individually rare, their cumulative incidence is relatively high, as conservative estimates are 1 in 1,500 to 1 in 5,000 live births for treatable IMDs and more than 1 in 1,000 individuals for all different IMDs. (1,2)

Inborn errors of metabolism (IEM) can present at any age and affect any organ system. A failure to suspect and diagnose the inborn errors leads to lost opportunities for intervention, possibly resulting in harm to the child and family. The appropriate integration of approaches for diagnosing IMDs in paediatric populations yields some of the highest benefits in medicine. The analysis of carnitine and amino acid profiles in dried blood spots (DBSs) using tandem mass spectrometry (MS/MS) for new-born screening (NBS) enables clinicians to screen for a wide range of previously unscreened IEMs using a single test. (3)

The application of NBS using MS/MS technology as a routine part of the care for all new-borns started in our region within the last several years, but according to National (Spanish) and Regional Public Health policies, a restricted disease panel for phenylketonuria (PKU), glutaric aciduria type 1 (GA-1), medium-chain acyl-CoA dehydrogenase (MCAD) deficiency and long-chain 3 hydroxy acyl-CoA dehydrogenase (LCHAD) deficiency is examined. Other potential diagnostic results are suppressed and are not reported. (4) As a second screening platform/confirmatory screen, we use a urine gas chromatography/mass spectrometry (GC/MS)-based analysis as an inexpensive technique (Centro de Diagnóstico y Estudios Metabólicos Avanzados de Cantabria, CDEMAC).

Considering the clinical relevance of a prompt diagnosis of treatable IEMs, we incorporated the urine GC/MS-based analysis in our University Hospital-CDEMAC as one-step metabolomics analysis for symptomatic patients with highly suspected of having IEM using conservative criteria, which can be thus considered a non-systematic but selective screen for diagnosing symptomatic patients. When a highly clinical suspicion of an IMD was present based on clinical symptoms and/or biochemical data, we performed a GC/MS-based analysis for the simultaneous detection of > 200 marker metabolites found in characteristic pattern in the

urine of patients with IMDs. Thus, we can diagnosis or discard an extensive and treatable IEM in symptomatic patients.

We recently published our results from a GC/MS-based analysis that enables quick detection, accurate identification, and precise quantification of a wide range of urinary markers that may not be discovered using DBS-NBS programmes. (5,6) The present study highlights the feasibility of using GC/MS with a one-step metabolomics analysis for symptomatic screening in regions with restricted NBS programmes but also reinforces the two-tier analysis system applied to expanded NBS programmes.

2. Materials and methods

2.1. Participants

This descriptive study examined patients diagnosed during the period of January 2015 to December 2018 in Cantabria, a northern Spanish region with a population of 582,200 inhabitants (ICANE 2016) and approximately 12,915 new-borns' during the period. All patients suspected of having a potential IMD were recruited from the Neonatal and the Metabolic Units of the Paediatric Department and the Neurology Department of the University Hospital Marques de Valdecilla in Santander, Spain. All studies were performed in hospitalized patients included in any of the following clinical scenarios: acutely ill patients; patients with a failure to thrive with or without the presence of vomiting; patients with an abnormal neurological system, including children or young people (less than 30 years old) with stroke; and children with multi-organ failure. The only exclusion criteria were related to the quality of the collected samples (insufficient or contaminated). Secondary lactic acidosis was not considered a positive result. The expanded NBS using MS/MS technology as a routine part of the care of all new-born's started in our region in November 2014, but according to the national recommendations for neonatal screening in Spain and our regional health policy, only a restricted metabolic disease panel for PKU, GA-1, MCAD and LCHAD is analysed, and any other diagnostic results are required to be suppressed and not reported. As the screen depends on the regulations of the Public Health System of Cantabria and local regulations of the University Hospital, the results of amino acid or acylcarnitine profiles from DBS are not available.

