EXPANDED NEWBORN SCREENING BY MASS SPECTROMETRY: NEW TESTS, FUTURE PERSPECTIVES

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Keywords: newborn screening; tandem mass spectrometry; dried blood spot; lysosomal storage disorders; severe combined immunodeficiencies

I. INTRODUCTION

The use of mass spectrometry (MS) in clinical laboratories is very much increased on the outset of the 21st century. This development is obviously due to great advances in MS hardware, software and research in the last 25 years. MS permits a very rapid measurement of many metabolites in different biological specimens by using filter paper spots or directly on biological fluids. Its use in NBS gives us the chance to identify possible treatable metabolic disorders even when asymptomatic and the benefits gained by this type of screening is now recognized worldwide. Today the use of MS/MS for second-tier tests and confirmatory testing is promising especially in the early detection of new disorders such as some lysosomal storage disorders, ADA and PNP SCIDs, X-adrenoleucodistrophy (X-ALD), Wilson disease, guanidinoacetate methyltransferase deficiency (GMAT), and Duchenne muscular dystrophy. The new challenge for the future will be reducing the false positive rate by using second-tier tests, avoiding false negative results by using new specific biomarkers and introducing new treatable disorders in NBS programs. © 2015 Wiley Periodicals, Inc. Mass Spec Rev 35:71–84, 2016.

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Tandem mass spectrometry (MS/MS) has become a leading technology used in clinical chemistry and has shown to be particularly sensitive and specific when used in newborn screening (NBS) tests. The success of tandem mass spectrometry is due to important advances in hardware, software and clinical applications during the last 25 years. MS/MS permits a very rapid measurement of many metabolites in different biological specimens by using filter paper spots or directly on biological fluids. Its use in NBS gives us the chance to identify possible treatable metabolic disorders even when asymptomatic and the benefits gained by this type of screening is now recognized worldwide. Today the use of MS/MS for second-tier tests and confirmatory testing is promising especially in the early detection of new disorders such as some lysosomal storage disorders, ADA and PNP SCIDs, X-adrenoleucodistrophy (X-ALD), Wilson disease, guanidinoacetate methyltransferase deficiency (GMAT), and Duchenne muscular dystrophy. The new challenge for the future will be reducing the false positive rate by using second-tier tests, avoiding false negative results by using new specific biomarkers and introducing new treatable disorders in NBS programs. © 2015 Wiley Periodicals, Inc. Mass Spec Rev 35:71–84, 2016.

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I. INTRODUCTION

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MS permits a very rapid measurement of many metabolites in different biological specimens by using filter paper spots or directly in biological fluids. Because of its high sensitivity and specificity, this technique can be used for qualitative and quantitative analysis of many analytes, such as amino acids and acylcarnitines, organic acids, homocysteine, orotic acid, purines and pyrimidines, steroids, and vitamin D to name a few, with appropriate internal standards.

Newborn screening (NBS) is definitely one of the most successful applications of MS in clinical chemistry in the recent years. The measurement of amino acids and acylcarnitines enables the identification of many inborn errors of metabolism (IEM) few days after birth. If they are not diagnosed and early treated, most of them might cause mental and/or growth retardation, severe permanent sequelae and in some case death.

The history of NBS as population-based test started in 1960s when Robert Guthrie developed a simple and cheap bacterial inhibition assay on filter paper spot able to identify the phenylketonuria (PKU), the most frequent aminoacidopathia (Guthrie & Susi, 1963). The following development of electro-spray tandem mass spectrometry (ESI-MS/MS) in more recent years (1990s) has permitted the introduction of this new technology in clinical chemistry laboratories, in particular for newborn screening purposes (Rashed et al., 1995).

Originally, fast atom bombardment (FAB) and thermospray (Yergey et al., 1984; Millington et al., 1991) were used to measure carnitine and acylcarnitines in plasma samples for the detection of fatty acid disorders.

Later on, advancements in MS/MS and research demonstrated that the analysis of acylcarnitines could be used to identify fatty acid oxidation defects in a neonatal population from dried blood spots. In fact, carnitine and its derivatives were ideal molecules for a MS/MS screening technique since they all presented a common product ion (85 m/z) containing a quaternary ammonium function. These structural characteristics enabled the detection of a panel of saturated, unsaturated, and hydroxylate acylcarnitines with high specificity and sensitivity.

More or less in the same years, FAB-MS/MS was also used for the first attempt to measure phenylalanine and tyrosine for PKU diagnosis (Chace et al., 1993). The procedure required esterification (as butyl esters) to enhance surface ionization in a glycerol matrix. The butylation “quenched” the negative charge on the acid moiety of phenylalanine increasing ionization efficiency and sensitivity.

During the first tests other amino acids were identified deducing a common neutral product, the butylformate, presenting with a mass of 102 Da. Since then, the common neutral loss of 102 Da is the basis for the MS/MS assay of several key butylated amino acids in newborn screening.

While butyl esterification assays are now routine, in the last 10 years new methods were developed able to detect amino acids and acylcarnitines as their native free acids (underivatized)
in an attempt to simplify analytical operations and to minimize the use of butanol in chloridic acid.

Derivatized and underivatized methods with some modifications with respect to the original version are now worldwide used in clinical chemistry labs and we should consider that many millions of newborns are today screened for a variable number of diseases by meaning of that test.

