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**Expanded Newborn Screening by Tandem Mass Spectrometry: new tests and future perspectives.**

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## Abstract

Tandem mass spectrometry (TMS) has become a leading technology in clinical chemistry and in particular in newborn screening (NBS) program showing to be sensitive, specific and versatile. This result is due to great advances in hardware, software and clinical applications during the last twenty-five years. TMS permits a very rapid measurement of many metabolites in different biological specimens using filter paper spots or directly in biological fluids. Its use in NBS gave the chance to identify treatable metabolic disorders possibly when asymptomatic and the evidence of benefits is now worldwide demonstrated. Today the use of TMS for second tier tests and confirmatory testing is greatly promising as well as 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 (GAMT) and Duchenne muscular dystrophy.

Reducing the false positive rate by using second tier tests, avoiding false negative results by using new specific biomarkers and introducing in NBS programs new treatable disorders is the new challenge for the future.

## I. INTRODUCTION

The use of mass spectrometry (MS) in clinical laboratories is very much increased on the outset of the 21th century. This development is obviously due to great advances in MS hardware, software and research in the last twenty-five years.

MS permits a very rapid measurement of many metabolites in different biological specimens 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. It is known to be a biochemical test enabling 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

electrospray 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 (Millington et al, 1991; Yergey et al, 1984) 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 aminoacids 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 amino acids in newborn screening.

While butyl esterification assays are now routine, in the last ten years new methods were developed able to detect aminoacids 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 screening 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 et al, 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 (Frazier et al 2006; Wilcken et al 2003).

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 overrules 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 computerisation, 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 in newborns with abnormal screening result. It is considered a confirmatory test performed on the original blood spot, in order to find a target analyte under the optimum operating conditions. The introduction of second-tier test, in newborn screening programs, has allowed to reduce the number of false positives causing unnecessary parental stress, increased laboratory analyses and personnel costs for repeat tests. However, second-tier tests cannot be used as primary screening test because of the low-throughput assay and the laboratory's cost (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 test allows to 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, they meet fully to the definition of second tier tests, in order to distinguish true from false positives and to improve positive predictive value of primary NBS results. Using this strategy several two-tier tests have been developed as for succinylacetone (SUAC), specific marker for Tyrosinemia I (Tyr I, OMIM 276700), recently included in the NBS panel (la Marca et al., 2008; Turgeon et al., 2008). Although, in our experience, false positive rate for SUAC is very low (la Marca et al., 2011), a sample contamination cannot excluded. A newborn falsely suspected for Tyr I cause high cost of hospitalization, without considering parental stress. To account for this, the specificity of some important diagnostic markers as SUAC, requiring immediately hospitalization, should have the highest possible specificity.

In the other case, second tier tests identify diagnostic metabolites, not available by routine NBS, 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 test. An high C3 (also >10μmol/L) is not real diagnostic marker contrariwise the presence of free organic acids has a pathognostic 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 test 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). Alloisoleucine (allo-Ile) is the pathognomonic marker of MSUD, but it cannot be differentiated in flow injection from isobaric 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 isobaric compound, permitting unequivocal interpretation of NBS results (Oglesbee et al., 2008).

Second tier steroid profiling by TMS 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 the second tier test by using TMS improves the specificity of NBS results (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 and colleagues (Sarafoglou et al, 2012). These authors suggested that molecular testing as second tier test could have a major efficacy but cost-effectiveness has not yet been evaluated.
However, TMS 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. A list of second tier tests MS based has been 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 results 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 a 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 signs and let to 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 showed to be 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, 2011b).
SCID show all the characteristics required by neonatal screening programmes and they were introduced in 2008 in Wisconsin first by T-cell receptor excision circles (TREC) assay; in 2009 Massachusetts joined and than 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).
The 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 et al., 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 his screening algorithm resulting in a great variability between areas (Chase, Verbsky & Routes, 2011) .

High false-positive TREC assay rate 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 and deoxyadenosine triphosphate, which inhibit the activity of ribonucleotide reductase and ultimately DNA synthesis. The incidence of ADA deficiency is estimated to be between 1 in 375,000 and 1 in 660,000 live births (Sauer & Aiuti, 2009) but it might be underestimated because of early death from infective disease 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 showed 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, 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, 2014). 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 et al., 2013).

At the beginning of 2013, Purine nucleoside phosphorylase deficiency (PNP, OMIM 613179) was also included in the TMS newborn screening panel of Tuscany region (la Marca et al., 2013).
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 deoxyinosine to their respective purine bases and pentose-1-phosphates (Hershfield & Mitchell, 2001). Lack of PNP function determines a intracellular accumulation of deoxyguanosine triphosphate derived from deoxyguanosine, toxic to lymphoid cells and resulting in various neurologic abnormalities (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.
TMS can easily identify abnormal metabolites in DBS for PNP patient 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, 2014) and have a combined incidence of about 1 in 1500 to 7000 live births (Stone & Sidransky, 1999; Fletcher, 2006).
Most affected newborn seems to be healthy at birth but they can present with 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 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 (Escolar et al., 2005; Urbanelli et al., 2011).