2.2. Clinical sample collection

Urine samples were collected in the hospital using sterile perineal bags or clean containers and then adsorbed on Whatman 903 filter paper (Ahlstrom-Munksjo TFN, Barenstein, Germany). Urine samples could dry properly at room temperature for at least 2 hours before being shipped to the analysing laboratory (Centro de Diagnóstico y Estudios Metabólicos Avanzados de Cantabria, CDEMAC in Santander, Spain). Samples were inspected during receipt at the laboratory and only processed according to the sample acceptance protocol. Written informed consent was obtained from the parents of all subjects or from adult subjects themselves.

2.3. Urine sample processing for GC/MS

The laboratory protocol involves pre-treating the sample with urease, followed by deproteinization with alcohol and derivatization with silylation, as described in the study by Kuhara et al., (7,8 replace with Hampe Et.al) with slight modifications. Briefly, 100 µl of the sample that had been eluted from dry urine on filter paper using distilled water were incubated with urease to remove the urea, followed by the addition of the internal standard (heptadecanoic acid). After deproteinization with ethanol and centrifugation, the supernatant was evaporated with a vacuum concentrator (Eppendorf) and the resulting residue was derivatized with BSTFA/TMCS. The analysis of the extract was performed with a GC/MS (GC/MS Instruments, 7890B/5977A, Agilent, USA) and the Windows GCMS Solution Version 2.5 software. The resulting data were processed by a computer using an algorithm. Urinary creatinine levels were measured with the Jaffe kinetic method using an ARX-235 analyser (Micro lab Instruments, Ahmedabad, India). The recovery standards were added together with the internal standard during the extraction process for QC verification. (9) As a control for calibration and internal verification, a previously retained sample with the corresponding controls was analysed in each batch. A mixture of n-alkanes was periodically used to verify the retention time (RT) of each metabolite.

2.4. Computer-assisted interpretation of the GC/MS data

The GC/MS data were annotated by comparing the mass fragments and the retention times with the reference standards included in the spectral databases of the NIST MS search 2.0 software at a similarity of 80%, choosing more than two ions for a given objective. The levels of each marker are expressed as µmol/mol of creatinine. After correcting for the creatinine concentration, each value was compared with the reference ranges for the same age. We use reference ranges from the literature (10-13) that were adjusted for each age range according to our own cut-off values based on the Spanish population and many normal samples recruited from a screening programme in India.(5) For each metabolite, the mean and standard deviation (SD) were calculated from our sample database after adjusting for age (CDEMAC). Reference ranges are reviewed periodically (every 6 months) by adjusting the analysis software (PreventiNe). Metabolite excretion in the range of mean values + 2SD and average values + 3SD is considered a mild elevation; the mean value + 3SD is considered significant excretion. The significant excretion of any metabolite is reported in terms of multiples of standard deviations (nXSD). All samples were checked manually with two spectral libraries, NIST MS version 2.0 and AMDIS version 2.7. Positive samples with a characteristic fingerprint for any of the IEMs were classified as selected positive IEMs and were manually verified. Samples with an ambiguous metabolic pattern and abnormal profiles that suggest a possible disease were re-evaluated manually and analysed again, if necessary. Because lactic acidosis is not exceptional in critically ill newborns, the diagnosis of primary lactic acidaemia is established when the condition of lactic aciduria is maintained on two or more occasions. All diagnoses were clinically and biochemically correlated with