But the high number of disorders covered in expanded newborn screening corresponded also to the increase in the recall rate (Tarini, Christakis, & Welch, 2006). False-positive screening results may cause unnecessary parental stress and influence the child–parent relationship (Gurian et al., 2006). On the other hand, a number of false-negative cases, have been reported by several newborn screening laboratories (Wilcken et al., 2003; Frazier et al., 2006).

In fact, some important metabolites in expanded newborn screening programs, markers of diseases, have proven to have poor specificity, causing a critical impact on the population due to the high number of recalls. The best way to decrease the recall rate, due to false positive results in the primary screening test, is represented by the introduction of the second-tier test, performed on the same NBS specimen with no necessity to additionally contact patient family. Second-tier test result over-rules the primary result.

Since for some metabolites there is an overlap between controls and patients ranges, second-tier test proved its superiority in discriminating the two populations thanks to the high specificity.

The choice of reference values for NBS test should be a good compromise between sensitivity and specificity; a good sensitivity could result in a high false positive rate while having high specificity could cause false negative results. Adjusting cutoff values for each metabolite should consider the availability of a second-tier test; if it is available, primary test cut-off should be reduced to capture presumptive positive cases (Chace & Hannon, 2010).

Near the proliferation of new second-tier tests, advances in computerization, automation, and sensitivity of analytical instruments have resulted in the expansion of potential new NBS tests in recent years. Furthermore, improvements in treatments, such as gene therapy, bone marrow transplantation, enzyme replacement, and chaperone therapy, have raised the status of some disorders as candidates for NBS. The availability of a effective treatment is the worldwide approved criterion to include a disorder in a newborn screening panel.

In the last years NBS tests have been proposed for lysosomal storage disorders, Duchenne muscular dystrophy, GAMT, peroxisomal disorders, SCIDs, and Wilson’s disease.

While pilot projects have demonstrated the potential of these tests, it remains to be discussed how effectively they can be applied in most NBS centers and how effective the newer treatments are. Each new test appends an additional complexity to the NBS program and some of the therapies are costly and long-term, often lifelong.

II. SECOND-TIER TESTS IN EXPANDED NEWBORN SCREENING

Expanded NBS by tandem mass spectrometry is normally performed by a flow injection analysis; since it does not require prior chromatographic separation, potential interference is possible. Second-tier testing is performed on newborns with abnormal screening result. It is considered a second level test performed on the original blood spot, in order to find a target analyte under optimum operating conditions. The introduction of second-tier testing, in newborn screening programs, has allowed the reduction of the number of false positives that were not only the cause of unnecessary parental stress but also resulted in the increase of laboratory analyses and personnel costs due to repeat tests. However, second-tier tests cannot be used as primary screening tests because of the low-throughput assay and laboratory costs (Chace & Hannon, 2010).

There are two categories of second-tier tests: a test looking for the same analyte identified in NBS analyses or a test looking for additional diagnostic markers not detectable among NBS analytes (Fig. 1).

In the first one, second-tier tests will obtain a more high specificity than the NBS method, so a potential false-positive interference from NBS process may be excluded. Some authors consider these methods part of primary screening instead of a real second-tier test (Chace & Hannon, 2010). However, these tests meet fully the definition of second-tier tests, as they distinguish true from false positives and improve the positive predictive value of primary NBS results. Using this strategy, several two-tier tests have been developed such as succinylacetone (SUAC), a specific marker for Tyrosinemia I (OMIM 276700), recently included in the NBS panel (la Marca et al., 2008; Turgeon et al., 2008). Although, in our experience, false positive rates for SUAC are very low (la Marca et al., 2011), a sample contamination cannot be excluded. A newborn falsely suspected for Tyr I causes a high cost of hospitalization, without considering the parental stress involved. To overcome this problem, the specificity of some important diagnostic markers of diseases which require immediate hospitalization should have the highest possible specificity.

In the other case, second-tier tests identify diagnostic metabolites, not available by routine NBS, by strongly supporting the presence of disorder.

The identification of free methylmalonic or propionic acid on DBS, after an abnormal propionylcarnitine (C3) result, has been revealed one of the most effective second-tier tests. A high C3 (also >10 μmol/L) is not a real diagnostic marker contrary-wise the presence of free organic acids has a pathognomonic importance. By using this second-tier test, the recall rate for C3 has been drastically reduced as previously reported (la Marca et al., 2007).

In the other cases, separation of isomers by second-tier testing can resolve multiple diagnostic possibilities. High levels of the branched-chain amino acids, revealed on NBS process, could be due to total parenteral nutrition or to maple syrup urine disease (MSUD, OMIM 615135). Alloisooleucine (allo-Ile) is the pathognomonic marker of MSUD, but it cannot be differentiated in flow injection from isobasic amino acids (leucine, isoleucine, and hydroxyproline; la Marca et al., 2005). Hydroxyprolinemia (OMIM 23700) is a benign condition for which recall is not recommended. A second-tier test has been shown to be able to identify each isobasic compound, permitting unequivocal interpretation of NBS results (Oglesbee et al., 2008).