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 favourably modify the natural history of the disease (Chien et al., 2009; Muenzer, 2014).

An additional argument for inclusion of LSDs in NBS program 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; Wittmann et al., 2012; Paciotti 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 since 2006 (Duffner et al., 2009).

In Tuscany region, Italy, a pilot project screening program for Pompe, Fabry and Mucopolysaccharidosis type I will start in September 2014.

Going to technical procedures, the pioneering work of Chamoles (Chamoles et al., 2004) first established the possibility to 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, Hurler-like LSDs, Niemann-Pick A/B disease, Tay-Sachs disease and Sandhoff disease (Chamoles, Blanco & Gaggioli, 2001; Chamoles et al., 2002a, 2002b). However, the incorporation of a chromophore or fluorophore into the substrate caused false negative results and had the 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 FIA-tandem mass spectrometry .

Later on, an expansion of simulateously 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, 2010b; Khaliq et al., 2011; Wolfe et al., 2011; Chennamaneni et al., 2014). Moreover in order to facilitate the introduction of LSD in newborn screening panels, the Centers 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 and colleagues to make it more suitable for newborn screening laboratories (Zhang et al., 2008). 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 and collegaues 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 (la Marca et al., 2009). As an example a chromatogram from a LC-MS/MS combined 6 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 an 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 and colleagues have recently published on a short-incubation mass spectrometry-based protocol (Mechtler et al, 2012).

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 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 normal population (Lugowska et al, 2000; Emre et al, 2000).

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

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 samples collection should be required for a reliable newborn screening program for metachromatic leukodystrophy.

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

Considerations should be made about the best strategy to treat presymptomatic individuals with positive screening results, the best mode 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 missing of Fabry affected female. 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 project 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 to the expanded newborn screening by using butyl esterification assays (Carducci et al., 2001). However, a high number of false positive is reported from newborn screening because of a 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 Amidinotransferase 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, OMIM 300100) 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 & 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 the 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 are 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 has benefit if the patient has not so far reached a stage with irreversible brain damage. Thus an early diagnosis is critical for improving 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 e 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 minutes per sample making it inconvenient for newborn screening (Hubbard, 2006). The method was slightly modified in 2009 (Hubbard, 2009) reducing run time analysis to 7 minute/sample.

Recently, other methods have been proposed to make newborn screening feasible: in 2012, Sandlers and colleagues (Sandlers, 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 (Sandlers, 2012). In a prospective study, nearly 5000 newborn blood spot samples were analysed by this method, providing evidence that it can be applied as a high throughput method for X-ALD newborn screening (Theda, 2014). In order to further improve the efficiency of newborn screening for X-ALD, Haynes and De Jesus (2012) developed a valve-switching system able to acquire acylcarnitines and aminoacids 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, remains 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).

In Tuscany region in the period 2004-2010 newborn screening sample preparation procedure has been performed by using derivatized (butylation) method and acylcarnitine profile was obtained by precursor ion scan of 85 m/z. During this period two peroxisomal storage disorders have been clinically diagnosed. A retrospective analysis on neonatal DBS showed abnormal concentration of C16DC, C18DC, C24 and C26 acylcarnitines in a Zellweger syndrome (OMIM 214100) according to Rizzo and colleagues (Rizzo et al., 2003); on neonatal DBS of X-ALD patient an increase of C24 and C26 (and not C16DC and C18 DC) acylcarnitine was identified (unpublished data) (Fig. 4).

**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.

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 1/30000 live births but in some populations it is much more common (Zappu et al., 2008; Figus et al., 1995).

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 transpantation (Loudianos et al., 2014; Schilsky, 2009)

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 whit genetic testing of ATP7B gene (Hahn, 2014). However

level of ceruloplasmin in infant 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 successful screening of WD 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 deWide et al (deWilde et al., 2008). However this new approach requires a too long sample preparation due to trypsin digestion step (8h) and a too 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).

**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 et al., 2004) resulting in the structural variant hemoglobin S (HbS). The mutation causes sickling of hemoglobin rather than reduced amount of beta globin which causes beta-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 coinherited with another haemoglobin mutant (e.g. HbC, HbD Punjab/Los Angeles or HbE).

SCD is associated with high mortality in early childhood and a tempestive diagnosis by newborn screening could increase patient survival. Nowadays treatments help reduce the severity and frequency of complications of sickle cell disease; among them, some drugs for reducing HbS polymerization or for stimulating 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 (Khoury & Abboud, 2011; Styles & Vichinsky, 1994) and recently studies are focused on gene therapy (Sadelain et al.,2008).

Newborn screening programs for haemoglobin 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).
Recently tandem mass spectrometry is considered an alternative screening approach for the characterization of haemoglobin 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. developed a method in which automated direct surface sampling of the DBS is coupled to top-down MS of the intact globin chains (Edwards et al., 2014).
TMS 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.

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