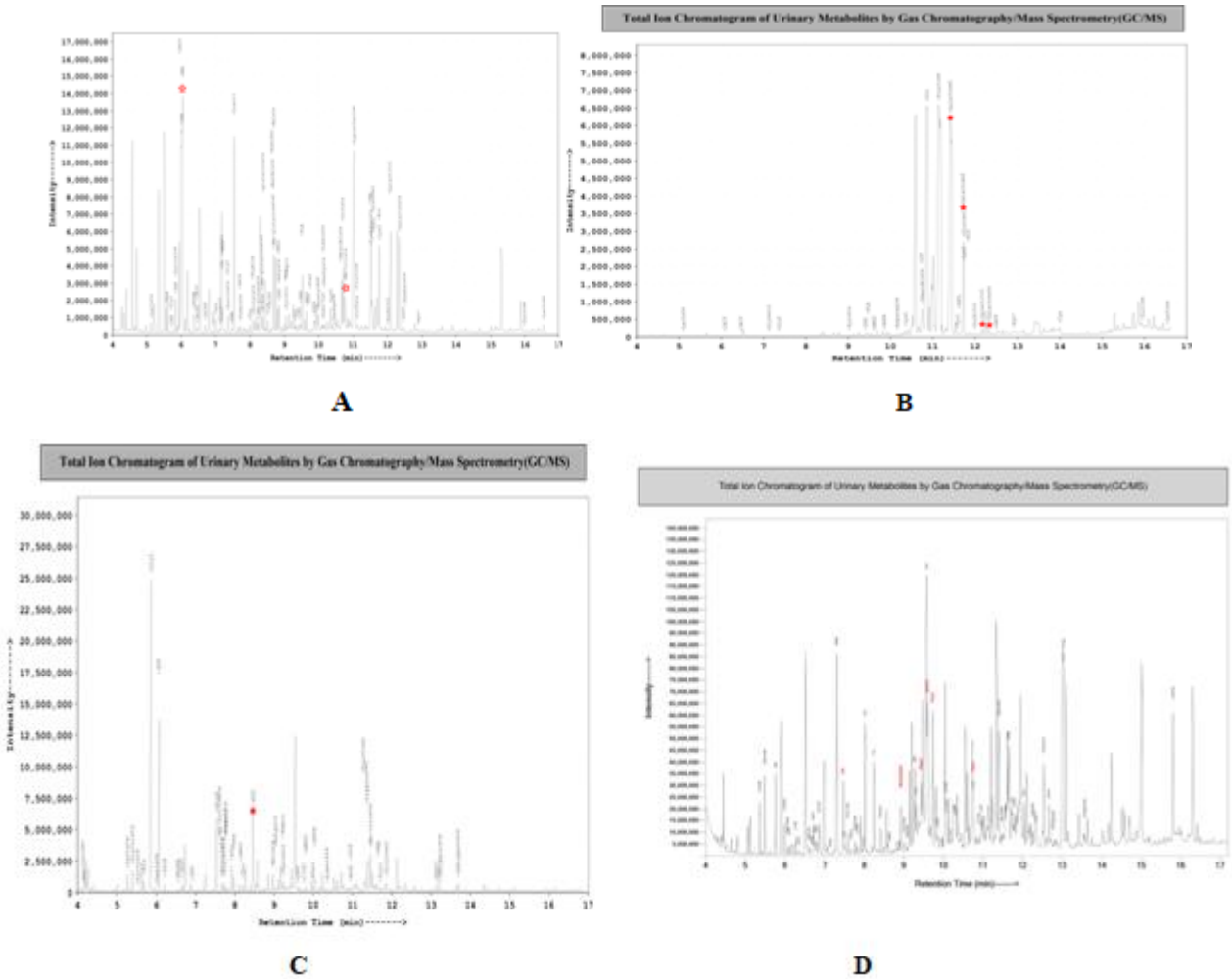
the amino acid, organic acid and acylcarnitine profiles, according to the expected metabolic pattern, and later confirmed by genetic analyses.

3. Results

During the study period, DBS screening was performed in all newborns (12,9,15), and the GC/MS-based analysis was performed on urine samples from 134 patients with a highly clinical suspicion of an IEM (mainly from the Paediatric Department but one from the Neurology Department) or with results in the DBS that were positive in the first tier (7 studies). Only one patient with a metabolic disorder (PKU) was diagnosed by the DBS, whereas eight new cases of symptomatic IEMs, which were later confirmed by the corresponding metabolic and/or genetic analysis, and three patients with secondary, but symptomatic, transient methylmalonic aciduria (MMA) due to a maternal B12 deficiency were diagnosed. Patients with secondary lactic acidosis excluded from the study, thus were not considered in the analysis. Urine samples from the seven positive patients, based on the DBS amino acid and carnitine profiles analysed using MS/MS, were subjected to our GC/MS-based analysis as a second screening platform/confirmatory screen, and all but one patient with PKU were negative in this screen.