Second tier steroid profiling by MS/MS can be also performed for congenital adrenal hyperplasia (CAH, OMIM 201910), for which NBS programs are fluorescence based. The false positive rate of CAH screening is much higher than other
screens and by using MS/MS in the second-tier tests, the specificity of NBS results improve (Schwarz et al., 2009), although not completely eliminating false-positive results but this benefit is opposed by the increased risk of false negatives as reported by Sarafoglou et al. (2012). These authors suggested that molecular testing as a second-tier test could have a major efficacy but cost effectiveness has not yet been evaluated.

However, MS/MS is a powerful technique that has very high analytical sensitivity and selectivity and so its poor effectiveness as second-tier test for CAH screening could be due to the lack of a consistent algorithm system, which determines the positive or negative results.

Many countries perform newborn screening to detect classic galactosemia (GALT deficiency, OMIM 230400) due to a deficiency of the galactose-1-phosphate uridyl transferase (GALT) enzyme. The first screening test can be performed either by fluorometric assay (Fujimoto et al., 2000) or by MS/MS (Jensen et al., 2001). In particular some false positive results by fluorimetric assay have been reported in DBS exposed to high temperature and humidity (Fujimoto et al., 2000; Ohlsson et al., 2012). In recent years, some second-tier tests able to reduce false positive rate have been developed, including multiplex enzyme testing by UPLC-MS/MS (Ko et al., 2011).

The combination of first and second-tier testings for many metabolic disorders can be considered the best working practice to achieve the most benefit out of newborn screening programs.

A list of MS-based second-tier tests is reported in Table 1.

III. SEVERE COMBINED IMMUNODEFICIENCY (SCID)

Severe combined immunodeficiency (SCID, OMIM 615615) is currently a genetically and clinically heterogeneous group of disorders belonging to primary immunodeficiency disorders (PIDDs) that result from thymic dysplasia and arrest in T lymphocyte maturation. There is also variable expression of B and natural killer (NK) cells, and patients are categorized into either SCID with absence of T lymphocytes but presence of B lymphocytes (T-B⁺ SCID) or SCID with absence of both T and B lymphocytes (T-B⁻ SCID) (Somech & Etzioni, 2014). These diseases can be inherited either in an X-linked or an autosomal recessive manner.

Infants with SCID might be difficult to diagnose in the neonatal period because in most cases they are initially asymptomatic, (Geha et al., 2007; Slatter & Gennery, 2008), but a prompt identification and treatment may lead to less severe clinical symptoms and avoid fatal consequences.

Valid therapeutic options are represented from bone marrow transplantation (Slatter & Gennery, 2008; Gaspar et al., 2014) and enzyme replacement therapy. Recently, gene therapy has shown to be a highly effective treatment for infants with Adenosine Deaminase deficiency (ADA-SCID, OMIM 102700).
and X-linked SCID (X-SCID, OMIM 300400; Aiuti et al., 2009; Hacein-Bey-Abina et al., 2010; Gaspar et al., 2011a,b).

SCID shows all the characteristics required by neonatal screening programs and they were first introduced in 2008 in Wisconsin by T-cell receptor excision circles (TREC) assay and Massachusetts joined in 2009 and then several other states (Chase, Verbsky, & Routes, 2011).

TREC are DNA biomarkers of normal T cell development (Somech & Etzioni, 2014) and their quantification by real-time PCR on DBS currently represents the only test available to screen for SCID in routine NBS programs (Gaspar et al., 2014).

This test is shown to be a cost-effective test for SCID, due to the low cost of the TREC assay and to the extremely high costs associated with delayed identification and treatment (Chan, Verbsky, & Routes, 2011).

The introduction of NBS for SCID using the TREC assay showed important advantages in early diagnosis and treatment of patients (Routes et al., 2009; Hale et al., 2010; Accetta et al., 2011); each state developed its own screening algorithm resulting in a great variability between areas (Chase, Verbsky, & Routes, 2011).

High false-positive TREC assay rates are generated from infants born prematurely (Routes et al., 2009).

ADA-SCID represents the second most common form of SCID (Buckley, 2004; Booth et al., 2007; Gaspar et al., 2009); adenosine deaminase dysfunction causes accumulation of metabolites, including 2-deoxyadenosine (DAdo) and deoxyadenosine triphosphate, which inhibit the activity of ribonucleotide reductase and ultimately DNA synthesis. The incidence of ADA deficiency is estimated to be between one in 375,000 and one in 660,000 live births (Sauer & Aiuti, 2009) but it might be underestimated because of early death from infective diseases or disorders caused by toxic metabolites (Azzari, la Marca, & Resti, 2011).

Different ADA deficiency phenotypes have been described depending on the clinical onset and on the severity of the disease: the most severe form, presenting in infancy and usually resulting in early death, is “early onset” ADA-SCID; “delayed” onset concerning about 10–15% of patients; “later” onset characterized by less severe infections and “partial” ADA deficiency occurs in a subset of immunocompetent individuals (Arredondo-Vega et al., 1994). The incidence of late-onset ADA deficiency is also underestimated, even more than that of early onset ADA-SCID (Arredondo-Vega et al., 1998).

SCID NBS by TREC assay can identify newborns with early-onset ADA-SCID but it has been shown to be unable to identify newborns with delayed or late-onset ADA-SCID (la Marca et al., 2013).

In 2010, our group set up a MS/MS based method to detect ADA-SCID biomarkers, 2-deoxyadenosine and adenosine (Ado), on DBS from newborn screening cards. Results demonstrated that patients with ADA-SCID can be easily diagnosed at birth by using the existing underivatized acylcarnitine and aminoacid method (Azzari, la Marca, & Resti, 2011; Fig. 2). A two step extraction derivatized method is also possible (la Marca, 2014a). The method seemed to be promising and a pilot project for a population-based neonatal screening in Tuscany started in September 2010. The method resulted applicable also to premature infants whose mean levels of metabolites do not differ from those found in full-term babies. In 2011, the first diagnosis of ADA-SCID was made through expanded newborn screening including ADA metabolites quantification. The patient was confirmed by molecular analysis as a late onset phenotype. Normal TREC were detected on neonatal DBS (la Marca, 2014b).

At the beginning of 2013, Purine nucleoside phosphorylase deficiency (PNP, OMIM 613179) was also included in the MS/MS newborn screening panel of the Tuscany region (la Marca et al., 2014).

PNP deficiency is a rare form of SCID (Markert, 1991; Al-Saud et al., 2009; Somech et al., 2013); the enzyme catalyses the phosphorolysis of guanosine, deoxyguanosine, inosine, and

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### TABLE 1. Second tier tests performed by mass spectrometry

<table>
<thead>
<tr>
<th>NBS MARKER</th>
<th>NBS ASSAY</th>
<th>MARKER II-TIER TEST</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propionylcarnitine (C3)</td>
<td>TMS</td>
<td>free methylmalonic and propionic acids on blood</td>
<td>la Marca et al., 2007</td>
</tr>
<tr>
<td>Succinylacetone</td>
<td>TMS</td>
<td>Succinylacetone</td>
<td>la Marca et al., 2011</td>
</tr>
<tr>
<td>Methionine (†) for homocystinuria Methionine (‡) for MTHFR</td>
<td>TMS</td>
<td>Homocysteine</td>
<td>Turgeon et al., 2010</td>
</tr>
<tr>
<td>17-Hydroxyprogesterone</td>
<td>immunoassay</td>
<td>17-hydroxyprogesterone, cortisol, androstenedione</td>
<td>Peter et al., 2008</td>
</tr>
<tr>
<td>Branched-chain amino acids</td>
<td>TMS</td>
<td>alloisoleucine, leucine, isoleucine, valine, OH-proline</td>
<td>Oglesbee et al., 2008</td>
</tr>
<tr>
<td>Isovalerylcarnitine (C5)</td>
<td>TMS</td>
<td>free isovaleric acid or isovalerylcarminie separated from 2-methylbutyrylacarnitine and pivalic acid</td>
<td>Forni et al., 2010</td>
</tr>
<tr>
<td>Isobutyrylcarnitine (C4)</td>
<td>TMS</td>
<td>free ethylmalonic acid or isobutyrylcarnitine separated from butyrylcarnitine</td>
<td>Forni et al., 2010</td>
</tr>
<tr>
<td>Creatine and Guanidinocacete</td>
<td>TMS</td>
<td>creatine and guanidinocacete</td>
<td>Pasquali et al., 2014</td>
</tr>
<tr>
<td>Total galactose and/or GALT activity</td>
<td>immunoassay</td>
<td>Galactose-1-phosphate, UDP-galactose, and UDP-galactose</td>
<td>Ko et al., 2011</td>
</tr>
</tbody>
</table>

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deoxyinosine to their respective purine bases and pentose-1-phosphates (Hershfield & Mitchell, 2001). Lack of PNP function determines an intracellular accumulation of deoxyguanosine triphosphate derived from deoxyguanosine, toxic to lymphoid cells and resulting in various neurologic abnormalities (Hershfield, 2004; Myers et al., 2004; Al-Saud et al., 2009). As for ADA-SCID, incidence and prevalence remain unknown because of misdiagnosis and insufficient available laboratory testing.

MS/MS can easily identify abnormal metabolites in DBS for PNP patients at birth as shown by retrospective studies on DBS samples from patients with genetically confirmed PNP deficiency.

When ADA and PNP SCID were added to the expanded newborn screening panel, a cost-effectiveness evaluation was performed. SCID metabolites inclusion was carried out without any additional operator intervention, sample preparation stages and any additional instrumentation beside that necessary for routine neonatal screening. Therefore, the cost was calculated for reagents only and rounded up to a few euro cents per newborn.

IV. LYSOSOMAL STORAGE DISORDERS

The lysosomal storage disorders (LSDs) are rare inherited diseases caused by a deficiency of one or more specific lysosomal enzymes, activator protein or membrane protein, resulting in deficient enzymatic activity. The defect causes the progressive accumulation of the substrate interfering with normal cellular activity and possibly resulting in cellular death.

LSDs represent a group of more than 50 different diseases (Filocamo & Morrone, 2011; la Marca, 2014c) and have a combined incidence of about 1 in 1,500–7,000 live births (Stone & Sidransky, 1999; Fletcher, 2006).

Most affected newborn seems to be healthy at birth but they can develop a wide range of clinical manifestations within the first few years of life. If left undetected and untreated, these diseases can lead to different clinical symptoms depending on the specific disorder. An early treatment could prevent these serious problems.