Over this three-year period, the most common IEMs diagnosed in our study group were disorders of vitamin B12 (cobalamin) metabolism. In addition to the three patients with secondary methylmalonic aciduria, 3 of 9 (33%) patients were diagnosed with methylmalonic aciduria with homocystinuria Cbl-C type; 2 patients, a new-born from the DBS neonatal screen and a five-year-old child with autism born in a country with no neonatal screening programme, were diagnosed with PKU; one child was diagnosed with propionic acidaemia; one child was diagnosed with vitamin B12-responsive methylmalonic aciduria; one presented with classic galactosaemia with hepatic failure at the time of diagnosis; and, finally, 1 adult was diagnosed with homocystinuria due to cystathionine β synthase. All results were reported within the first 24 hours after samples were received at the laboratory. Fig. 1 presents the chromatographic fingerprints of the disorders diagnosed in our laboratory (methylmalonic acidaemia with Cbl-C type, propionic acidaemia (PA), classic galactosaemia + hepatic failure and PKU).

Figure 1: Chromatographic fingerprint of the different disorders diagnosed in the present study: (A) methylmalonic acidaemia with, Cbl-C type; (B) classic galactosaemia + hepatic failure; (C) propionic acidaemia (PA); and (D) PKU



The age of the study population ranged from as young as 9 days (1 patient with Cbl-C and 1 patient with PKU) to 28 years old (1 patient with classic homocystinuria). The most common symptoms in the diagnosed case population were neurological symptoms in 7 of 11 (63%) patients, followed by failure to thrive in 6 of 11 (54%) patients and vomiting in

5 of 11 (45%) patients. Neurological manifestations encompassed altered sensorium or encephalopathy, as well as stroke in one patient. The most significant data obtained from all patients with IEM at the time of diagnosis are summarized in Table 1.

Table 1: Urine metabolic markers in positive cases

No.	Name of IEM	Age	Primary Markers	Secondary Markers	Clinical Presentation
1	Phenylketonuria (PKU)	7 days	Phenylalanine, Mandelate, 2-Hydroxyphenylacetate	4-Hydroxyphenyllactate, Phenyllactate, Phenylacetate, Phenylpyruvate	DBS screening, asymptomatic
2	Methylmalonic acidaemia with homocystinuria, Cbl-C type	12 days	Methylmalonic acid	3-Hydroxypropionate, Methylcitrate, Propionyl glycine	Sleepiness, failure to thrive, vomiting
3	Classic galactosaemia	18 days	Galactose, Tyrosine	Galactitol, Galactonate, 4Hydroxyphenyllactate, 4Hydroxyphenylpyruvate 4Hydroxyphenylacetate	Sleepiness, failure to thrive, vomiting, jaundice
4	Methylmalonic acidaemia	1.5 months	Methylmalonic acid	3-Hydroxypropionate, Methylcitrate, Propionyl glycine	Failure to thrive, refusal to feed, vomiting, hypotonia
5	Methylmalonic acidaemia with homocystinuria, Cbl-C type	2 months	Methylmalonic acid	3-Hydroxypropionate, Methylcitrate, Propionyl glycine	Failure to thrive, dystrophy, refusal to feed, vomiting, hypotonia
6	Methylmalonic acidaemia with homocystinuria, Cbl-C type	2.5 months	Methylmalonic acid	3-Hydroxypropionate, Methylcitrate, Propionyl glycine	Failure to thrive, dystrophy, refusal to feed, vomiting, hypotonia
7	Propionic acidaemia	3.5 years	Propionylglycine	2-Hydroxybutyrate, 3Hydroxypropionate, 3Hydroxyisobutyrate, 3-Hydroxyisovalerate, 4Hydroxyphenyllactate, Methylcitrate and Tiglylglycine	Cyclic vomiting, anorexia
8	Phenylketonuria (PKU)	5 years	Phenylalanine, Mandelate, 2-Hydroxyphenylacetate.	4-Hydroxyphenyllactate, Phenyllactate, Phenylacetate, Phenylpyruvate	Pervasive disorder
9	Classic homocystinuria	28 years	Homocysteine	Methionine	Stroke