Thanks to the new effective therapeutic options available for some LSDs (Escolar et al., 2005; Urbanelli et al., 2011; Parenti et al., 2013; Ortolano et al., 2014) and to the development of new analytical methods to test enzyme activity on DBS specimens, these disorders have become strong candidates for inclusion in future mandatory screening panels.

Although in 2006 the American College of Medical Genetics (ACMG) Newborn Screening Expert Group declared LSDs as not appropriate for newborn screening because they did not meet the current evaluation criteria, more recently the Discretionary Advisory Committee on Heritable Disorders in Newborns and Children (DACHDNC) recommended to include Pompe disease to the Recommended Universal Screening Panel (RUSP) for all north American newborns (http://www.hrsa.gov/advisorycommittees/mchbadvisory/heritabledisorders/recommendations/correspondence/uniformpanel060313.pdf).

There are net evidences that presymptomatic diagnosis and early treatment for some LSDs can favorably modify the natural progression of the disease (Chien et al., 2009; Muenzer, 2014).

An additional argument for inclusion of LSDs in NBS programs is the relative prevalence of these conditions. The incidence of LSDs is not known but, surprisingly, pilot NBS programs for Fabry disease revealed an incidence of 1 in ~3,100
in Italy (Spada et al., 2006) and 1 in ~1,250 in Taiwan (Hwu et al., 2009) in male newborns.

Some countries already include some LSDs into their newborn screening panels, and local or pilot programs have been launched in others (Chien et al., 2008; Piaciotti et al., 2012; Wittmann et al., 2012; Inoue et al., 2013; Liao et al., 2014).

The state of New York was the first state to perform population screening for the lysosomal disorder Krabbe disease starting in 2006 (Duffner et al., 2009).

In the Tuscany region of Italy, a prospective pilot project screening program for Pompe, Fabry, and Mucopolysaccharidoses type I has started in November 2014.

Going to technical procedures, the pioneering work of Chamoles et al. (2004) first established the possibility of assay lysosomal enzyme activity on DBS using fluorescence because many of these enzymes are still active in re-hydrated DBS. Fluorometric methods have been developed for many LSDs such as Pompe disease, Gaucher disease, Fabry disease, mucopolysaccharidosis (MPS), Niemann-Pick A/B disease, Tay–Sachs disease and Sandhoff disease (Chamoles, Blanco & Gaggioli, 2001a,b; Chamoles et al., 2002a,b). However, the incorporation of a chromophore or fluorophore into the substrate caused false negative results and had limitations of specificity and limited capacity for multiplexing. In the last year, new technology for simultaneous screening of several enzyme activities related to lysosomal storage disorders from DBS was developed replacing the old methods enabling the assay of single enzyme activity (Li et al., 2004; Zhang et al., 2008).

Li et al. firstly developed a direct multiplex assay of lysosomal enzymes for Gaucher, Pompe, Krabbe, Fabry, and Niemann-Pick A/B diseases in DBS by flow injection analysis (FIA)-tandem mass spectrometry.

Later on, an expansion of simultaneously diagnosable disorders by MS has been reported (Orsini et al., 2012; Spacil et al., 2013).

Novel substrates, internal standards and assays have been developed over the years for testing enzyme activity of Krabbe, Pompe, Niemann-Pick A and B, Gaucher, Fabry, MPS-I, MPS-II, MPS-III, MPS-IVA, and MPS-VI (Gelb et al., 2006; Wang et al., 2007; Blanchard et al., 2008; Duffey et al., 2010a,b; Khaliq et al., 2011; Wolfe et al., 2011; Chemmannaneri et al., 2014). Moreover in order to facilitate the introduction of LSD in newborn screening panels, the Center for Disease Control and Prevention distribute substrates and internal standards to interested newborn screening centres; QC programs are also available (De Jesus et al., 2009).

The substrates used are structurally closer to the natural substrates, the enzyme products are specific to each enzyme reaction and are quantified against internal standards with known concentrations for the measurements of enzyme activities (Li et al., 2004).

The multiplex assay developed by Li et al. was later refined by (Zhang et al. (2008) to make it more suitable for newborn screening laboratories. These methods required liquid or solid-phase extraction and subsequent FIA-MS/MS analysis to determine the concentration of each reaction product based on the intensity of internal standards.

La Marca et al. (2009) simplified Zhang’s assay eliminating the liquid/liquid and solid-phase extraction steps by using an online trapping-and-cleanup liquid chromatography/mass spectrometry method. As an example a chromatogram from a LC-MS/MS combined six enzyme assay on DBS of a Pompe patient is reported in Figure 3.

Furthermore, the introduction of the separation of analytes by liquid chromatography (LC) coupled to the tandem mass spectrometer (LC-MS/MS) was crucial (la Marca et al., 2009; Kasper et al., 2010; Spacil et al., 2011). In fact it has been demonstrated that a too high declustering potential value in the mass spectrometer could cause a in source breakdown of the substrate with an undue appearance of the product (la Marca et al., 2009). Chromatographic separation allowed the distinction of the real enzymatic product from the one derived by the source breakdown of the substrate.

In the last years, new sample preparation methods reporting on a combination of substrates and internal standards into a single buffer, for a triplex assay (Duffey et al., 2010c; Scott et al., 2013) or a multiplex assay have been reported (Spacil et al., 2013).