In all GC/MS-positive results for any of the diseases, the results were validated within the appropriate metabolic (blood and urine) and genetic assays. In all cases, urine and blood amino acid, blood acylcarnitine and organic acid profiles were measured. Other biochemical or haematological analyses (i.e., vitamin levels or red blood cell counts) were performed when necessary. According to our results, the cumulative incidence of newly diagnosed IEMs in new-borns for the period in our region was estimated as 1 in 1,435 live births.

The results for the DBS amino acid and carnitine profiles obtained using LCMS/MS universal neonatal screening (limited to four IEMs) were true positives in 1 in 12,915 live births and 1 in 1,C5 new-borns for the GC/MS-based analysis of symptomatic patients. Considering the three infants with secondary MMA due to a maternal B12 deficiency, the estimate of positive cases for the limited but symptomatic screening was 1 in 1,076 new-borns.

4. Discussion

We present our regional results obtained during a three-year period in which we detected twelve treatable patients, nine with IEMs (eight from the symptomatic GC/MS screen and one from the universal MS/MS screen) and three with secondary metabolic defects, all of which are potentially treatable diseases after diagnosis. Based in our strategy of screen by GC/MS the urines of highly suspected of having IEM patients, we achieved an 8.9% positive diagnosis rate for the 134 received samples. With our results, we can estimate that the cumulative incidence of intermediary

treatable IEMs for the period is 1:1413, like the estimates from other series. (1)

Additionally, three infants who were fully breast-fed and presented with a symptomatic vitamin B (12) deficiency were also examined during the period. Vitamin B (12) deficiency should be avoided or diagnosed as early as possible since the administration of supplements to the mother and child has been shown to reverse the neurological symptoms of the baby. (18) We emphasized the utility of symptomatic screening using a GC/MS analysis of urine samples; thus, we excluded secondary lactic acidosis detected in neonates with a severe illness that might cause confusion in interpreting the results.

Screening the healthy neonate for IEMs is not simply a process of testing, but it is an organized, systematic approach to ensure that every infant identified as positive in the screen will undergo a diagnostic evaluation and, when necessary, treatment and management. In 2005, the American College of Medical Genetics (ACMG) proposed up to 29 core conditions that are considered appropriate for healthy NBS in developed countries, because a screening test, an efficacious treatment, and an adequate knowledge of natural history are available. (14) However, each country and state determine the conditions included on their NBS panel, and different countries are introducing the technology at different rates and for different disease panels. In Cantabria in northern Spain, the Regional Ministry for Health and Social Security prescribed a restricted disease panel, with the instruction that any other diagnostic results are to be suppressed and not reported. (14-16) The lack of even broad concordance at the level of national policy is extremely disturbing, as dramatic differences across Spanish regions have been noted.

In addition to neonatal screening policies for IEMs directed towards the healthy neonate, an investigation of IEMs in a symptomatic patient begins with a careful examination of routine metabolic/biochemical parameters followed by specific studies, such as the proposed GC/MS urine analysis. Patients with IMDs may present with acute symptoms, such as lethargy, hypotonia, tachypnoea, convulsions, and vomiting, or may present with a developmental delay or mental retardation and could die of 'obscure causes', even though a number of these patients might potentially achieve normal growth and development if early detection and intervention were feasible. (17)