Mechtler et al. (2012) have recently published on a short-incubation mass spectrometry-based protocol.

Metachromatic leukodystrophy (MLD) is a disorder caused by the deficiency of the enzyme arylsulfatase A (ASA) and resulting in the accumulation of 3-O-sulfogalactosyl ceramides (sulfatides) in tissues.

Recently, a noteworthy interest for newborn screening of MLD has arisen due to the development of potential therapies (Biffi et al., 2013).

However, application of direct assay of lysosomal enzyme activity in DBS seems to be difficult for MLD because trace amounts of ASA activity are sufficient to minimize disease severity (Renaud, 2012) and pseudodeficiency in ASA is a relatively frequent condition in the normal population (Emre et al., 2000; Lugowska et al., 2000).

Barcenas et al. (2014) in 2014 proposed a UHPLC/MS/MS method to quantify sulfatides in DBS and dried urine spots (DUS) for newborn screening of metachromatic leukodystrophy.

However, they show that analysis of sulfatides on DBS is problematic because their levels appear to be increased in some but not in all DBS patient samples, while there was a good separation between MLD and non-MLD samples using DUS. These results suggest that urine sample collection should be required for a reliable newborn screening program for metachromatic leukodystrophy.

Talking about neonatal screening for LSDs, some ethical considerations must be made.

Considerations should be made about the best strategy to treat presymptomatic individuals with positive screening results, the best way to inform parents of the potential outcomes of the affected individual and risks for future pregnancies.

Furthermore, newborn screening could potentially identify adult-onset variants or variants of uncertain significance and some patients with these variants may never develop symptoms or require therapy. It must be also considered that effective therapies are available only for few LSDs.

In addition LSD newborn screening could identify, as previously described for ASA, enzymatic pseudodeficiency, in fact there are some individuals showing a reduced enzyme activity but they remain clinically healthy (Filocamo & Morrone, 2011).
If a newborn screening panel would include Fabry disease, a complication could be the potential false negative of a female affected with Fabry disease. In fact, Fabry disease is an X-linked disorder and random X-inactivation results in highly variable activity of alpha galactosidase in female patients with Fabry disease. A negative newborn screening for Fabry disease in female should be interpreted with caution (Linthorst et al., 2005).

V. GUANIDINOACETATE METHYLTRANSFERASE DEFICIENCY

Guanidinoacetate methyltransferase (GAMT, OMIM 601240) deficiency is a rare creatine synthesis disorder resulting in a developmental delay and intellectual disability. It is characterized by a depletion cerebral creatine and an accumulation of guanidinoacetate (GAA) in the brain and body fluids. Early diagnosis and treatment with creatine, ornithine, and an arginine-restricted diet reduces the risk of neurological compromise (Gordon, 2010; El-Gharbawy et al., 2013). For this reason, GAMT deficiency might be a good candidate for newborn screening.

In the last years, some pilot projects have been started for newborn screening of GAMT deficiency (Mercimek-Mahmutoglu et al., 2012; Pasquali et al., 2014). GAA and creatine measurements can be included in the expanded newborn screening by using butyl esterification assays (Carducci et al., 2001). However, a high number of false positives is reported during newborn screening because of an overlap between the maximum value of GAA in healthy newborns and the lowest value in GAA patients. This problem can be solved by a second-tier test which allows to achieve the positive predictive value to 100% (Pasquali et al., 2014). A reliable method for the detection of Arginine: Glycine Amidino-transferase Deficiency (AGAT, OMIM 612718; low levels of both GAA and creatine) is still not present, given the variability of creatinine levels with the time of collection and the significant overlap of low levels with the normal population.

The detection of GAMT in the neonatal period has been demonstrated to improve the severe neurological outcome encouraging, with a minimal additional cost, the inclusion of GAMT deficiency within the routine newborn screening (El-Gharbawy et al., 2013).

VI. X-LINKED ADRENOLEUKODYSTROPHY

X-linked adrenoleukodystrophy (X-ALD) is a genetic disorder caused by mutations in ABCD1 gene on the X-chromosome resulting in the failure of peroxisomal oxidation of very long chain fatty acids (VLCFA; Ferrer, Aubourg, & Pujol, 2010; Kemp, Berger, & Aubourg, 2012). X-ALD is estimated to affect 1 in every 17,000 newborns (Bezman and Moser, 1998); however, the true prevalence can be known only from the newborn screening data.

The disease primarily affects the nervous system and the adrenal glands with onset of symptoms at variable ages (Moser, Mahmood, & Raymond, 2007). Phenotypes include rapidly progressive childhood, adolescent, and adult cerebral forms;
adrenomyeloneuropathy, which presents as slowly progressive paraparesis in adults and Addison disease without neurological manifestations.

X-ALD could be added to the list of disorders that could benefit from newborn screening because new therapies have been developed in the last years and they show encouraging promise for clinical outcomes (Moser, Raymond, & Dubey, 2005). In particular, hematopoietic stem-cell transplantation is the most effective treatment that is often recommended but is beneficial only if the patient has not already reached a stage with irreversible brain damage. Thus an early diagnosis is critical for improving the outcome of these patients.

The biochemical marker of XALD is the accumulation of saturated very long-chain fatty acids (VLCFA) in tissues and body fluids particularly of hexacosanoic acid (C26:0).