An understanding of basic principles, presentations and approaches to IMDs will increase the likelihood of prompt recognition, diagnosis and appropriate management of patients with IMDs and their families. Typically, an IEM is suspected as a result of a suggestive combination of acute clinical symptoms without warning that may present from the new-born to adult stages in a variety of ways. However, clear recommendations concerning the high-risk or symptomatic metabolic screens that might be much more efficient than NBS are unavailable. Moreover, some techniques have been reported to be as valid as NBS tests and are currently ongoing, manly in Asia. (5,6) When a patient presents with a potential acute manifestation of an IMD, the urine tests are very informative for most treatable IEMs. Using GC/MS, we were able to identify up to 200 different markers that enabled a one-step metabolomics analysis for a symptomatic screen. The possibility of diagnosing any treatable IEM as soon as possible is a vital process that identifies infants with serious disorders that are usually correctable by dietary or drug interventions before they suffer significant morbidity or mortality. (19) Although IEMs have usually been considered paediatric diseases, they can present at any age and affect any organ system. (20) A disease does not necessarily appear/manifest at a certain age, but the age at which the screen or diagnosis is performed can vary. In the expanded new-born screen, a single test enables the early detection and treatment of many disorders and it potentially prevents serious consequences. (21-23)

Notably, in our cohort study, all except one (classic galactosaemia) of the patients who were diagnosed by the GC/MS-based urine analysis for symptomatic screening should have been suspected of having the disease based on the carnitine and amino acid profiles obtained by MS/MS in the pre-symptomatic state, if it would not have been limited by the use of the official panel. Galactosaemia has been screened using MS/MS technology, but measurements of galactose–1-phosphate levels are not being performed simultaneously with other tests using MS/MS. The elevation of levels of some acylcarnitine species (such as C3 for MMA and PA) in the neonatal period might alert the clinician to initiate a second-tier metabolic study, at least for patients with PA, MMA and Cbl-C. Although a suspicion of homocystinuria might be noted based on the DBS carnitine profile obtained using MS/MS, in our case, this strategy is only a theoretical approach as an adult aged 28 years had this disorder. Finally, most NBS programmes are based on a blood analysis, and very few programmes use urine as a primary screening sample.

A combined screen represents a good choice to minimize false positives. The application of expanded a two-tier method that simultaneously analyses carnitine and amino acidprofiles in dried blood spots (DBSs) using tandem mass spectrometry (MS/MS) followed by a urine metabolic analysis using gas chromatography mass spectrometry (GC/MS) hasbeen reported to be a suitable method for implementation in routine IMD screening programmes. In our experience, the simultaneous two-tier metabolome test as a screen for IEMs is more comprehensive and delivers confirmatory results with significantly reduced turnaround times, thus reducing recall rate by eliminating false positive results and helping to prevent unnecessary anxiety in parents. (24-26)

We propose to incorporate a one-step metabolomics analysis for symptomatic screening of IMDs as a simple but strong, non-invasive and efficient tool to minimize the delay in the time of diagnosis of symptomatic and treatable IMDs. The stability of the samples that are easily collected during the acute phases of the disease and the feasibility for shipment and/or storage makes the method very suitable to implement in regional hospitals without advanced biochemical departments. With the one-step metabolomics analysis, we are able to diagnose or screen amino acidopathies, organic acidaemia's, fatty acid oxidation defects, primary lactic acidaemia's, sugar metabolism defects, peroxisomal

disorders of the Zellweger spectrum, purine and pyrimidine defects and urea cycle disorders. Most of these disorders are life threatening and some of them treatable if detected in the initial phases of metabolic decompensation. Because the number of potential screening tests is expanding and because the costs of these tests is also decreasing, we anticipate that parents will face bewildering choices. Rather than a broad symptomatic metabolic screening, we also propose a combined blood and urine screen for the healthy new-born as an efficient method to reduce the number of false positives obtained from DBS by examining the dried urine samples or by expanding the spectrum of screened diseases to more than 100 IEM disorders.

5. Conclusions

Most NBS programmes are based on a blood analysis, and very few programmes use urine as a primary screening sample. However, a urine analysis using GC/MS significantly improves the efficacy of neonatal screening programmes, indicating the need for more comprehensive screening programmes and improved throughput with advancement in computerized MS data handling. Using non-invasive urine sample collection on a dry matrix, easy sample transport, sample stability, and precise metabolome profiling, GC/MS has provided an efficient platform for symptomatic screening of IEMs, assisting with IMD diagnoses.

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