In 2006, an LC-MS/MS method has been developed to measure lyso-phosphatidylcholines (L-PC) C20:0, C22:0, C24:0 and C26:0 on dried blood spots, in which C26:0 and its ratios appeared the potential diagnostic marker for X-ALD. However, this method required a gradient analysis that had a chromatographic run time of 13 min/sample making it inconvenient for newborn screening (Hubbard et al., 2006). The method was slightly modified in 2009 (Hubbard et al., 2009) reducing run time analysis to 7 min/sample.

Recently, other methods have been proposed to make newborn screening feasible: in 2012, Sanders et al. (2012) reported on a modified method from Hubbard 2009 for quantitation of L-PC 26:0 able to reduce both sample preparation and analysis time and to perform simultaneous extraction of acylcarnitines. In a prospective study, nearly 5,000 newborn blood spot samples were analysed by this method, providing evidence that it can be applied as a high throughput method for newborn screening for X-ALD newborn screening (Theda et al., 2014). In order to further improve the efficiency of newborn screening for X-ALD, Haynes and De Jesús (2012) developed a valve-switching system able to acquire acylcarnitines and amino acids in positive ion mode and L-PC 26:0 in negative ion mode to eliminate an interfering compound that could result in false positives.

An early diagnosis of X-ALD through newborn screening offers to these patients the best chance for treatment, thus an implementation of NBS programs could be desirable. Presently, heterozygous females, who are frequently symptomatic, remain an unresolved problem. Carrier females have a reduced L-PC 26:0 accumulation and that could lead to false negative in blood investigations (Berger & Gärtner, 2006).

Two peroxisomal storage disorders have been clinically diagnosed as X-ALD and Zellweger syndrome (OMIM 214100) from 2004 to 2010 in Tuscany. A retrospective analysis of butylated acylcarnitines on their neonatal DBS has been performed by extending precursor ion scan of 85 m/z up to 600 Th (range was set from 200 to 600 m/z) and by adding acylcarnitine C23 as internal standard to quantify very long chain acylcarnitines. Their profiles revealed abnormal concentration of C16DC, C18DC, C24, and C26 acylcarnitines in the Zellweger syndrome neonate DBS, as reported by Rizzo et al. (2003) and an increase of C24 and C26 (but not C16DC and C18 DC) acylcarnitines in X-ALD sample (Fig. 4). The ratio C26/C22 was also informative. Normal cutoff ranges for C16DC, C18DC, C22, C24, and C26 were than calculated on 20,000 NBS samples; no abnormal values of these markers were found (unpublished data).

Given the current poor data further evaluation should be done in order to develop a reliable, sensitive, and specific screening test for X-ALD patients.

VII. WILSON DISEASE

Wilson disease (WD; OMIM# 27790) is an autosomal recessive disorder caused by mutations in the gene encoding the copper transporting ATPase, ATP7B, that transports citosolic copper into the lumen of the trans-Golgi network for incorporation in ceruloplasmin (CP).

The enzyme defect reduces the incorporation of copper into ceruloplasmin causing copper accumulation mainly in the liver and in the central nervous system (deWilde et al., 2008) and a reduction in ceruloplasmin levels in the bloodstream.

WD incidence is reported to be about 1/30,000 live births but in some populations it is much more common (Figuš et al., 1995; Zappu et al., 2008).

The pharmacological treatments are represented by copper chelators to promote its excretion from the body, zinc salts to reduce its absorption in the intestine and liver transplantation (Schilsky, 2009; Loudianos et al., 2014)

Due to the severity of the disease and to the existence of an effective therapy approach, an early diagnosis is fundamental.

Usually the diagnosis of WD is performed through the measurement of copper in liver tissue followed by molecular confirmation with genetic testing of ATP7B gene (Hahn, 2014). However level of ceruloplasmin in infants and newborns are considerably lower than in adults and this complicates a newborn screening programs due to a high rate of false positive rate.

A measurement of ceruloplasmin on DBS through ELISA assay, even if it is able to distinguish WD patients from healthy control individuals, is critical for a successful screening program because of the variation due to ceruplasmin antibody binding (Kroll et al., 2006). An antibody-independent method using LC-MS/MS to quantify ceruloplasmin on DBS was developed by deWilde et al. (2008). However this new approach requires a time consuming sample preparation step (8hr) due to trypsin digestion and long chromatographic run for the separation of specific CP peptides (7.5 min).

Therefore, nowadays, due to the absence of an effective and efficient approach for NBS, Wilson disease is not included in newborn screening programs (Matern, Oglesbee, & Tortorelli, 2013).

VIII. SICKLE CELL DISEASE

Sickle cell disease (SCD) is an inherited disorder caused by a replacement of a glutamic acid to a valine at position six on the β-globin chain (Stuart & Nagel, 2004) resulting in the structural variant hemoglobin S (HbS). The mutation causes sickling of hemoglobin rather than reduced amount of β globin which causes β-thalassemia (Galanello & Origa, 2010).

In homozygous state, HbS (HbSS) is responsible for anemia and tissue damage due to the occlusions of blood vessels caused by the abnormal red cells (Odièvre et al., 2011).

Heterozygous state associated with normal hemoglobin A (HbAS) is phenotypically silent, while different disease phenotypes occur in compound heterozygotes in which HbS variant is
co-inherited with another haemoglobin mutant (e.g., HbC, HbD Punjab/Los Angeles, or HbE).

SCD is associated with high mortality in early childhood and an early diagnosis by newborn screening could increase patient survival. Nowadays treatments help reduce the severity and frequency of complications of SCD; among them, some drugs that reduce HbS polymerization or stimulate HbF synthesis reactivation (Brugnara et al., 1996; Fathallah & Atweh, 2006; Migliaccio et al., 2008), such as hydroxyurea (Tang et al., 2005), have proved to be effective. Furthermore other important therapeutic options are represented by stem cell transplantation and transfusion programs (Styles & Vichinsky, 1994; Khoury & Abboud, 2011) and recently studies are focused on gene therapy (Sadelain et al., 2008).

Newborn screening programs for hemoglobin disorders can be performed on cord blood or on DBS by isoelectric focusing (IEF) or cation exchange HPLC (ceHPLC) HPLC–UV; these methods frequently produce results of difficult interpretation and data must be confirmed by second line testing using DNA sequencing or amino acid analysis by tandem mass spectrometry (Old, 2003).

Tandem mass spectrometry has recently been considered an alternative screening approach for the characterization of hamoglobin variants; different methods are used including the detection of the intact globin chains masses or analysis of tryptic peptide fragments (Boemer et al., 2008). Edwards et al. (2014) developed a method in which automated direct surface sampling of the DBS is coupled to top–down MS of the intact globin chains.

MS/MS for diagnosis of clinically significant Hb variants could be easily transferred for use in neonatal hemoglobinopathy screening due to the reasonable running cost, analytical sensitivity, and specificity and speed of analysis.

**IX. CONCLUDING REMARKS**

The NBS program is a complex process that is dependent on a multidisciplinary team which includes pediatricians, geneticists, dieticians as well as biologists, chemists, and technicians with expertise in mass spectrometry. The collaboration of all these professionals has contributed to the success of NBS for over 20 years and consequently to the beneficial impacts on public health care. Present trends in NBS are aimed at expanding testing panels in order to cover a wide range of conditions. Research including more testing in NBS and new therapeutic strategies has made noteworthy steps forward in this field.
However, the expansion of NBS raises some questions and the discussion of which disorders should be added to a NBS panel is still ongoing worldwide.

In any case, there are no doubts that the model of newborn screening and MS in preventive medicine may be exemplary in developing other clinical applications that go beyond metabolic disorders. Although there will be many challenges to the further expansion of MS in clinical chemistry, it is clear that this technique will play a fundamental role in the future.

**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADA</td>
<td>adenosine deaminase</td>
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<tr>
<td>Ado</td>
<td>adenosine</td>
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<tr>
<td>AGAT</td>
<td>arginine-glycine amidotransferase</td>
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<tr>
<td>ASA</td>
<td>arylsulfatase A</td>
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<tr>
<td>CAH</td>
<td>congenital adrenal hyperplasia</td>
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<tr>
<td>CP</td>
<td>ceruloplasmin</td>
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<td>C3</td>
<td>propionylcarnitine</td>
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<tr>
<td>D/Ado</td>
<td>2-deoxyadenosine</td>
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<tr>
<td>DBS</td>
<td>dried blood spot</td>
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<tr>
<td>DUS</td>
<td>dried urine spot</td>
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<tr>
<td>ESI-MS/MS</td>
<td>electrospray tandem mass spectrometry</td>
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<td>FAB</td>
<td>fast atom bombardment NBS newborn screening</td>
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<td>FIA</td>
<td>flow injection analysis</td>
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<tr>
<td>GAA</td>
<td>guanidinoacetate</td>
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<tr>
<td>GAMT</td>
<td>guanidinoacetate methyltransferase</td>
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<tr>
<td>Hb</td>
<td>hemoglobin</td>
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<tr>
<td>IEF</td>
<td>isoelectric focusing</td>
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<td>IEM</td>
<td>inborn errors of metabolism</td>
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<tr>
<td>L-PC</td>
<td>lysophosphatidylcholines</td>
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<td>LSD</td>
<td>lysosomal storage disorders</td>
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<tr>
<td>MLD</td>
<td>metachromatic leukodystrophy</td>
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<td>MPS</td>
<td>mucopolysaccharidosis</td>
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<td>MSUD</td>
<td>maple syrup urine disease</td>
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<td>NK</td>
<td>natural killer</td>
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<tr>
<td>PKU</td>
<td>phenylketonuria</td>
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<tr>
<td>PNP</td>
<td>purine nucleoside phosphorylase</td>
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<tr>
<td>SCD</td>
<td>sickle cell disease</td>
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<tr>
<td>SCID</td>
<td>severe combined immunodeficiency</td>
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<tr>
<td>SUAC</td>
<td>succinylacetone</td>
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<tr>
<td>TREC</td>
<td>T-cell receptor excision circles</td>
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<tr>
<td>VLCFA</td>
<td>very long chain fatty acids</td>
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<tr>
<td>X-ALD</td>
<td>X-linked adrenoleukodystrophy</td>
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<td>WD</td>
<td>Wilson disease</td>
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**REFERENCES**


